COP9 SIGNALOSOME PROMOTES NEOINTIMAL HYPERPLASIA VIA DENEDDYALTION AND CSN5-MEDIATED NUCLEAR EXPORT

Samiksha Giri

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COP9 SIGNALOSOME PROMOTES NEOINTIMAL HYPERPLASIA VIA DENEDDYALTION AND CSN5-MEDIATED NUCLEAR EXPORT

By
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ABSTRACT

Neointimal hyperplasia (NH) is a common pathological response to vascular injury and mediated primarily by vascular smooth muscle cell (VSMC) migration and proliferation. The COP9 signalosome (CSN) is formed by 8 canonical subunits (CSN1 through CSN8) with its de neddylation activity residing in CSN5. Each or some of CSN subunits may have de neddylation-independent function but this is not well established. Despite the CSN being known to be a key regulator of protein degradation, its role in vascular biology remains obscure. The present study was conducted to fill these critical gaps. Our immunohistochemistry analyses revealed substantially higher CSN5 levels in the neointimal VSMCs of the pulmonary arteries of human pulmonary hypertension (PAH) than in the VSMCs of control pulmonary arteries. Left common carotid artery (LCCA) ligation induced NH and significantly increased the mRNA and protein levels of CSN subunits in the LCCA wall of adult wild type mice. Adult mice with smooth muscle cell-restricted CSN5 knockout (CSN5-SMKO) impaired cullin de neddylation and the nuclear export of p27 in vessel walls and markedly inhibited VSMC proliferation in mice. On the contrary, CSN8 hypomorphism (CSN8-hypo) significantly exacerbated NH and VSMC proliferation in vivo and in cellulo. Cytoplasmic CSN5 mini-complexes and the nuclear export of p27 were significantly increased in CSN8-hypo mouse vessels and cultured CSN8-hypo VSMCs. Nuclear export inhibition with leptomycin attenuated the PDGF-BB induced increases in VSMC proliferation in both CSN8-hypo and control VSMCs. Further, genetically disabling CSN5 nuclear export but not disabling CSN5 de neddylation activity suppressed the hyperproliferation and restored p27 nuclear localization in CSN8 hypomorphic VSMCs. Interestingly, CSN de neddylation inhibition by CSN5i-3 did not alter the hyperproliferation of cultured CSN8-hypo VSMCs but suppressed wild type VSMC proliferation in vivo and blocked neointimal formation in wild type mice. Thus, we have established both de neddylation by the CSN and nuclear-export by CSN5 mini-complex as the underlying mechanisms for the promotion of VSMC proliferation and NH by CSN5.

Dissertation Advisor: Xuejun Wang, M.D., Ph.D.
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LIST OF ABBREVIATIONS

BCA: Bicinchoninic acid

BSA: Bovine serum albumin

BW: Body weight

CAND1: Cullin associated and neddylation dissociated 1

CDK: Cyclin-dependent kinase

CDKI: Cyclin-Dependent Kinase Inhibitor

CO: Cardiac output

CRLs: Cullin-RING ligases

CSN: The COP9 signalosome

CTL: the control group

Cul: Cullin

CVD: Cardiovascular disease

DMEM: Dulbecco’s modified eagle’s medium

DMSO: Dimethyl sulfoxide

DUB: Deubiquitinating enzymes

E1: Ubiquitin activating enzyme

E2: Ubiquitin conjugating enzyme
E3: Ubiquitin ligase

Echo: Echocardiography

ECL: Enhanced chemiluminescence

EDV: End diastolic velocity

EF: Ejection fraction

eIF3: Eukaryotic initiation factor

FBS: Fetal bovine serum

FS: Fractional shortening

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

HECT: Homologous to E6-associated protein carboxyl terminus

HR: Heart Rate

JAMM: JAB1-MPN-domain metalloenzyme

K: Lysine, Lys

KO: Knockout

L.C. Loading Control

LCCA: Left common carotid artery

LMB: Leptomycin

LV: Left ventricle
LVID; d: End-diastolic left ventricular internal diameter

LVPW; d: End-diastolic left ventricular posterior wall thickness

Lys: Lysine

NAE: NEDD8-activating enzyme

NEDD8: Neuronal precursor cell expressed developmentally down-regulated protein 8

NES: Nuclear export signal

NTG: non-transgenic

PAH: Pulmonary hypertension

PBS: Phosphate-buffered saline

PBST: PBS containing 0.1% tween-20

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase chain reaction

PCI: Proteasome lid-CSN-initiation factor

PI: Pulsatility index

PQC: Protein quality control

PSV: Peak systolic velocity

RCCA: Right common carotid artery

RI: Resistivity Index
RING: Really interesting new gene

RT-PCR: Semi-quantitative reverse transcriptase-PCR

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

siRNA: Small interference RNA

SREF: Substrate receptor exchange factor

SRM: Substrate receptor module

SV: Stroke Volume

UPS: Ubiquitin proteasome system

VSMC: Vascular Smooth Muscle Cell

VTI: Velocity time integral

WT: Wild type
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INTRODUCTION

1. Vascular Smooth Muscle Cells

1.1 Structure and function of vascular smooth muscle cells

Blood vessels play an important role in promoting the exchange of oxygen and nutrients in tissues across the body. Anatomically, a blood vessel consists of three layers, the outermost layer of the vessel tunica adventitia, the middle layer tunica media, and the innermost layer tunica intima. Endothelial cells along with the basal membrane and collagen fibers form tunica intima and act as a barrier between the blood carrying lumen and the wall of a vessel. The tunica media neighbors tunica intima and is primarily composed of vascular smooth muscle cells (VSMCs) along with elastin and collagen fibers laying in between them. The outermost layer of the vessel is formed primarily by the connective tissue consisting of extracellular matrix (ECM), fibroblasts and progenitor cells. All three layers are mechanoresponsive and are capable of remodeling during disease progression; hence, a healthy interaction between all components of the vessel wall is required to preserve vascular health.

VSMCs are the predominant constituent of all blood vessels except capillaries. They are the main cells in the media layer of an artery and play an important role in the physiology and pathophysiology of the vasculature. Moreover, the function of smooth muscle can be extended on a much broader scale depending on the organ and system it regulates. Smooth muscle functions as an involuntary muscle, mostly found in hollow and tubular organs. SMCs are uninuclear, spindle shaped cells embedded and enwrapped in large amounts of ECM. The cytoplasm of SMCs contains actin and myosin, the key proteins in muscle contraction. Smooth muscle does not contain sarcomeres but uses the contraction of filaments of myosin and actin to ensure directional movement of body fluids and regulation of organ shape and size.
Lineage tracing studies suggest that VSMCs originates from multipotent precursors from a number of developmental origins. These stromal cells of the vascular wall are responsible for regulating arterial tone, blood pressure and the blood supply to the tissues. An increasing body of evidence also suggests that SMC embryonic lineage plays an important role in determining the location and presentation of disease. Crucial for maintaining vascular homeostasis, VSMCs adapt the mechanism of mechanical coupling, which triggers strong and synchronized contractions at a low energy cost. In the arterial wall, VSMCs get exposed to plethora of mechanical cues, including stretch and matrix stiffness, which regulates VSMC contraction.

1.2 Phenotypic switching of VSMCs

VSMCs, unlike many other mature cells, are not terminally differentiated but retain phenotypic plasticity. In healthy adult vessels, a vast majority of VSMCs exhibit a quiescent non-proliferative, contractile phenotype. This differentiated phenotype is characterized by the expression of a repertoire of SM-specific contractile and associated proteins. However, under stimulating or mechanical damage to the blood vessel such as vascular injury, VSMCs can undergo “phenotypic switching”. They make a transition from their original contractile phenotype to a proliferative or synthetic phenotype, primarily characterized by the increased VSMC proliferation and migration, in concert with decreased expression of contractile markers. The concept of phenotypic switching was first described in 1979 and historically has been considered a bidirectional process. This phenotypic modulation appears to be governed by numerous signaling pathways including those triggered or mediated by integrins, contractility, mechanic forces, cytokines, growth factors and inflammatory mediators. Mechanistic studies continue to emerge trying to apprehend the pathways that influence VSMC
phenotypic plasticity and its effects on vascular remodeling, but the underlying mechanisms are still not fully understood.19

1.2.1 Role of p27 in phenotypic switching

Cell division is one of the predominant physiologic processes in tissue homeostasis. Phenotypic switching of VSMCs from the contractile state to the synthetic state requires the reentry into the cell cycle. The cell cycle process is highly conserved and precisely controlled to govern the genome duplication and cytokinesis. It consists of four distinct and ordered phases, termed G0/G1 (gap 1), S (DNA synthesis), G2 (gap 2), and M (mitosis) phases; and multiple checkpoints are instigated in the cell cycle control to ensure faithful replication in the S phase and the exact aggregation of the chromosomes into daughter cells.20 In a normal quiescent state, the VSMC mostly exists in the G0 phase of the cell cycle, but under appropriate stimuli for proliferation, VSMCs have to progress through the cell cycle. Advancement through the cell cycle is coordinated by expression of multiple holoenzymes composed of a cyclin-dependent kinase (CDK) and cyclin-regulatory subunits. The kinase activity of the CDKs is negatively regulated by CDK inhibitor proteins (CDKIs). As such, CDKIs are essential for cell cycle progression and the proliferation of cells. An important group of CDKIs is the KIP/CIP family consisting of p21cip1/waf1, p27kip1 and p57kip2 that regulate the phases of the cell cycle serving different functions.21

Encoded by the CDKN1B gene in humans, CDK inhibitor 1B (p27kip1) is commonly referred to as p27, known as KIP1. It primarily targets cyclin E-CDK2 or cyclin D-CDK4 complexes and thereby controls cell cycle progression at G1.22 p27 has been implicated as a regulatory factor in vascular proliferative response in disease.21 While the transcription of p27 is
constitutively steady, protein levels of p27 are found at a higher level during the G0 phase, mostly detected in nucleus and effectively inhibiting the cell cycle. The p27 protein level subsequently decreases as the cell progresses through the cell cycle. This decrease in the protein level follows the export of p27 from the nucleus to cytoplasm and correlates with the increased degradation by the ubiquitin proteasome system (UPS). Evidence suggests that degradation of p27 is regulated by two distinct RING (Really Interesting New Gene)-finger type ubiquitin ligase complex: during the G0-G1 transition, p27 degradation is promoted through a Kip1 ubiquitination-promoting complex (KPC) in the cytoplasm; however, at the S/G2 interface, p27 levels are regulated by the cullin-RING ligase complex, SCF^{Skp2}, in the nucleus. Phenotypic switching triggered by vascular injury was associated with a decrease in the expression of p27, whereas VSMC proliferation was found increased in p27 null mice. Likewise, reduced level of p27 hastened the progression of atherosclerosis, and similarly, in angiotensin induced hypertension, increased matrices for VSM proliferation correlated with reduced p27. Moreover, overexpression of p27 in injured rat carotid arteries reduced VSMC proliferation and intimal hyperplasia. Collectively, studies have suggested that p27 is an important control modulator of phenotypic switching, where its expression inversely correlates with the increase in VSMC proliferation.

1.3 VSMC phenotypic switching in cardiovascular disease

The plasticity nature of SMCs has been extensively studied in vitro; thus, the concept of phenotypic switching is widely accepted. Imbalanced VSMC plasticity results in maladaptive phenotypic alterations that ultimately lead to progression of multiple VSMC-driven pathologies.
Cardiovascular disease (CVD) remains the leading cause of death worldwide and accounts for the death of one person in every 34 seconds in the United States. Moreover, CVD is an umbrella term, which holds to cover multiple related diseases, such as peripheral artery disease, hypertension, pulmonary hypertension, coronary heart disease and stroke. A majority of CVD are due to vascular dysregulation, for example, caused either by impaired VSMC proliferation or vascular rarefaction. As such, phenotypic switching of the VSMCs contributes to the progression of a large subset of CVD.

1.3.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory condition characterized by the development of plaques and the narrowing of vascular lumen. It is considered one of the major causes of CVD. During the genesis of atherosclerotic lesions, the VSMCs are stimulated in response to surrounding growth factors and cytokines, and because of their plasticity nature, the VSMCs then undergo phenotypic switching. The phenotypic switching of VSMCs is long considered a hallmark for atherosclerosis. The VSMCs proliferate and migrate to endothelium resulting in the secretion of extracellular matrix (ECM) that participates in the formation of fibrous cap, a clinical determinant of atherosclerosis. Moreover, in humans, atherosclerotic lesions have been shown more prone to develop at sites of preexisting intimal hyperplasia. Intimal hyperplasia is considered a precursor lesion for atherosclerosis.

1.3.2 Restenosis and neointimal hyperplasia

Restenosis occurs when the treated vessel becomes blocked again. The principle biological mechanism of restenosis is neointimal hyperplasia (NH) which is a pathological
process whereby various coagulation and inflammatory factors stimulate VSMC proliferation and migration at the site of injury and thus causes a reduction in the diameter of the vessel lumen. As the major cellular determinants of the arterial wall pathology, VSMC proliferation and neointima formation represent an important event in the pathophysiological courses of atherosclerosis and restenosis.\textsuperscript{42,43}

1.3.3 Aortic aneurysms

Aortic aneurysm (AA) are balloon-like bulges that cause the gradual weakening of the aortic wall. It is the second most prevalent aortic disease following atherosclerosis. Studies have suggested that VSMCs phenotype is altered before or early during the establishment of aneurysms.\textsuperscript{44} VSMCs dysfunction correlates with the loss of structural integrity of aortic wall and VSMC phenotypic switching contributes to the development of AA.\textsuperscript{45}

1.3.4 Hypertension and pulmonary hypertension

Hypertension is a major risk factor for heart disease, stroke, and renal disease. The genesis of hypertension, the leading risk factor for premature CV death worldwide is a major event of VSMC, where rapid proliferation of VSMC and subsequent lumen narrowing represents a pathological hallmark change during the progression of hypertensive vascular remodeling contributing to peripheral vascular resistance.\textsuperscript{46,47}

VSMC proliferation and NH are prominent features of pulmonary arterial hypertension (PAH). All vessel layers (intima, media, and adventitia) are affected in PAH. However, it is widely recognized that the most prominent feature of PAH is the aberrant proliferation of SMCs.
As the disease progresses, SMC phenotype changes from contractile to proliferative, and participates in cell migration, and neointima formation. This subsequently results in obstruction of the lumen, elevation of pulmonary arterial pressure and ultimately leading to right ventricular failure and premature death. Neointima formation represents a key hallmark denoting the irreversibility of PAH.48,49

In addition, VSMCs phenotypic change has been suggested to contribute to promote vascular aging and calcification.37 Likewise, Marfan syndrome, a monogenic disorder of connective tissue involves the phenotyping switching of VSMC to a more differentiated state.36

The remodeling of the vascular wall represents a dominant process in vascular disease. Over the past 20 years, great progress has been made in investigating the role of VSMC phenotypic changes in vascular disease.37 VSMC phenotype change provides a new perspective to understand the pathogenesis of vascular diseases. As such, innovative technologies developed over the years like cell lineage passage, single-cell RNA sequencing, and human genome have been integrated to get insights into the molecular reprogramming of VSMCs in experimental as well as clinical studies. Nevertheless, we still lack a comprehensive understanding of VSMC phenotypic switching and the ability to precisely manipulate it. Thus, a better understanding of the molecular and cellular mechanisms that govern the phenotypic switch of VSMCs and regulate the formation of NH is of utmost importance to selectively manipulating VSMCs in disease states, and for the development of treatment and therapeutics.
2. Neointimal Hyperplasia

In healthy mature vessels, the innermost intimal layer is mainly composed of endothelial cells, while the amount of contractile VSMCs in the layer is insignificant. As the vessel suffers injury, the endothelium suffers damage, the subendothelial collagen becomes exposed followed by a large amount of platelet adhesion and aggregation. While the inflammatory factors converge to the damaged endothelium, the activated VSMCs primarily undergo dedifferentiation resulting in proliferation and gradually migration to the tunica intima where they further proliferation and secrete ECM. At the end, while the endothelial cells are regenerated covering the intima, a new neointima is formed. This pathological process is known as neointimal hyperplasia (NH).

NH thickens arterial wall and decreases luminal patency. A huge body of evidence supports the idea that VSMC phenotypic switching is a key event in the pathogenesis of vascular intimal hyperplasia. Vascular injury-induced phenotypic modulation of VSMCs promotes the repair of the lesion, but failure to appropriately resolve the healing process leads to NH. The condition is considered the sole or major devastating post-operational complication for interventions like angioplasty, coronary artery bypass conduits, stenting, and other surgical repair. The uncontrolled proliferation of VSMCs and the consequent narrowing of the lumen ultimately also contributes to pulmonary hypertension (PAH), diabetic vascular complications, and transplantation arteriopathy.

3. The Ubiquitin-Proteasome System (UPS)

It is postulated that almost 80-90% of intracellular proteins are degraded by the UPS. Degradation of proteins by the UPS involves two distinct steps: i) covalent attachment of multiple ubiquitin to a target protein molecule and ii) degradation of the ubiquitin-conjugated
target protein by the 26S proteasome. The former is accomplished by a process known as ubiquitination that requires a cascade of enzymes in a successive manner, namely E1, ubiquitin activating enzyme, E2, ubiquitin conjugating enzyme and E3, ubiquitin ligase.\textsuperscript{61,62} The ubiquitin gets activated through an ATP dependent E1 that binds ubiquitin via a thiol ester linkage, which is then transferred to E2 and thereafter interacts with E3. The E3 brings E2 and the substrate together. E2 can then either directly or indirectly conjugate ubiquitin to the substrate protein which then tags the protein for degradation by the 26S proteasome.\textsuperscript{63} In the human body, the genome encodes 2 E1s, 30-50 E2s, and more than 600 E3 ubiquitin ligase.\textsuperscript{64}

The 26S proteasome is found in both the nucleus and cytoplasm of eukaryotic cells and accounts for approximately 1-2\% cell mass.\textsuperscript{65} The 26S complex is composed of a barrel-shaped 20S proteasome and a 19S regulatory particle associated with one or both ends of the 20S. Because of the presence of many deubiquitinating enzymes (DUBs) in the cell which belong to either cysteine-protease or metalloprotease families and are capable of reversely modifying the ubiquitinated protein by cleaving the ubiquitin chain on substrate proteins, ubiquitination is considered a reversible posttranslational modification.\textsuperscript{66} Aberrations in protein homeostasis (synthesis and degradation) cause cellular anomalies and even cell death, and is an underlying cause of a plethora of diseases.\textsuperscript{67}

### 3.1 Cullin-RING ligases, the largest family of ubiquitin E3 ligases

The ubiquitin E3 ligase confers the vital role of selecting which substrates to ubiquitinate and therefore, as a rate-limiting step has proven to play a pivotal role in the degradation system and subcellular signaling cascades in eukaryotes.\textsuperscript{68} Hence, malfunctioning E3 ubiquitin ligase tend to inflict adverse effects on human health. Based on the structure, chemistry, and
mechanism, the E3 ligases are divided into three major categories: the RING (Really Interesting New Gene)-finger E3 ligases, HECT (Homologous to E6-associated protein carboxyl terminus) domain E3 ligases, and the U-box domain proteins. While RING finger motif containing E3 and U-box E3 acts as scaffold to bring the ubiquitin-charged E2 to the substrate, the activated ubiquitin is transferred from the E2 to a cysteine residue of the HECT domain E3 before the conjugation of ubiquitin to the substrate.\textsuperscript{69,70}

RING-finger E3 ligases, characterized by the RING domain are the largest family of ubiquitin ligases. The members of this family can function as monomers, dimers, or multi-subunits complexes. Cullin-RING ligases (CRLs) are multi-protein complexes belonging to the RING E3 ligase category and are implicated in regulation of a diverse array of eukaryotic function.\textsuperscript{71} Human genomes encode 7 cullins (Cul1, Cul2, Cul3, Cul4A, Cul4B, Cul5, and Cul7), which form ~300 distinct CRL complexes in different subfamilies. All CRLs share a similar core structure where the cullin serves as the central scaffold that incorporates a C-terminal portion to recruit RING-box protein which in turn interacts with an E2. The N-terminal portion binds to an adaptor protein that interacts with variable substrate receptors, specific to cullin class.\textsuperscript{72} Accordingly, each cullin can assemble with numerous substrate receptors to recruit different substrates and despite the considerable diversity, all classes of CRLs share a similar regulatory machinery.

The archetypical CRL the SCF (Skp1-Cul1-Fbox) complexes or CRL1 E3 ligase is the most well characterized CRLs. In CRL1, cullin 1 serves as the scaffold; the amino terminus of Cul1 is associated with Skp1 which in turn binds to the variable F-box proteins for substrate recruitment whereas the carboxyl terminus binds to RING-finger protein, Rbx1 which can interact with a specific ubiquitin-charged E2 enzyme. The F-box proteins contain the substrate-
binding motif which specifically binds to the substrates, which confers CRL1 E3s the substrate specificity.\textsuperscript{73}

\section*{3.2 Activation of CRLs by cullin neddylolation}

The neddylolation pathway is highly conserved from yeast to humans. Neddylolation is a ubiquitination-like post translational modification that conjugates Nedd8, a ubiquitin-like protein, to target proteins. It is executed by a cascade of enzymatic reactions analogous to ubiquitination but relies on its own E1, E2 and E3.\textsuperscript{74} Neddylolation begins with the activation of the mature Nedd8 by the NEDD8-activating enzyme E1 (NAE), through forming an NAE-S\textendash Nedd8 thioester-bond in an ATP-dependent manner, which is then transferred to a Nedd8-conjugating enzyme E2, Ubc12 through a trans-thiolation reaction and finally, Nedd8 E3 ligase interacts with the Nedd8 charged E2 which subsequently transfers Nedd8 to the substrate.\textsuperscript{75} Nedd8 molecule is linked to its substrates by formation of an isopeptide bond between its C-terminal and the $\varepsilon$-amino group on the side chain of a lysine residue of the target protein.

The best characterized substrate for neddylolation is cullin in the CRLs. Nedd8 conjugation with the lysine residue at C-terminal side of cullin proteins change the conformation of cullins, an essential mechanism regulating the activity of CRLs. CAND1, a substrate receptor exchange factor (SREF) binds the unmodified SCF complexes and promotes the dissociation of Cul1 and Skp1. The SREF activity of CAND1 is regulated by Nedd8. The conjugation of Cul1 to Nedd8 causes the dissociation of CAND1 from Cul1, thereby promoting the binding between cullin and substrate receptors.\textsuperscript{76,77} Neddylolation of cullin down the line activates the E3 ligase which suggests that Nedd8 could potentially also facilitate the positioning of the E2 enzyme and
subsequent ubiquitin transfer. Once Nedd8 is removed, the CRL complex is susceptible to the potent SREF activity of CAND1.78

Neddylation is counter-balanced by the action of deneddylases via a process known as deneddylation. Cullin deneddylation inactivates the E3 ligase and also allows CRLs to be partially disassembled which are then recycled to form a new CRL with different substrate receptor.79 Deneddylation of CRLs is mediated specifically by the COP9 signalosome (CSN).80-82

4. The COP9 Signalosome

The COP9 (COconstitutive Photomorphogenesis 9) signalosome (CSN) is a highly dynamic multiprotein complex consisting of 8 canonical unique protein subunits termed CSN1 through CSN8 in a holocomplex. Originally discovered in *Arabidopsis thaliana* as an essential regulator of light-mediated development, the CSN has been found to be evolutionary conserved from plants to mammals. Because of the similarity in electron microscopic morphology and domain structure, the CSN is often considered a paralogue of the lid complex of the 26S proteasome and has been found highly homologous to eukaryotic initiation factor 3 (eIF3).83,84,85 Among the 8 CSN subunits, 6 CSN subunits (CSN1, CSN2, CSN3, CSN4, CSN7, CSN8) contains the proteasome lid-CSN-initiation factor (PCI) domain characterized by a helical repeats followed by winged-helix subdomain. The remaining two, CSN5 (also known as Jab1) and CSN6 are the 1 Mpr-Pad1-N-terminal (MPN) domain containing subunits.

4.1 Deneddylation

The CSN regulates the CRLs, the largest family of ubiquitin ligases, and exerts its function by catalyzing the removal of NEDD8 from the CRL Cullin subunit, a function known as
deneddylation. Hence, the CSN is critical in regulation of CRL-dependent ubiquitination and a vital regulator of the UPS.\textsuperscript{79} The enzymatic activity of the CSN is harbored in CSN5 and is mediated by the zinc containing JAMM (JAB1-MPN-domain metalloenzyme) motif of this subunit.\textsuperscript{86} The discovery that the CSN copurifies Cul1 subunit of SCF isolated from animal cells and triggers changes in Nedd8 modification has provided an important hint to the biology of the CSN.\textsuperscript{87} However, although the catalytic activity resides in CSN5, fundamentally, Cullin deneddylation requires all 8 canonical subunits of the CSN.\textsuperscript{79} The active site of CSN5 is autoinhibited when alone and only exerts the deneddylation activity when its congregated into the fully assembled CSN holocomplex composed of all 8 CSN subunits. Downregulation of even one of the subunits of the complex has been shown to deplete other subunits to various degree and impair the deneddylation activity of the CSN.\textsuperscript{88,89} The CSN has been recognized to play a vital role in a wide variety of cell processes and signaling pathways like cell cycle progression, DNA repair, nuclear export, and immune response.\textsuperscript{84,90,91}

4.2 The CSN: beyond deneddylation

Interestingly, in fact, the first discovery of CSN5 as reported by Claret and colleagues identified CSN5/JAB1 as a coactivator that binds to c-Jun and increases the specificity of Activator protein 1 (AP-1) transcription factors.\textsuperscript{92} CSN5 effects on c-Jun pathway was reassessed once it was discovered that it is a CSN subunit. This ultimately led to discovery of the serine/threonine kinase activity associated with the CSN; and the first of these was identified to phosphorylate inositol trisphosphate 5/6 kinase and IκBα.\textsuperscript{93,94} Curcumin, which is known to have anti-tumorigenic and anti-angiogenic properties, was able to effectively suppress this activity's specificity for c-Jun and IκBα defined signalosome-dependent phosphorylation.\textsuperscript{95} In HeLa cells,
de novo CSN synthesis brought about by excessive CSN2 production was shown to stabilize c-Jun and boost AP-1 activity. These findings revealed the presence of a COP9 signalosome-directed c-Jun signaling pathway.\textsuperscript{93} Besides, the CSN-specific phosphorylation of the tumor suppressor protein p53 was found to promote the degradation of p53 by the UPS; inhibition of this phosphorylation, either by curcumin or a phosphorylated p53 peptide, stabilized p53 and resulted in accumulation of the endogenous protein \textit{in vivo}.\textsuperscript{96} It has been suggested that in order for substrates of the COP9 signalosome kinase activity to be phosphorylated, they must attach to one of its subunits.\textsuperscript{96,97} In addition, studies over the past years have shown that the CSN is associated with many other protein kinases such as Protein kinase D, casein kinase 2, and phosphoinositide 3-kinase.\textsuperscript{98} Interestingly, CSN subunits can be phosphorylated by associated or non-associated kinases.

Furthermore, the CSN can also mediate deubiquitination. This action, which is mediated by the CSN-associated Ubp12/USP15, has been hypothesized to stabilize E3 ligase subunits by inhibiting promiscuous autoubiquitination.\textsuperscript{99} This was recently demonstrated to extend to E3 ligase substrates, whose stability is reliant on the CSN. Following signal-dependent IκB\textit{α} degradation and subsequent nuclear localization of NF-κB, CSN-dependent USP15-mediated deubiquitination was found to stabilize synthesized IκB\textit{α}, reducing NF-κB activity.\textsuperscript{100,101}

4.3 The postulated existence of CSN minicomplexes and free CSN subunits

Studies mostly \textit{in cell cultures} have suggested that CSN subunits may exist in two different forms, a holocomplex consisting of all the 8 subunits and mini complexes which are composed of reduced number of canonical CSN subunits or even free CSN subunit.\textsuperscript{102} Initially, the minicomplex theory came from an observation in \textit{S. pombe}, where knockout of CSN1 and
CSN2 but not of other subunits resulted in delayed S-phase of the cell cycle leading to an assumption of viable smaller complexes of the CSN but the complex was never identified.\textsuperscript{103} Likewise, \textit{in vitro} mass spectrometry from recombinant human CSN or expression in a bacterial reconstitution system further suggested a subset of symmetrical mini complexes namely CSN1/2/3/8 and CSN4/5/6/7 but failed to be detected in cells.\textsuperscript{104} However further interpretation by glycerol-gradient pulldowns showed the existence of CSN4-8 minicomplex mostly localized in cytoplasm in proliferating fibroblast.\textsuperscript{105} Similar observation was later made in mouse embryonic fibroblasts (MEFs) and HEK 293 cells where CSN5 proteins were found as both component of the CSN complex and also outside of the holocomplex.\textsuperscript{88,106}

The idea of CSN5 existing outside the holocomplex was further corroborated by a study by Peth \textit{et al.} in HeLa cells which showed that the knockdown of CSN1 and CSN3 reduced the steady level of other subunits as well as CSN holocomplex, downregulation of CSN5 on the other hand did not affect the holocomplex nor the abundance of other CSN subunits suggesting that maybe the unique periphery nature of CSN5 allows the subunit to also exist in free forms.\textsuperscript{107}

Though a definite route and assembly mechanism of minicomplex is not quite clear yet, it is conjectured that the formation of the minicomplex requires the destruction of the fine-tuning mechanism of the holocomplex or the disintegration of holocomplex in general. Another speculation that exists is that since all the subunits are initially produced in free form before they assemble into the holocomplex, it is conceivable to hypothesize that the formation of minicomplex happens during such assembly. The significance of CSN/CSN5 minicomplex or free CSN has not been established in animals and it is yet to be understood if they play a role in any (patho)physiological condition. Thus, the understanding of these minicomplex or free CSN subunits remains largely elusive.
4.4 Function linked with CSN minicomplexes

The CSN minicomplex integrates only a subset of CSN subunits, it is thus considered incapable of compensating for the function of the holocomplex. CSN5 alone does not have affinity for the NEDD8 and is inefficient in processing its precursor form. In line of that, free CSN5 is actually unable to cleave the isopeptide bond between cullin and Nedd8. Further, adding free CSN5 to native CSN complex does not impact the CSN deneddylation activity but however, binding of free CSN5 interfered the essential cellular processes. Besides, while overexpression of CSN5 has been reported in multiple cancer types, it is unclear if the increase is characterized by the free CSN5 or the CSN holocomplex.

In proliferating fibroblasts, CSN4-8 minicomplex were reported to be involved in the downregulation of p27. Another study by Yoshida et al. showed that in NIH3T3 cells free CSN5 functions through CDK2 and is involved in regulation of premature senescence. Possibly, the propensity of the CSN complex to change and adapt its subunit composition might underlie its ability to perform multiple functions.

5. Balance between Neddylation and Deneddylation is Required for Cellular Homeostasis

Defects in protein quality control are increasingly being recognized as prominent pathogenic factors in many forms of disease. Functional stability of proteins involved in the post-translational modification is pivotal to maintaining proteome homeostasis. Neddylation has emerged as a key player in control of cellular functions, a major regulatory pathway for ubiquitination and hence, its importance in health and disease are being explored. Studies have suggested the role of Nedd8 in progression of many diseases and pathogenic condition such as atherogenesis, tumor metastasis, neurodegenerative disease, and CVD. Dysregulation of
neddylation and overexpression of enzymes implicated in the neddylation pathway are closely related with occurrence of diseases mainly, tumors and correlates with the disease progression and poor patient survival.\textsuperscript{115,116} Thus, the overactivated Cullin neddylation pathway has emerged as a promising target for anti-tumor strategy.\textsuperscript{117} MLN4924, also known as pevonedistat, is a first-in-class molecular inhibitor of NAE. It covalently binds with NEDD8 forming an irreversible adduct which remains tightly bound to NAE and thus NAE is unable to process CRL conjugation preventing cullin neddylation, leading to accumulation of CRLs substrates and triggering cellular responses such as cell cycle arrest, apoptosis, senescence, and autophagy in a cell-type specific manner.\textsuperscript{118-121} Indeed, MLN4924 has been evaluated in Phase I/II clinical trials for patients with advanced nonhematological malignancies, acute myeloid leukemia, and myelodysplastic syndromes.\textsuperscript{122-124}

Theoretically, it was considered that neddylation activates while deneddylation by CSN inhibits the CRLs function by promoting the cleavage of NEDD8-CUL1 conjugates. However, this paradox was refuted; studies have suggested that in fact deneddylation by the CSN protects the CRL component from auto-ubiquitination and aids in the recycling of the CRLs receptor component.\textsuperscript{125,126} Therefore, the CSN fundamentally is responsible for regulating cullin-based E3 ligase mediated responses. The unneddylated cullin-RBX1 heterodimer can bind to the adaptor-substrate receptor (SR) module or the paralogous regulatory factor CAND1/2; and once there is a substrate to be degraded, deneddylate cullin-RBX1 heterodimer would exchange CAND1/2 for an adaptor-SR module. This newly formed CRL goes into a cycle of neddylation-deneddylation on cullins; cullin neddylation activates the SCF complex. The CSN would transform the neddylated CRL into a deneddylated one to inactivate it, while the deneddylated CRL returns to be bound to the CAND1/2, until the next cycle with a different adaptor-SR module.\textsuperscript{127-129} Hence,
though the CSN biochemically inhibits CRL activity, genetically it is indispensable for normal CRL functions.

Consistent with this notion, it was reported that the deneddylose CSN is essential for the integrity of the structure and function of postnatal hearts in mice. Perinatal cardiomyocyte-restricted loss of CSN8, the smallest subunit of the CSN led to an accumulation of neddylated cullins and many non-cullin proteins as well as premature death of neonatal mice, confirming an essential role of CSN8/the CSN in deneddylation and perinatal cardiac development and functioning. Similarly, in adult mouse hearts, impaired deneddylation caused by the cardiac loss of CSN8 resulted in rapid heart failure and premature death. Persistent neddylation of cullins led to destabilization of CRL components and accumulation of their substrates in non-cardiac cells as well. In addition, loss of CSN8 reduced the expression of the mRNA or protein levels of several F-box genes.

In line with this, perturbation of the ratio of cellular CSN/CAND1 showed impaired plasticity and exacerbated CRL auto-degradation. Moreover, different ratios of CSN/CAND1 differentially affected the cell specific regulation of proteolysis. Overall, studies suggest that a tight balance between neddylation and deneddylation is crucial for maintenance of normal homeostasis.

6. The Potential Role of the CSN in VSMC Proliferation

The cell cycle reentry is critical for the phenotyping switching of VSMCs. Thereby, CDKs and CDKIs as key regulators in cell cycle control are pivotal players in this proliferative process. When we talk about the CSN and VSMC proliferation in the big picture, the understanding is obscure. However, evidence has suggested that the CSN is a critical regulator of
cell cycle; the corroboration for the regulation comes mostly from the studies in non-vascular cells, where the CSN has been shown to both positively and negatively regulate the cell cycle.

Arguably, the best studied association of the CSN in cell proliferation is the regulatory role of CSN5 in p27 degradation. CSN5 promotes the degradation of p27 and thereby is a negative regulator of p27. Overexpression of CSN5 caused the accelerated degradation of p27 in mouse fibroblasts. Moreover, it was reported that CSN5 contributes to p27 degradation by mediating the p27 shuttling between the nucleus and cytoplasm in a CRM1 dependent manner. However complicating the interpretation, subsequent work showed that in proliferating fibroblasts the downregulation of p27 was mediated by CSN4-8 minicomplex primarily located in the cytoplasm. Shedding more light on the independent function of CSN5, Yoshida et al. showed that free CSN5 could interact with CDK2 and ectopic expression of CSN5 could rescue NIH3T3 cells from p27 induced cell cycle arrest. Similarly, in NIH3T3 cells, overexpression of CSN6 released the cells from p57 induced G1 arrest.

In HTC116 and HEK293T cells, depletion of CSN6 resulted in reduction of E3 ligase, COP1 and elevated the level of p27. Likewise, knockdown of CSN4 or CSN5 reduced the S phase entry in HeLa and HEK293T cells and in thymocytes knockdown of CSN5 arrested the cell cycle in S/G2/M phase. Providing more evidence on the role of the CSN in proliferation, deletion of CSN subunits was found embryonically lethal at least in part due to impaired cell proliferation. In HeLa cells, reduction of CSN5 caused a marked attenuation of cell proliferation whereas the reduction of CSN8 in the same cell accelerated the cell growth. Similarly, CSN8 hypomorphic MEFS exhibited a more robust increase in cell proliferation. On the other hand, conditional knockout of CSN8 in postnatal hepatocytes caused massive hepatocyte apoptosis at baseline and impaired hepatocyte proliferation upon liver injury. Collectively, these studies
suggested that the CSN can both promote and inhibit cell cycle, presumably the net effect is CSN subunit-specific and tissue/cell type specific.

SCF, a subfamily of CRLs which is in part controlled by the CSN, is known to be involved in the control of cell cycle by regulating the ubiquitination and degradation of CDKIs. Substantial evidence shows that an SCF complex containing the F Box protein SKP2 (SCF^{Skp2}) contributes to the regulation of VSM proliferation by controlling the turnover of CKDIs. Skp2, the substrate recognition component of SCF^{Skp2} has been shown to recognize p27,\textsuperscript{141,142,143} p27 related p21,\textsuperscript{144} and p57.\textsuperscript{145} Overexpression of Skp2 in rat aortic SMCs triggered a reduction in p27 and increased the proliferative indices of these cells.\textsuperscript{146} Conversely, suppression of Skp2 in rat thoracic aorta smooth muscle resulted in an increase in p27.\textsuperscript{147} In quiescent VSMCs, both Skp2 and p27 are expressed in a low level.\textsuperscript{148} Vascular injury (balloon injury) that promotes VSMC proliferation caused an increase in the level of Skp2 that correlated with the reduction in p27;\textsuperscript{149} on the other hand, Skp2 null mice exhibited reduced neointima thickening in response to vascular injury.\textsuperscript{150}

Another piece of indirect evidence for the association of the CSN in VSMC proliferation could come from the role of CSN5 as a coactivator that binds to c-Jun and increases the specificity of AP-1 transcription factors. AP-1 regulates the inflammatory gene expression in response to various stimuli including cytokine and growth factors; and activation of AP-1 has been observed in VSMCs proliferation \textit{in vitro} and in response to arterial injury \textit{in vivo}.\textsuperscript{151,152} Knockdown of AP-1 inhibited the proliferation of cultured rat SMCs,\textsuperscript{153} and transfection of AP-1 decoy in rat carotid arteries suppressed NH after vascular injury.\textsuperscript{152}

In summary, multiple control points of regulation between CSN and cell proliferation have been studied; and the findings of these studies together convincingly indicate a pivotal role
played by the CSN in cell proliferation. Though the direct evidence is still missing, the current
studies and findings are sufficiently intriguing and encourage more mechanistic investigation
into the regulation of VSMC proliferation by the CSN.

7. A Need to Better Understand Neointimal Hyperplasia

Regarding 82.6 million people live with one or more forms of CVD, and it continues
to be the leading cause of death, accounting for nearly 17% of death annually. VSMC
contribution to the pathology of CVD and its vascular remodeling characteristics mainly arise
from extensive hyperproliferation of VSMCs, a hallmark of NH.

One of the most frequent pathogenic mechanisms of CVD is atherosclerosis, a chronic
inflammatory disease where arteries become clogged and narrower with cholesterol plaques
causing them to lose their elasticity. Atherosclerosis in the coronary arteries has attracted
rightfully more attention because it is a major underlying cause of heart attack, but it can affect
most of other arteries in the body as well. Over the years, surgical procedures to open the
clogged arteries like percutaneous transluminal intervention have been established for the
symptomatic treatment of atherosclerosis. However, a long lasting success and benefit of these
procedures is drastically limited by the process of NH, which is the most common cause of
failure to maintain long-term patency. The recurrence rate of the disease after the surgical
intervention remains at 20% within the first 12 months, which varies between different vascular
regions.

NH limits all forms of vascular grafts including both venous and prosthetic conduits used
in arterial bypass and arteriovenous fistulae (AVF) created for hemodialysis access. Vascular
access remains the lifeline for the hemodialysis patient. Over the last decades, advancement
in surgical techniques and vascular grafts have permitted the reconstruction of large and small
vessels. However, millions of these bypass grafts fail due to vascular remodeling, the narrowing of the lumen caused by NH, constituting a real burden for the healthcare system. It is reported that the patency rate of venous graft is 80% in one-year, lowers to 60% at five years; and NH is more prominent in 30-60% of AV graft failure following vascular thrombosis. In addition, when a bypass fails, blood supply becomes much worse than before the bypass surgery. In a clinical landscape with ever increasing and more aggressive bypass procedures, improving our understanding of the molecular and cellular mechanisms governing arterial remodeling is absolutely necessary and expected to facilitate the search for more effective measures to prevent or better treat these complications.

Revascularization procedures are one of the greatest achievements in interventional cardiology but are hampered by the occurrence of restenosis, which remains a major clinical problem. For example, carotid endarterectomy (CEA) is a procedure to remove build-up of fatty deposits to prevent the narrowing of carotid artery. CEA provides a means of restoring blood flow to affected area and reduces the incidence of subsequent stroke. Nevertheless, operative repair for this procedure does not come without complications, one of which is the occurrence of restenosis caused by NH. Further, failure of carotid reconstruction due to NH can present with transient ischemic attack or postoperative cerebrovascular deficits or stroke symptoms. Similarly, in percutaneous coronary interventions such as stenting or transluminal angioplasty, NH presents itself as one of the major causes of the failure of the surgical procedures. Coronary stents prevent constrictive arterial remodeling but stimulate NH in the regions next to both ends of the stent. Therefore, reduction in the restenosis after stent placement is required to prevent or reduce recurrent ischemic events and improve the clinical outcomes, which necessitates the development of new approaches to inhibit NH.
Pulmonary arterial hypertension (PAH) is a fatal vasculopathy where the pulmonary vasculature undergoes remodeling causing progressive increases in pulmonary artery pressure and resistance.\textsuperscript{167} PAH lacks effective clinical treatment beyond lung transplantation.\textsuperscript{168} All forms of PAH in its more severe form demonstrate the formation of neointima lesions, eventually occluding the vessel lumen and leading to right heart failure.\textsuperscript{169-171}

NH is an important clinical entity in vascular surgery. Many vascular interventions put the vessels at risk of injury leading to the activation of inflammatory cascades and cellular environment and ultimately leading to restenosis. Therapeutic targeting of VSMC phenotypic modulation is critical to combating the massive burden of CVD on human health. Although several approaches have been used to prevent or mitigate VSMC phenotype change, they have limitations. Lack of understanding of the mechanism of this proliferative process limits the ability to fully intervene this dysfunction. Therefore, an improved understanding of the molecular mechanisms governing NH would be extremely important to unveil new molecular targets for prevention and intervention.
8. Hypothesis and Specific Aims

The overarching goal of this project is to understand the role of the CSN in VSMC proliferation and NH. VSMC proliferation plays a predominant role in NH; NH remains a prevailing clinical problem globally. The isopeptidase activity of the CSN resides in CSN5; but the de neddylation function requires formation of the CSN holoenzyme consisting of all the 8 canonical CSN subunits. Studies mainly using non-vascular cells have demonstrated that the CSN/CSN5 is a critical regulator of the cell cycle. In line with that, the significance of the CSN5 has been very well-studied in cancer and the CSN de neddylase inhibitor has shown very promising antitumor effects. Moreover, it is essential to note that studies using cell cultures have suggested the existence of CSN minicomplexes or free CSN subunits, which may have de neddylation independent functions; however, not only this remains to be established in animals but also the pathophysiological significance of these minicomplexes is yet to be demonstrated. As such, the role of the CSN in VSMC proliferation and NH remains obscure.

Indeed, my preliminary results indicate that the steady state protein and mRNA levels of CSN subunits were increased following vascular injury, suggesting that the homeostatic level of CSN is altered during vascular remodeling and the CSN may play an important role in NH. Moreover, at least CSN5 was found to be increased in the NH VSMCs of both an animal model and human tissues. Thus, this study is aimed to test the hypothesis that both CSN de neddylation and CSN5-minicomplex mediated nuclear export play an important role in VSMC proliferation and NH via the pursuit of following two specific aims:

AIM 1: To determine the necessity of vascular smooth muscle CSN5 in the control of neointimal hyperplasia.
This is to test the hypothesis that CSN5 in VSMCs is increased upon vascular injury and required for the injury triggered VMSC proliferation and NH. Smooth muscle cell-restricted ablation of the CSN5 gene (CSN5-SMKO) induced in adult mice will be used for assessing the impact on NH. Left common carotid artery (LCCA) ligation will be used for modeling in vivo vascular injury and induce NH. Further pharmacological interrogation specific to CSN deneddylase function will be employed to understand the necessity of CSN5 in NH and VSMC proliferation.

**AIM 2: To establish the mechanistic involvement of the CSN5 minicomplex in neointimal hyperplasia.**

Using a genetic model of CSN8 downregulation where the CSN holocomplex is diminished resulting from reduced CSN8 proteins but the CSN5 minicomplex is not, we sought to unveil the role of CSN5 minicomplex in the VSMC remodeling process. The CSN8 hypomorphic mice serve as the genetic model for in vivo CSN8 downregulation. Both in vivo and in cellulo approaches will be employed to test the role of CSN5 minicomplex in injury-triggered VSMC proliferation. VSMCs from CSN8 hypomorphic and control mice will be used in cell culture experiments, in which CSN5 nuclear exclusion and CSN5-dennedylase activity will be interrogated using both genetic and pharmacological methods. Injury-induced VSMC proliferation in cellulo will be simulated by treating the cultured cells with platelet-derived growth factor BB (PDGF-BB), a cytokine known to participate in vascular response to injury.
Figure 1. An illustration of the central hypothesis for AIM 2.
9. Significance of the Study

Vascular disease is the dominant component of CVD, and adaptive and maladaptive remodeling of the vascular wall represents key processes in vascular disease. VSMC proliferation and resultant NH limit seriously the long-term effectiveness of surgical and endovascular intervention and are considered a culprit lesion and basic etiology of occlusive vascular diseases. However, the mechanisms involved in this proliferative process remain to be fully elucidated, which limits the ability to effectively intervene this dysfunction. The CSN by virtue of its ability to control CRL-regulated ubiquitination, is suggested to play an important role in modulation of CDK inhibitors. In addition, studies in cultured cells have postulated that each or some of the CSN subunits may possess deneddylation-independent function. Despite many lines of evidence linking the CSN to cell cycle regulation, no reported studies have determined the regulatory role of the CSN in VSMC proliferation or the significance of CSN minicomplexes in animals. Through both in cellulo and in vivo approaches, this study has tested the novel hypothesis that both CSN deneddylation and CSN5 minicomplex-mediated nuclear export mediate the promotion of VSMC proliferation and NH by CSN5 after vascular injury. Methodologically, this study represents the first to use the novel mouse model of SMC-restricted CSN5 knockout. The knowledge established from this study will not only improve our understanding on the biology of the CSN in VSMC proliferation and NH but also provide new information essential to the development of new strategies for selective intervention of VSMC proliferation in various disease states, such as restenosis, atherosclerosis, and pulmonary hypertension. Indeed, our data compellingly unveil the CSN as a major regulator of vascular remodeling after injury and identify a novel strategy for NH prevention and treatment.
MATERIALS AND METHODS

Animal models

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Dakota and conform to the NIH Guide for the Care and Use of Laboratory Animals.

Smooth muscle cell-restricted CSN5 knockout mice

CSN5 (also known as JAB1) is encoded by the Cops5 gene (COP9 Signalosome Subunit 5) localized at chromosome 1 in mouse and chromosome 8 in humans. Mouse with a Cops5-floxed allele contains exon 2 flanked by 2 LoxP sites inserted in the introns sandwiching exon 2. Cre-mediated recombination effectively removes exon 2, resulting in an early frameshift and translation termination. The Myh11-CreERT2 transgenic mouse was obtained from Jackson Laboratory, # 019079 (B6.FVB-Tg (Myh11-icre/ERT2)1Soff/J). The generation of CSN5^floxed/floxed::Myh11-CreERT2 was achieved though crossbreeding Myh11-CreERT2 transgenic mice with CSN5^floxed/floxed in C57BL/6 genetic background. Further, to achieve homozygous smooth muscle-restricted CSN5 knockout (CSN5-SMKO) in adult mice, we treated CSN5^floxed/floxed::Myh11-CreERT2 mice with tamoxifen (intraperitoneal injection), with the Myh11-CreERT2 mice as a control. The mice were routinely checked after the inducible knockout. The Myh11-CreERT2 transgenic mouse harbors in its Y-chromosome a transgenic cassette that expressed a mutant estrogen receptor sandwiched Cre recombinase (CreERT2) under the control of the promoter of the myosin heavy chain 11 (Myh11); the latter is SMC-specific. Hence, limited by the Myh11-CreERT2 transgene insertion location (Y chromosome), only male mice
were suitable for this part. So far, no reported studies have investigated CSN5\textsuperscript{flox/flox::Myh11-CreERT2}.

**CSN8 hypomorphic mice**

Generation of CSN8/Cops8 targeted alleles has been previously described\textsuperscript{89}. Briefly, Cops8\textsuperscript{neoflox} allele contains a neomycin resistant cassette in intron between exon 3 and 4 while CSN8 knockout allele (CSN8\textsuperscript{-}) has a deletion of exon 4 to 6. The C57BL/6J Cops8\textsuperscript{neoflox/+} and Cops8\textsuperscript{+/-} mice were backcrossed into FVB/N background for at least six generations. The homozygous Cops8\textsuperscript{neoflox/neoflox} mice were then mated with Cops8\textsuperscript{+/-} mice to produce Cops8\textsuperscript{neoflox/-} and Cops8\textsuperscript{neoflox/+} mice, which were used as CSN8 hypomorphic mice and control mice, respectively. The neomycin gene inserted in an intron of a CSN8-floxed allele (CSN8\textsuperscript{neoflox/+}) reduces CSN8 gene expression but this reduction of CSN8 gene expression does not cause CSN8 protein reduction until the other CSN8 allele is deleted (CSN8\textsuperscript{neoflox/-}); hence, the CSN8\textsuperscript{neoflox/-} mice have been confirmed as CSN8 hypomorphic (CSN8\textsuperscript{hypo}) and the littermate CSN8\textsuperscript{neoflox/+} mice were used as controls (CTL). Hypomorphic mice were viable, fertile, normal in size and did not display any gross physical or behavioral abnormalities\textsuperscript{177}.

**Tamoxifen administration for smooth muscle cell restricted CSN5 knockout mice (CSN5-SMKO).**

To achieve homozygous CSN5-SMKO in adult mice, we treated CSN5\textsuperscript{flox/flox::Myh11-CreERT2} mice with tamoxifen (Catalog\#HY-70062; MedChem Express, Monmouth, NJ) with the Myh11-CreERT2 mice as a control. Tamoxifen was dissolved in 100% ethanol through
extensive vortexing and mild heating (<40°C) to solubilize completely. The tamoxifen-ethanol solution was mixed with autoclaved sunflower oil at the final concentration of 10µg/ul and stored in light blocking vessel. The tamoxifen-oil mix (1mg tamoxifen/mouse) was intraperitoneally (IP) injected in adult mouse. Each animal went through 2 rounds of IP for 5 days with 2 days break between, with total of 10 injections. Mice were closely monitored for any adverse reaction to the treatment. After the last IP, we waited for at least 2 weeks to wash off the side-effects of exogenous tamoxifen as previously described.178

Ligation of the left common carotid artery (LCCA)

To produce NH in vivo, LCCA ligation was performed on adult mice as described.179 Briefly, mice were anesthetized using 2.5% isoflurane, the left carotid bifurcation was located and then ligated with 6.0 silk suture (Catalog#XS-N618R11, AVTEC Surgical LLC, Mount Pleasant, SC) immediately approximal to the bifurcation. The mice were monitored until recovery and buprenorphine SR (1.0 mg/kg, subcutaneous injections) was used as an analgesic. Animals were then observed each day until tissue collection for pain or any health-related problems. After either 4 or 1 week after ligation, the mice were sacrificed and the ligated LCCA section was collected for further processing. The right common carotid artery (RCCA) was used as the uninjured intra-animal control vessel.

Cell culture

VSMCs were enzymatically isolated from abdominal aortas of adult mice as described.180 Briefly, mice were euthanized using a continuous flow of carbon dioxide and the skin was
opened to expose the abdomen and thorax using surgical scissors. The entire abdominal aorta up
the renal bifurcation was dissected and cleaned off the surrounding fat and adventitial tissue. The
cut pieces of aorta were digested in collagenase II for 6 hours at 37°C and then were grown in
DMEM supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution at
37°C in a humified atmosphere of 5% CO2. VSMCs at passage 3-6 were used for all
experiments. Before each experiment, the cells were serum starved and then treated with PDGF-
BB, 10 ng/ml (Catalog#220-BB; R&D biosystems, Minneapolis, MN) or the vehicle control,
PBS- without calcium and magnesium (Catalog#55-031PC, Corning, Glendale, AZ).

Immunohistochemical assessment of CSN5 in human pulmonary arteries

Human lung tissue samples were taken from archived surgical pathology paraffin
embedded tissue acquired as standard of care. The control tissue represents sections of normal
segment of lung tissue taken during surgical resection of lung tumors. The PAH tissue was
acquired from explanted lungs from patients with idiopathic PAH undergoing transplantation.
The demographic information of the patients is shown in Table 2. Immunohistochemistry
detection was performed with VitroView Universal 1-step polymer-based IHC/DAB kit
(Catalog# VB-6023D, VitroVivo Biotech, Rockville, MD) as per manufacturers instruction with
minor optimization. Paraffin embedded sections were deparaffinized with xylene (3X5min) and
rehydrated through series of ethanol (100% ethanol for 2X2mins, 95% ethanol for 2x2 mins,
70% ethanol for 2 mins and 50% ethanol for 2 mins) prior to histological staining. Antigen
retrieval was performed by microwaving the sections in citrate buffer (10 mM, pH 6) with 0.05%
Tween-20 for 15 mins. Paraffin sections were then permeabilized using 0.3% Triton X-100 for
10 mins, washed with 1XPBS, and blocked for non-specific binding of immunoglobulin in normal
Protein extraction and western blot analysis
The surrounding adventitia of the collected carotid arteries was gently removed. Collected arteries or cultured VSMCs were lysed in lysis buffer (41 mM tris-HCl, 1.2% SDS, and 8% glycerol), subsequently sonicated for about 3-4 seconds, about 2-3 times at the power of around 180 watts, incubated in 100°C boiling water for 5 mins and centrifuged at 10,621 x g for 10 mins at 4°C. The supernatant was collected for western blot analyses and protein concentration was quantified using the Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (Catalog#23225, ThermoFisher scientific, Waltham, MA). Equal amounts of protein were loaded in SDS-polyacrylamide gel (8-16%), transferred to polyvinylidene difluoride (PVDF) membrane, and incubated with primary antibodies against the protein of interest, overnight at 4°C. The incubated PVDF membrane was washed (1X PBST) to remove unbound primary antibodies, followed by the incubation with horseradish peroxidase (HRP) conjugated secondary antibodies and again, washed (1X PBST) to remove unbound antibodies. The bound secondary antibodies were detected using the enhanced chemiluminescence detection reagents. Blots were imaged by ChemiDoc MP Imaging System (Bio-Rad) and quantified using the Image Lab software (Bio-Rad). Either anti-GAPDH or the total protein content derived from the stain-free protein imaging technology was used as in-lane loading control. The antibodies used are detailed in Table 1.

**Cytoplasmic and nuclear fractionation**

Cytoplasmic and nuclear fractions were extracted using the Epiquik nuclear extraction kit (Catalog #OP-0002-1; Epigentek, Farmingdale, NY) according to the manual provided by the manufacturer. For tissues, 3 carotid arteries were combined per group. In brief, tissues or cultured cells were washed or collected using PBS and centrifuged to collect the pellet. The pellet was then suspended in cytoplasmic extraction buffer, incubated in ice for 10 minutes after
which it was vortexed vigorously for 10 sec and finally, centrifuged to collect the supernatant, cytoplasmic fraction. The remaining pellet was then washed with ice-cold 1X PBS two times, suspended in nuclear extraction buffer, and incubated on ice for 15 mins (vortexing the tube every 3 mins). The tube was then centrifuged for 10 mins at 20,817 x g and the supernatant, nuclear fraction was collected. Both cytoplasmic and nuclear fractions were stored at -80°C until further use. Anti-GAPDH and anti-Histone H3 were used as a marker for cytoplasmic and nuclear fractions respectively.

Native gel electrophoresis

Total protein isolated from carotid arteries, or the homogenates isolated using nuclear and cytoplasmic kit were used for running native gel. For the former, 2 carotid arteries were combined per group. The tissue was then homogenized on ice in an extraction buffer (50 mM Tris-HCL, pH 7.5, 1mM ATP, 5 mM MgCl2, 1 mM DTT, 250 mM Sucrose) and centrifuged at 4°C for 30 min (15000 x g). Protein concentration was determined with Pierce BCA Protein Assay Kit (Catalog#23225, ThermoFisher scientific, Waltham, MA) and the samples were diluted with 4X native gel loading buffer [200mM Tris-HCL, pH 6.8, 60%(v/v) glycerol, 0.05%(w/v) bromophenol blue]. Equal amounts of protein (20mg) were separated on a 4% gradient gels at 100V and 4°C in the SDS-free running buffer for about 3-4 hours at room temperature or at 4°C. Following, conventional western blot analysis was performed using antibodies specific for indicated CSN subunits.

RNA isolation, cDNA synthesis and quantitative PCR
Total RNA was extracted from ligated LCCA and unligated RCCA from wildtype mice using the TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The concentration of RNA was determined using Agilent RNA 6000 Nano assay (Agilent technologies Inc., Germany) following the manufacturer’s instruction. For reverse transcription (RT) reaction, 1 µg of RNA was used as a template to generate complementary DNA using the high-capacity cDNA reverse transcription kit (Catalog #4368814; ThermoFisher Scientific, Waltham, MA), and the RT was performed by following the manufacturer’s instructions. Transcripts of interest were visualized and quantified using conventional semi-quantitative PCR (RT-PCR) and quantitative real-time PCR (qPCR) with the SYBR-Green assay respectively. For the conventional RT-PCR, 2 µl of solution from the RT reaction and specific primers toward the target gene and GAPDH were used. The mRNA levels of the gene of interest were visualized by PCR at the minimum number of cycles (15 cycles) capable of detecting the PCR products within the linear amplification range.

Further, the relative quantification of mRNA expression was carried out using ~100ng of cDNA per reaction. qPCR was performed in technical duplicates in 20µl of reaction volume containing 200 nM of specific primers and 5 ul of SYBR using real-time detection system. Level of gene expression was normalized to internal control, GAPDH, calculated as ΔCt and ΔΔCt, and plotted as $2^{-ΔΔCt}$ (fold-change). Mouse CSN8, CSN5, CSN6 and GAPDH primer (5′-3′) sequences are listed below:

CSN8
Forward 5′-GTCAGTTGGACAGCGAATCT-3′,
Reverse 5′-CGTCTCCTTTHTTGCATCTCTAA-3′

CSN5
Histological processing and immunostaining of mouse carotid artery sections

Carotid artery sections for Hematoxylin and Eosin (H&E) staining were embedded in paraffin blocks. The tissue was cut to obtain 5 µm sections using a cryostat, placed on glass slides, air dried and heat fixed overnight. Sections were deparaffinized and rehydrated through xylene and series of decreasing alcohols. Following, the sections were stained with hematoxylin and rinsed with tap water. After counterstaining with eosin, the sections were dehydrated and mounted with xylene. The imaged sections were then visualized (Olympus IX71) and analyzed using Image J (https://imagej.nih.gov/ij/) for morphometric parameters. Immunohistochemical assessment of CSN5 in mouse tissue was processed similarly as of human paraffin lung sections as mentioned above. Immunofluorescence staining and confocal microscopy were performed on cryosections from ligated LCCA, unligated RCCA or cultured VSMCs. 4% paraformaldehyde-fixed VSMCs on cover glass or tissue cryosections (7µm) were washed thrice for 5 min with
PBS, incubated with 1% glycine in PBS for at least 30 min. The tissue or cells were incubated with 0.1% Triton-X-100 for 10-15 mins and subsequently, blocked with 2% BSA at room temperature for 1 h. Primary antibodies were then added to specimen and incubated overnight at 4°C. Unbound antibodies were removed via 5 min x 3 washes with room temperature 1XPBS before incubation with appropriate secondary antibodies at room temperature for 1 h. The specimens were then rinsed with PBS 3 times for 5 min. DAPI (Catalog#0100-20, Southern Biotech, Birmingham, AL) was used for staining nuclei. The stained sections were covered by glass coverslips, sealed with nail polish, and kept in -20°C prior to confocal imaging analyses. The fluorescence staining was visualized and imaged using a confocal microscope (Leica DMi8). Ligated and unligated carotid arteries were stained for CSN5 or CSN8 and SM22α to examine the expression of the CSN in neointima VSMCs. In addition, carotid arteries and cultured VSMCs were also stained for SM22α, DAPI and Ki-67. From each ligated LCCA, 5 sections were stained and the colocalization between the SM22α and Ki-67 with the nuclei (DAPI) in the intimal area was examined. Immunofluorescence imaging was collected and processed similarly in the experimental and control groups. To minimize non-specific binding, the primary antibodies (Table 1) was used at an optimized concentration for the immunostaining.

**Plasmids**

EYFP-JAB1 was a generous gift from Johannes A. Schmid (Addgene plasmid # 111213; http://n2t.net/addgene:111213; RRID: Addgene_111213). Plasmid amplification was carried out using maxiprep/midiprep/miniprep kit in accordance with the manufacturer’s instruction. Restriction enzymes, BamHI (Catalog# R0136S; New England Biolabs, Ipswich, MA) and HINDIII Catalog# R0104S; New England Biolabs, Ipswich, MA); and T4 DNA ligase (Catalog#
M0210S; New England Biolabs, Ipswich, MA) were used in accordance with the manufacturer’s protocol. Transformation into *E. coli* was achieved with Subcloning Efficiency DH5α chemically competent cells (Catalog# 18265017; ThermoFisher Scientific, Waltham, MA) according to manufacturer’s instruction.

The mutants were constructed by a three-step site specific PCR approach. To achieve the constitutive-nuclear CSN5, primers containing mutation sites against leucine residues at 237, 238, and 240 were strategized and converted to alanine. The primers were used at a final concentration of 200nM. In the first step, a mutagenic primer was used as the 3’ primer and external primer was used as the 5’ primers. The latter 5’ external primer contains the BamHI recognition site for cloning. This step produced a mutant product. In the second step, a second PCR was carried out with the mutagenic primer as the 5’ primer (complement of the first mutagenic region) and an external primer at the 3’ regions. The 3’ end contained the HINDIII recognition site for cloning. After each PCR step, the mutant product was run on an agarose gel to confirm the presence of desired base pair of PCR product. A fusion overlapping PCR was then carried out to combine the above two PCR products (200ng of each) with the end primers BAMHI and HINDIII in order to obtain the desired CSN5 mutant. The primers were added after nine cycles of fusion PCR in order to prevent the amplification of multiple PCR products due to traces of primers present in the template. The CSN5 mutant product was then TA cloned (Catalog# K202020; ThermoFisher Scientific, Waltham, MA) following the manufacturers instruction and sequenced (sangers sequencing) to confirm the presence of desired mutation. The mutant construct (insert) was digested using restriction enzymes, and gel purified. The mutant CSN5 insert was then ligated into EYFP vector containing the CMV promoter using the T4 DNA
ligase, transformed, and amplified. The orientation and ligation were reconfirmed by restriction
digestion using BAMHI and HINDIII as recognition sites.

Similarly, for the deneddyylase-dead CSN5, histidine residues at 140 and 142 from the
protypical MPN-JAMM consensus sequence EX₇HSHX₇SXXD,⁴⁷ were targeted for mutation
and converted to aspartic acid using strategy as mentioned above. The mutants were constructed
using the primer pairs listed below.

CSN5-ΔNES
Forward 5’-CACCATCTTCCGCCGAGCGGATGCTGA-3’
Reverse 5’-TCAGCATCGGCTCGGCGGAAGATGGTGA-3’

CSN5-ΔMPN
Forward 5’-GCCAGGGTCGCTATCATACCACCCGA-3’
Reverse 5’-TCGGGTGATGTATGATAGCGACCCTGGC-3’

External primers
BAMHI 5’-GATCCGGTGGATCTAAGAG-3’
HIND-III 5’-TCGAGCTCAAGCTTCCATGGC-3’

The control EYFP was prepared by gel cutting the backbone obtained by restriction
digestion with BAMHI and HINDIII. Following the 3’ and 5’ end were blunted using the
Klenow fragments (Catalog# M0210S; New England Biolabs, Ipswich, MA) as per
manufacturers instruction. The ends were ligated and further amplified.

**Cell transfection**
Transient transfection was achieved with lipofectamine 3000 transfection reagent (Catalog# L3000001; ThermoFisher Scientific, Waltham, MA) using manufacturers protocol. In brief, cells were plated until it reached 70-80% confluency. Lipofectamine 3000 was diluted in Opti-MEM medium. Then, a master mix was prepared by diluting DNA in Opti-MEM medium and the enhancer reagent, P3000TM was added, mixed, and incubated at room temperature for 15-20 mins. The DNA-lipid complex was then added to the cells. The media was replaced after 6-8 h and checked for the transfected cells 48-72 post-transfection.

**CSN5i-3 administration in vivo**

CSN5i-3 (Catalog# HY-112134; MedChem Express, Monmouth, NJ) was formulated in 90% autoclaved corn oil and 10% DMSO at a concentration of 20 mg/kg. Starting, young mice were injected with CSN5i-3 intraperitoneally (IP) and 3 h of the first injection, ligation of LCCA was performed. Following, mice were injected each day until the last day of treatment and closely monitored for any health-related issues or overt phenotype. On day 7, post 3 hours of injection, samples were collected for respective experiments.

**MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay**

To determine the cell viability and cell growth, an MTT assay was used. VSMCs were seeded on a 24 well plate and cultured in DMEM containing 10% FBS. The medium was then replaced with serum-free media for 24 hours and serum starved VSMC were then stimulated with PDGF-BB (10 ng/ml). MTT working solution was prepared by diluting the stock solution in PBS, 5 mg MTT in 1 ml 1X PBS. MTT solution was added to each well (1ml for each well)
and was incubated at 37°C. The medium was removed after 3 hours of incubation and solubilized completely using 200ul of acidic isopropanol. Next, 100ul of the dye solution was transferred into a colorless 96 well plate and the absorbance of the converted dye was measured at a wavelength of 570 nm with a background subtraction at 650 nm.

**Cell-cycle analysis**

The cells were grown up to 70-80% confluency, washed with PBS and then fixed with 70% ethanol in a dropwise manner for 30 minutes at 4°C. The fixed cells were washed with 1X PBS (2X), centrifuged and the pellets were collected. Following, the cells with treated with ribonuclease and further stained with propidium iodide (PI). Avoiding light, the cells were then analyzed using the flow-cytometer (BD Accuri C6 Plus Flow Cytometer).

**Echocardiography**

Transthoracic echocardiography was performed using VisualSonics Vevo 3100 (FUJIFILM VisualSonics) system and a 40MHz probe to assess the cardiac morphometric and functional parameters as previously described.182 Mice were kept under light anesthesia with isoflurane at 1.5% in a room supplemented with 100% oxygen. A short axis view was recorded at the papillary muscle level and the guided M-mode was acquired through anterior and posterior walls.

**Doppler ultrasound**

The left common carotid artery (LCCA) was investigated by VisualSonics
Vevo 3100 (FUJIFILM VisualSonics) system and a 40MHz probe. Systolic and diastolic diameter was obtained from the B-mode imaging keeping the carotid artery as horizontal as possible. For the blood flow measurements, pulsed wave doppler mode was used and aliasing was used to identify the region of peak flow. The transducer angle and orientation were adjusted as necessary to obtain an angle between 45°- 50° throughout the experiment. Doppler spectral trace for blood velocity and diameters was calculated using the on-board software on the VisualSonics system, Vevo LAB 5.6.0.

**Statistical Methods**

Data were statistically analyzed using GraphPad Prism (version 9, GraphPad Software, San Diego, CA). All quantitative data are presented as Mean ± SEM unless otherwise indicated. Differences between the two groups were evaluated using two-tailed unpaired Student’s *t* test and, when difference between three or more groups were evaluated, two-way analysis of variance (ANOVA) or, when appropriate, three-way ANOVA followed by Tukey’s multiple comparison test was used. Serial doppler and echocardiography data were examined using two-way repeated measures ANOVA followed by Tukey’s multiple comparison. For non-parametric data sets, Mann-Whitney test was also used. Statistical tests used to assess the statistical significance are indicated in each figure legend and the p-value is provided in the graphs. The probability value <0.05 was considered statistically significant.
Table 1. Primary/secondary antibodies used in this study.

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RESULTS

PART I: Understanding the Significance of the CSN in Neointimal Hyperplasia

CSN subunits are frequently overexpressed in tumor tissues of multiple cancer types. However, no reported study has directly investigated if the expression of the CSN/CSN subunits is affected in response to vascular injury. Hence, we evaluated the changes in the CSN in mice following ligation of LCCA and in human neointima of PAH.

1.1 The effect of vascular injury on the expression of the CSN in mice

1.1.1 Changes in the protein level of the CSN in neointima of wild-type mice

To better understand the significance of the CSN in vascular biology, we assessed CSN expression in the wall of LCCA proximal to the ligation site in wild type (WT) mice 1 week after LCCA ligation. The steady-state protein levels of all 6 representative CSN subunits were significantly higher on the ligated side (LCCA) compared with the counter non-ligated side (RCCA) (Figure 2).
Figure 2. The protein levels of CSN subunits are upregulated after vascular injury in mice.

Adult wildtype mice were subject to LCCA ligation; 1 week later, the LCCA proximal to the ligation site as well as the corresponding segment of the right common carotid artery (RCCA) were collected. A and B, Representative images (A) and pooled densitometry data (B) of Western blot analyses for the indicated CSN subunits. L.C., loading control which is a segment of the in-lane loading control image derived from the stain-free total protein imaging technology, the same for all other figures. Scatter plots superimposed by Mean±SEM; each dot represents a mouse, Student’s t-test of indicated CSN subunits.
1.1.2 Changes in the mRNA level of the CSN in neointima of wild-type mice

Consistent with the protein data, RT-PCR and qPCR analysis revealed that the mRNA levels of all examined CSN subunits (CSN5, CSN6 and CSN8) were significantly increased in the ligated side of the WT mice compared to the counter side control, RCCA (Figures 3).
Figure 3. The mRNA levels of CSN subunits are upregulated in wildtype mice after ligation of the left common carotid artery (LCCA).

Adult wildtype mice were subject to LCCA ligation; 1 week later, the LCCA segment proximal to the ligation site as well as the corresponding segment of the right common carotid artery (RCCA) were collected for RNA extraction. A and B, Shown are representative images of conventional reverse transcriptase PCR (RT-PCR) (A) and real-time PCR (qPCR) data (B) of the indicated genes. Scatter plots superimposed by Mean±SEM; each dot represents a mouse, Student’s t-test.
1.1.3 Impact of vessel injury on the holocomplex and minicomplex

CSN subunits have been suggested to exist in two different forms of complexes, a larger molecular weight holocomplex consisting of all 8 canonical CSN subunits and a postulated minicomplex containing only a subset of CSN subunits. Our prior results demonstrated a marked increase in the CSN subunits after vascular injury. We next aimed to decipher if the increases were in the holocomplex, minicomplex, or both. Immunoprobing for CSN5, CSN6, and CSN8 in native proteins fractionated by native-PAGE, a technique separating native protein complexes based on their size, consistently revealed remarkable increases in the CSN holocomplex in the ligated LCCA (Figure 4).
Figure 4. The abundance of the CSN holocomplex is increased in the vessel wall after vascular injury in mice.

One week after the ligation surgery, carotid arteries (ligated and unligated) were collected from adult wildtype mice. Representative images of native-PAGE followed by Western blot analyses for the indicated CSN subunits.
1.1.4 The expression of the CSN in the VSMCs of neointima

To further determine if the increase in the CSN occurs in VSMCs, the ligated and unligated arteries were processed and immunostained to visualize the distribution of CSN5 and CSN8 among different cell types. Immunofluorescence confocal microscopy detected that the upregulated CSN5 (Figure 5A) and CSN8 (Figure 5B) were primarily localized in the neointimal VSMCs. Taken together, these data indicate that the expression of CSN subunits is increased in the SMCs of the neointima induced by vessel injury.
Figure 5. CSN5 and CSN8 are upregulated in the smooth muscle cells of the left common carotid artery (LCCA) in wildtype mice 1 week after LCCA ligation.

Shown are representative confocal micrographs of LCCA walls immunostained for CSN5 (A), CSN8 (B) and SM22α (green) as indicated. Nuclei were stained with DAPI (blue). Scale bar= 75 µm.
1.2 Assessing the expression of CSN5 in the neointima of human pulmonary hypertension

NH is a prominent lesion in the pulmonary arteries of PAH patients. Next, we were able to validate the CSN upregulation in human NH by immunostaining and quantification of CSN5 in the pulmonary arterial NH from PAH patients. The demographic information of the PAH patients is shown in Table 2. Immunohistochemistry (IHC) revealed that CSN5, the enzymatic subunit of the CSN, was remarkably higher in the neointimal VSMCs of the pulmonary arteries in the explanted lungs of humans with PAH than in the VSMCs of control pulmonary arteries (Figures 6C). Quantitative image analyses showed that both the percent of CSN5-positive areas and the percent CSN5-positive VSMC nuclei in the neointima of PAH tissues were significantly greater than those in the tunica media of control pulmonary arteries (Figure 6D, 6E).
Table 2. Demographic information of the patients for the human lung tissue samples with pulmonary arterial hypertension (PAH) and without PAH (CON).

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Figure 6. Immunohistochemistry (IHC) analysis for the expression of CSN5 in the pulmonary artery from human patients without Control) or with pulmonary arterial hypertension (PAH) lesions.
Paraffin sections of lung tissues from human patients without (Control) or with PAH were processed for H&E staining or IHC. A, H&E-stained images of sister sections from the same Control and PAH tissue samples used for the CSN5 IHC staining as shown in panel C. B, Lower magnification IHC images of the CSN5 (brown). The boxed area is shown at higher magnifications in panel C. C, Representative images of sections stained for CSN5 and negative control images of sister sections with the omission of the anti-CSN5 primary antibody. D and E, The percent CSN5-positive area (D) and the percentage of CSN5-positive nuclei (E) of smooth muscle cells in the tunica media (Control) or the neointima (PAH) of the pulmonary arteries. Scale bar=100 µm. Scatter plots superimposed by Median±95%CI (D) or Mean±SEM (E); each dot represents a pulmonary artery, 3 arteries/patient, and 6 patients/group are included. P values are derived from Mann-Whitney test (D) or nested t-test (E).
PART II: Defining the Importance of VSM CSN5 in Neointimal Hyperplasia

CSN5 harbors the isopeptidase catalytic center of the CSN complex. Although much has been studied about the role of CSN subunits and the knockout phenotypes in non-vascular tissues, specifically related with cancer, the regulatory effect of the CSN subunit, CSN5 in VSMCs has not been studied yet. To fill this critical gap, we aimed to understand the importance of CSN5 in VSMCs in this part of the study.

2.1 The effect of smooth-muscle restricted CSN5 knockout in neointimal hyperplasia

As the first approach to understanding the role of CSN5 in NH, we used a novel genetic model of smooth-muscle restricted CSN5 knockout (CSN5-SMKO) by coupling a CSN5-floxed allele with a Myh11 promoter-driven tamoxifen-inducible Cre expression in mice. The Myh11 gene encodes the smooth muscle myosin heavy chain (SMMHC), a contractile protein expressed only in SMCs.

2.1.1 Verification of tamoxifen induced CSN5-SMKO

To achieve homozygous CSN5-SMKO, adult Cops5fl/fl::Myh11-CreERT2 mice with littermate Myh11-CreERT2 mice as the control (CTL) were injected intraperitoneally with tamoxifen (1mg/day x 5 days/round x 2 rounds, 2-day break in between rounds). Tamoxifen can bind to the mutant estrogen receptors in the Cre-ERT2 sequestered in the cytoplasm and translocate the bound Cre-ERT2 into the nucleus where the Cre performs the site-specific recombination of floxed alleles. After the last tamoxifen injection, we waited at least 2 weeks to wash off the exogenous effect of tamoxifen, then a cohort of mice were sacrificed for the collection of tissues from SM and non-SM organs.
Western blot analyses revealed a drastic decrease in the protein level of CSN5 in SM-specific tissues vs. the non-SM tissues that did not show any change (Figure 7A). To further confirm the SMC-specific nature of the CSN5 knockout, we performed IHC staining for CSN5 in representative organs, which demonstrated an efficient knockout of CSN5 specifically in SMCs including VSMCs (Figure 7B). Together, we were able to achieve successfully tamoxifen-induced CSN5 SMKO in adult Cops5fl/fl::Myh11-CreERT2 mice.
Figure 7. Characterization of smooth muscle restricted CSN5 knockout (SMKO) in mice.

Two weeks after the last tamoxifen injection, tissues of the indicated organs were collected from the homozygous Cops5<sup>fl/fl</sup>::Myh11-CreERT2 mice and the control Myh11-CreERT2 transgenic (CTL) mice and processed for Western blot analysis (A) and IHC staining (B) for CSN5. Scale bar= 100µm.
2.1.2 Characterization of the blood vessel morphometric and functional parameters in CSN5-SMKO mice

So far, no reported studies have investigated the functional impact of CSN5-SMKO. To characterize the effect of CSN5-SMKO in mice at baseline, we performed serial doppler ultrasound evaluation for blood velocity, resistivity, and vessel diameters of the LCCA in CSN5-SMKO and the control mice at 0.5, 1, 2, 3, and 4 months after the last tamoxifen injection. Starting from 3 months post tamoxifen injection, a comparison of phenotypic differences between the groups showed that CSN5-SMKO mice developed a significant decrease in the end diastolic velocity (EDV) along with a decrease in the velocity time interval (VTI), though the modest tendency of difference was observed soon after a month of CSN5 knockout (Figure 8A-8D). In addition, the CSN5-SMKO mice showed reduced diastolic and systolic diameter; a significant difference in diastolic diameter and systolic diameter was observed at 3 months and 4 months after the last tamoxifen injection (Figure 8E,8F). Moreover, pulsatility and resistivity index were found to be significantly different between the two groups 3 months after the last tamoxifen injection (Figure 8G,8H). These together suggest that ablation of CSN5 in SMC impairs vascular function.
Figure 8. The time courses of changes in key parameters of doppler ultrasound for the left common carotid artery (LCCA) of CSN5-SMKO mice.

Starting from 2 weeks after the final tamoxifen injection, mice were subjected to doppler ultrasound evaluation at the indicated time points. Shown are the end diastolic velocity (EDV; A), peak systolic velocity (PSV; B), velocity time interval (VTI; C), mean velocity time integral (Mean VTI; D), diastolic LCCA diameter (LCCA Diam;d; E), systolic LCCA diameter (LCCA Diam;s; F), pulsatility index (PI; G), and resistivity index (RI; H).

Mean±SD; two-way repeated measures ANOVA followed by Tukey’s test; *p<0.05, **p<0.005, ***p<0.0001.
2.1.3 Serial echocardiographic characterization of cardiac functional and morphometric parameters in CSN5-SMKO mice

Echocardiography (Echo) was performed at 0.5, 1, 2, 3, and 4 months after the last tamoxifen injection to assess the morphometric changes including wall thickness, left ventricular (LV) mass, and functional parameters such as fractional shortening (FS), ejection fraction (EF), stroke volume (SV), and cardiac output (CO).

Up until 4 months after the last tamoxifen injection, we did not observe any significant difference in body weight between the control and CSN-SMKO mice (Figure 9A). Consistent with the doppler ultrasound, serial Echo analysis revealed that the functional abnormalities were substantially significant at later timepoints in the CSN5-SMKO group compared to the control. Although a modest tendency of change was discernible starting from 1 month post tamoxifen, CSN5-SMKO mice developed a significant decrease in the heart rate, FS, EF, SV, and CO starting at 3 months post tamoxifen injection (Figure 9B-9F). Nevertheless, no difference was observed in the Echo-derived LV mass (Figure 9G). This suggested that ablation of CSN5 in VSMCs differentially affects the cardiac functional parameters but however, does not substantially cause any change in the structural parameters in heart.
Figure 9. Baseline characterization of the cardiac morphometry and function of CSN5-SMKO mice.

Two weeks after the final tamoxifen injection, mice were subjected to serial echocardiography. 

A, Comparison of changes in body weight. B ~ G, Comparisons of the time course of the indicated perimeters derived from echocardiography. Mean±SD; two-way repeated measures ANOVA followed by Tukey’s test; *p<0.05, **p<0.005, ***p<0.0001.
2.1.4 Assessment of markers of cullin de neddylation

CSN5 harbors the catalytic center of the CSN isopeptidase activity that mediates the de neddylation of CRLs. Thus, we next sought to investigate the necessity of CSN5 in SMC by evaluating the effect on the de neddylation function and markers at baseline and after 1 week of LCCA ligation. Consistent with loss of cullin de neddylation activity, only the de neddylated forms of the examined Cul1 and Cul2 were discernible and, consistently with CRL1 dysfunction, Skp2 was substantially decreased in the vessel walls of both LCCA (ligated) and RCCA in CSN5-SMKO mice compared with littermate CTL mice (Figure 10A). Skp2 is an F-box protein that can serve as the substrate receptor in the Skp1-Cul1-Fbox complex (SCFSkp2) to target p27 for ubiquitination and subsequent degradation by 26S proteasomes. Moreover, the protein level of Skp2 was increased in control mice after the ligation of LCCA but nonetheless the increase was inhibited in CSN5 SMKO mice. In addition, the decrease of Skp2 was accompanied by a tendency of increased p27 (Figure 10A,10B) indicating that the activity of de neddylation as well as the expression of the CRLs and the substrates are altered by knockout of CSN5 in SMCs.
Figure 10. Smooth-muscle cell restricted CSN5 knockout (SMKO) mice exhibited increases in neddylated cullins and decreases in Skp2.

Myh11-CreERT2 transgenic (CTL) and homozygous SMKO mice were subject to LCCA ligation; the LCCA proximal to the ligation site and the corresponding segment of unligated RCCA were collected 1 week later. A and B, Representative images of Western blot analyses for the indicated proteins (A) and pooled densitometry data for p27 (B). Scatter plots superimposed by Mean±SEM; each dot represents a mouse; n= 3 mice/group; two-way ANOVA followed by Tukey’s tests.
2.1.5 Subcellular localization of p27 in CSN5-SMKO mice

Independent of the de neddylation, the suggested minicomplex in proliferating fibroblast was shown to mediate the nuclear export of p27.\textsuperscript{105} Thus, it was our interest to determine if ablation of CSN5 would affect the subcellular localization of p27 after vascular injury in mice. p27 in the nucleus inhibits CDKs, but as the cell enters the proliferative phase, nuclear p27 gets translocated to the cytoplasm.\textsuperscript{7} Our western blot analyses of nuclear and cytoplasmic fractions of mouse arteries revealed that LCCA ligation led to a significantly reduced nuclear p27 and increased cytoplasmic p27 protein level in the CTL mice, but the export of p27 to cytoplasm was suppressed in CSN5-SMKO mice (\textbf{Figure 11}). These data suggest that CSN5 in SMCs is required for the nuclear export of p27.
Figure 11. Nuclear-export of p27 is suppressed in Smooth-muscle cell restricted CSN5 knockout (SMKO) mice.

Myh11-CreERT2 transgenic (CTL) and homozygous SMKO mice were subject to LCCA ligation; the LCCA proximal to the ligation site and the corresponding segment of unligated RCCA were collected 1 week later. A ~ D, Representative images (A) and pooled densitometry data (B-D) of Western blot analyses for p27 in the nuclear (Nucl) and cytosolic (Cyto) fractions. RCCA was used as a baseline control. GAPDH and Histone H3 (H3) are probed as the loading control for cytosolic and nuclear proteins, respectively. Scatter plots superimposed by Mean±SEM; each dot represents a mouse; n= 3 mice/group; two-way ANOVA followed by Tukey’s tests or unpaired Student’s t-test.
2.1.6 Effect of CSN5-SMKO on neointimal hyperplasia and VSMC proliferation

To study the necessity of CSN5 for NH, we characterized the CSN5-SMKO and CTL mice one week after LCCA ligation. Western blot analyses demonstrated that the ligation-induced increase in the cell proliferation marker PCNA was significantly less in the CSN5-SMKO mice than that in the CTL mice (Figure 12A,12B). The decrease in the proliferation corresponded to the increased p27 observed prior (Figure 11B). We did double-immunofluorescence staining for cell proliferation marker Ki-67 and SMC marker SM22α to further investigate the effect on proliferation and neointima formation. Analyses of SM22α stained neointima using Image J revealed a significantly thinner neointima in CSN5-SMKO mice compared to the control (Figure 12C,12D,12E). Ki-67 is a widely accepted cell proliferation marker.186 Thus, we double immunostained the collected arteries with Ki-67 and SM22α and evaluated the colocalization between Ki-67 with SM22α and DAPI, which revealed fewer Ki-67-positive VSMCs per section and lower percentage of Ki-67-positive nuclei in the neointima (Figure 12C,12F,12G) in CSN5-SMKO mice, compared with those in the CTL mice. Taken together, these results demonstrate that CSN5 in SMCs is essential for VSMC proliferation and neointima formation after vascular injury.
Figure 12. Smooth-muscle cell restricted CSN5 knockout (SMKO) suppresses VSMC proliferation.

Myh11-CreERT2 transgenic (CTL) and homozygous SMKO mice were subject to LCCA ligation; the LCCA proximal to the ligation site and the corresponding segment of unligated RCCA were collected 1 week later. A and B, Representative images of Western blot analyses (A) and pooled densitometry data for PCNA (B). GAPDH was used as a loading control. C–G, Cross-sections of carotid arteries were immunofluorescence stained for SM22α (green) and Ki-67 (red) and counter-stained with DAPI to identify nuclei (blue). Shown are representative confocal images (C) and quantitative analysis (D–G) of neointima formed (D), total nuclei in
neointima (E), Ki-67 positive VSMC in neointima (F), and the percentage of Ki-67 positive VSMC (G) calculated by normalizing the Ki-67 positive VSMC/total nuclei in the neointima. Scale bar=50 µm. Scatter plots superimposed by Median±95%CI; each dot represents a section, 5 sections/mouse, 3 mice/group; Mann-Whitney test (E – G). Scatter plots superimposed by Mean±SEM; each dot represents a mouse; n= 3 mice/group (B, D); two-way ANOVA followed by Tukey’s tests (B) or unpaired student t-test (D).
2.2 Effects of pharmacological inhibition of CSN5 deneddylase in vivo

CSN5 is a vital subunit of the CSN and deneddylation plays an important role in maintaining cellular homeostasis. Previously, studies have shown that CSN5i-3, a novel chemical inhibitor of the CSN deneddylase suppresses cell proliferation and affects the viability of cancer cell lines.\textsuperscript{187,188} Hence, we sought to determine the importance of CSN5 in NH by testing if pharmacological inhibition of CSN deneddylase function using CSN5i-3 could repress neointima formation in mice after vascular injury.

2.2.1 Effects of the selective inhibition of CSN5 deneddylase activity on cardiac function of mice

A cohort of WT mice (equal number of males and females) were randomly assigned to either CSN5i-3 (20 mg/kg/day x 7 days, i.p.) or vehicle control treatment groups. Three hrs post the first dosage, ligation of LCCA was performed. A day before the terminal experiment, transthoracic echocardiography was performed on both drug and vehicle treated mice to assess the effect of deneddylase inhibition on cardiac function.

We did not observe any significant difference in body weight between the two groups throughout the days of treatment (\textbf{Figure 13A}) indicating that the doses were well-tolerated. Compared to the vehicle treated group, CSN5i-3 treated mice displayed impaired cardiac function phenotype as evidenced by the marked decreases in the heart rate, LV end-diastolic internal diameter (LVID;d), FS, EF, SV, and CO (\textbf{Figure 13B–13I}). The results of these echocardiograms show that inhibition of deneddylase by CSN5i-3 with this regime induces moderate cardiotoxicity.
Figure 13. Effects of CSN5i-3 on mouse LV function and morphometry.

A, The time courses of changes in mouse body weight measured daily throughout the treatment time. B–I, Comparisons of the indicated echocardiographic perimeters collected at the 6th day of the treatment. The mice injected with CSN5i-3 (20 mg/kg/day) and vehicle controls were subjected to echocardiography to assess the cardiac morphological and functional parameters. Mean±SEM; n= 8 mice/group (4 males, 4 females); Unpaired students t-test.
2.2.2 Effects of systemic administration of CSN5i-3 on its pharmacodynamic markers in carotid arteries

We investigated the effect of pharmacological inhibition of CSN deneddylase with CSN5i-3 on the carotid artery protein levels of neddylated Cul1 and Skp2, known pharmacodynamic markers for an inhibitor of CSN deneddylase. Carotid arteries were excised at 3 hours after the 7th daily injection of CSN5i-3 or vehicle. For the Western blot analyses for Cul1, proteins extracted from VSMCs treated with MLN4924, or CSN5i-3 were used as positive controls to identify native and neddylated Cul1 bands, respectively. MLN4924 is a NEDD8 activation enzyme (NAE) inhibitor, prevents the neddylation of cullins, thereby trapping cullins in their native form. Western blot analyses revealed an accumulation of neddylated Cul1a decrease in the protein level of Skp2, an F-box protein, in both RCCA and LCCA (ligated) of CSN5i-3 treated mice (Figure 14). These data show that the treatment with CSN5i-3 in mice was effective and intervened with the expression of the pharmacodynamic markers.
Figure 14. Assessment of pharmacodynamic markers following treatment with CSN5i-3.

Wild-type mice were administered with a dose of CSN5i-3 (20 mg/kg, i.p.) or vehicle control 3 h before and daily after LCCA ligation. LCCA and RCCA were harvested 3 hours after the 7th daily injection. Shown are the representative images of western blot analyses for indicated proteins. MLN4924 (neddylation inhibitor) and CSN5i-3 treated VSMCs were used to identify native and nedd8-Cul1 bands (the far-left and the far-right lanes), respectively.
2.2.3 Effect of CSN5i-3 on neointima formation in wildtype mice

Following the evidence of compromised deneddylase function by CSN5i-3 treatment, we next aimed to test the effect of CSN5i-3 on neointima formation. Western blot analyses showed that the increase in the expression PCNA after vascular injury as observed in the vehicle treated group was significantly suppressed in the CSN5i-3 treated group (Figure 15A,15B). Confocal microscopy for the immunofluorescence-stained SM22α in the carotid artery demonstrated that the NH was significantly decreased in the CSN5i-3 treated group compared to the control (Figure 15C,15D,15E). Consistent with our Western blot results, double immunofluorescence staining for Ki-67 and SM22α showed that the total number of Ki-67 positive VSMCs and the percentage of Ki-67 positive VSMCs were significantly decreased in the CSN5i-3 treated mice compared with the vehicle treatment group (Figure 15C,15F,15G). Together, these data compellingly demonstrate that the CSN5i-3 effectively suppresses NH.
Figure 15. CSN5i-3 suppresses neointimal formation in vivo.

Wild-type mice were administered with a dose of CSN5i-3 (20 mg/kg, i.p.) or vehicle control 3 hours before and daily after LCCA ligation. LCCA and RCCA were harvested 3 hours after the 7th daily injection. A and B, Representative images (A) and pooled densitometry data (B) of Western blot analysis for PCNA. MLN4924 (neddylation inhibitor) and CSN5i-3 treated VSMCs were used to identify native and nedd8-Cul1 bands (the far-left and the far-right lanes...
in A), respectively. Scatter plots are superimposed by Mean±SEM where each dot represents a mouse, and the p values are derived from two-way ANOVA followed by Tukey’s tests. C, Representative fluorescence confocal images of immunostained Ki-67 (red) and SM22α (green) in the cryo-sections of LCCA and RCCA. Nuclei were counter-stained with DAPI (blue). Scale bar= 50 µm. D, Scatter plots superimposed by Mean±SEM of the Sm22-positive neointima area in LCCA (2 mm proximal to the ligation site). Each dot represents a mouse; unpaired Students t-test. E~G, Scatter plots superimposed by Median±95%CI of total number of nuclei in neointima (E), Ki-67 positive VSMCs in neointima (F), and the percentage of Ki-67 positive VSMCs (G) derived from microscopy as illustrated by panel C. Each dot represents a tissue section; 5 sections/mouse and 4 mice/group were included; Mann-Whitney test.
PART III. Delineating the Role of CSN5 Minicomplexes in VSMC Proliferation and Neointimal Hyperplasia

Experimental results described in Part II demonstrate that loss of CSN deneddylation activity alone is sufficient to suppress VSMC proliferation and NH in mice and likely represents a main mechanism by which CSN5-SMKO suppresses NH. To decipher further whether and how CSN5 minicomplexes may play role in promoting VSMC proliferation and NH upon vascular injury, we turned to a genetic hypomorphic model of the smallest and non-catalytic CSN subunit, CSN8. Previously, CSN8 hypomorphism (CSN8-hypo) in MEF cells displayed an increased ratio of CSN5 minicomplex to CSN holocomplex and decreased deneddylation activity. Thus by using a model of CSN8 hypomorphism where the downregulation of CSN8 disables the formation of the CSN holocomplex and thereby increases the opportunity for other CSN subunits to form minicomplexes, we sought to understand the significance of minicomplexes. Therefore, we investigated the effect of CSN8 hypomorphism on NH and VSMCs proliferation in vivo and in cellulo.

3.1 To determine the in vivo effect of CSN8 hypomorphism in neointimal hyperplasia

We first turned to delineate the effect of CSN8 hypomorphism in mice at baseline and after the LCCA ligation.

3.1.1 Effect of CSN8 hypomorphism on neointimal thickening and proliferation

We first determined the effect of CSN8 hypomorphism on neointima formation. Four weeks after the ligation of LCCA, the ligated carotid arteries were collected, fixed, and paraffin-embedded.
Serial tissue sections were numbered and subsequently subjected to H&E staining. Sections with equal distance to the ligation site of different groups were imaged and compared. Histological examination showed that the neointimal thickening was markedly greater in CSN8-hypo mice as evidenced by the increased neointima thickness, and neointima to media ratio (Figure 16A-16C). Furthermore, this differential response was comparable between the male and female CSN8 hypomorphic mice (Figure 16G,16H). We did not observe any significant differences in the thickness of tunica media or vessel lumen at the location for morphometry between the groups (Figure 16D,16E).

To further test if the more severe neointimal thickening in CSN8-hypo mice triggered by injury was due to enhanced VSMC division, we examined markers of VSMC proliferation at 1 week after LCCA ligation. Consistent with our 4 weeks findings, even at 1 week after the ligation, neointimal thickening was more severe in CSN8-hypo mice in comparison to CTL. Further, PCNA protein levels in the LCCA of CSN8-hypo mice were more than doubled compared to the CTL mice although no difference was detected from the RCCA that was not ligated (Figure 17A,17B). These data indicate that CSN8 hypomorphism promotes vascular cell proliferation in response to vessel injury.

Immunofluorescence confocal microscopy confirmed that LCCA ligation induced a markedly greater increase in Ki-67 positive VSMCs (Figure 17C-17F) in the LCCA of CSN8-hypo mice compared with those in the CTL mice. Between CSN8-hypo and CTL groups, no difference in PCNA proteins was observed in the RCCA of LCCA-ligated mice (Figure 17A,17B) or the LCCA and RCCA of mice subjected to LCCA sham surgery (Figure 18). These results indicate that NH and VSMC proliferation are increased in CSN8-hypo mice after vascular injury.
Figure 16. CSN8 hypomorphic (CSN8-hypo) mice displayed exacerbated neointima formation following vascular injury.
LCCAs of CSN8-hypo and CTL mice were collected 4 weeks after LCCA ligation and processed for H&E staining and morphometric analyses. **A~G**, Representative serial section images (A) and morphometric analyses of the indicated parameters (B~G) from the H&E-stained cross-sections of paraffin-embedded LCCAs. Scale bar=200 µm; scatter plots superimposed by Mean±SEM; each dot = a mouse, n= 8 mice/group (4 males, 4 females); two-tail unpaired students t-test.
Figure 17. CSN8 hypomorphism (CSN8-Hypo) exacerbates LCCA ligation-induced neointimal thickening and vascular smooth muscle cell (VSMC) proliferation. CSN8-
hypo and control (CTL) mice were subject to LCCA ligation; LCCA (ligated) and RCCA were collected 1 week after ligation. A and B, Representative image (A) and pooled densitometry data (B) of Western blot analyses for PCNA. Stain-free total protein imaging was used for loading control (L.C.). Scatter plots superimposed by Mean±SEM; n= 4 mice/group; two-way ANOVA followed by Tukey’s tests. C, Representative confocal images of Sm-22 (green) and Ki-67 (red) immunofluorescence. SM22α was stained (green) to identify VSMCs and nuclei are counter-stained with DAPI (blue). Scale bar=50 µm. D~F, Quantitative data of Ki-67 positive VSMCs (D) and the total number of nuclei (E), as well as the percentage of Ki-67 positive VSMC (F) in the neointima of LCCAs. Scatter plots superimposed by median±95%CI; each dot represents a section; 5 sections/mouse; n=4 mice/group, Mann-Whitney test.
Figure 18. Representative image of Western blot analyses for PCNA in RCCA and LCCA tissues of CSN8-hypo (Hypo) and littermate controls (CTL) mice subjected to LCCA sham surgery. LCCA and RCCA were collected 1 week after the sham surgery.
3.1.2 Relative changes in the protein level of CSN subunits by CSN8 hypomorphism, in vivo

In order to study the influence of CSN8 downregulation on the stability of other CSN subunits, we performed western blot analyses for CSN5 and CSN6. CSN5 is the isopeptidase harboring subunit; CSN6 is strongly incorporated in the CSN complex, thus loss of CSN6 would lead to loss of structural integrity of the CSN complex. Our results revealed that downregulation of CSN8 was accompanied with a remarkable reduction in the protein levels of CSN5 and CSN6 in carotid arteries of CSN8-hypo mice. Corroborating our previous finding (Figure 2), the expression of CSN subunits were increased after vascular injury in the CTL mice, but however, this increase was not discernible in the CSN8-hypo mice (Figure 19), indicative of an essential role of CSN8 in stabilizing the holocomplex in mouse arteries.
Figure 19. CSN8 hypomorphism reduced the protein levels of other CSN subunits in mice. A–D, Representative images (A) and pooled densitometry data of the Western blot analyses for CSN5 (B), CSN6 (C) and CSN8 (D). In-lane total protein images of the SDS-PAGE gel from the stain-free total protein imaging technology were used as the loading control. Scatter plots superimposed by Mean±SEM; each dot represents a mouse; two-way ANOVA followed by Tukey’s tests.
3.1.3 Changes in subcellular distribution of p27 in CSN8 hypomorphic mice

To investigate if CSN8 hypomorphism affects the subcellular localization of p27, nuclear and cytoplasmic proteins were isolated from hypo and CTL mice, 1 week after LCCA ligation. In both LCCA and RCCA, nuclear p27 proteins were significantly lower, cytoplasmic p27 proteins were higher, and the nuclear p27 to cytoplasmic p27 ratios were significantly smaller in CSN8-hypo mice compared to CTLs (Figure 20A-20D). These findings are consistent with the increased VSMC proliferation induced by LCCA ligation in CSN8-hypo mice (Figure 17).
Figure 20. p27 is increased in the cytoplasmic fraction of CSN8-Hypo mice. LCCA and RCCA were collected from CSN8-hypo and the control mice 1 week after LCCA ligation. For nuclear fractionation, three carotid arteries of the same group were combined into one sample. 

A–D, Representative images (A) and pooled densitometry data (B–D) of Western blot analyses for cytoplasmic (Cyto) and nuclear (Nucl) p27. GAPDH and Histone H3 were probed as the loading control for cytoplasmic and nuclear fractions, respectively. Scatter plots superimposed by Mean±SEM; each dot represents a combined sample of 3 mice (A–D); unpaired Student’s t-test (B–D).
3.1.4 Prevalence of CSN5 in CSN8 hypomorphic mice

Besides being an obligatory subunit of the CSN, each CSN subunit may participate in the formation of mini-complexes. CSN5 is unique in partitioning as the ~500kDa holo-complex which is primarily located in the nucleus, and as the minicomplex mostly localized in the cytoplasm.\textsuperscript{107} Nuclear fractionation revealed an increased protein level of CSN5 in the cytoplasmic fraction of the CSN8-hypo mice. This was accompanied by a decrease in the nuclear CSN5 (\textbf{Figure 21A-21D}). We further subjected the homogenates from the nuclear and cytoplasmic fractions to non-denatured gel electrophoresis followed by immunoprobing for CSN5, which revealed a significant increase in the abundance of CSN5 minicomplex located primarily in the cytoplasmic fraction and, concomitantly, a significantly decrease in the level of CSN5-contained holocomplex in both RCCA and the ligated LCCA of CSN8-hypo mice compared with control mice (\textbf{Figure 21E-21G}). Taken together, these results demonstrated increased CSN5 mini-complexes in the cytoplasm of CSN8-hypo arteries, which is consistent with an augmented nuclear-export of p27, a likely mechanism underlying the enhanced VSMC proliferation and NH in CSN8-hypo mice upon vascular injury.
Figure 21. CSN5 is increased in the cytoplasmic fraction of CSN8-Hypo mice. LCCA and RCCA were collected from CSN8-hypo and the control mice 1 week after LCCA ligation. For nuclear fractionation, three carotid arteries of the same group were combined into one sample.

A–D, Representative images (A) and the summary of densitometry data (B–D) of Western blot analyses for cytoplasmic and nuclear CSN5. E–G, Representative image (E) and pooled densitometry data (F and G) of native-PAGE followed by Western blot for CSN5. Scatter plots superimposed by Mean±SEM; each dot represents a combined sample of 3 mice; unpaired Student’s t-test (B–D); two-way ANOVA followed by Tukey’s tests (F and G).
3.2 To determine the \textit{in cellulo} effect of CSN8 hypomorphism on VSMC proliferation

Our \textit{in vivo} findings have compellingly unveiled that CSN8-hypo mice show an exacerbation of NH. Thus, we next aimed to understand if this could be VSMCs autonomous. Pharmacological as well as genetic approaches were used in this part of the study to further investigate the mechanism governing the phenomenon.

3.2.1 Isolation and characterization of VSMCs from mouse aorta

VSMCs were isolated from mouse aorta and were further characterized. Figure 22A presents the brightfield image showing the morphology of murine adult aortic passage 1 (P1) VSMCs grown undisturbed for 6 days. To determine the purity of the isolated VSMCs, we stained the VSMCs in P1 and P2 with SM22α, an SMC marker (Figure 22B). In average, 97-99% of VSMCs stained positive for SM22α. Additionally, we also did western blot analysis using the cultured cells from P1 and P2 to check for the expression for SM22α and desmin. As controls to validate the SMC specific markers, we also isolated adventitial fibroblasts and used the lysates from aorta and hearts as a positive control for SM22α and desmin, respectively. Western blotting revealed the expression of SM22α and desmin in the cultured VSMCs; however, desmin was not expressed in the adventitial fibroblasts (Figure 22C). Fibroblasts are desmin negative whereas VSMCs express desmin.\textsuperscript{189,190} Overall, our results indicated a successful isolation and culture of VSMCs from mice.
Figure 22. Validation of cultured vascular smooth muscle cells (VSMCs) isolated from mouse aorta.

A, A representative bright field phase-contrast micrograph of passage 1 (P1) VSMCs in culture. Scale bar=100 µm. B, Representative micrographs showing the VSMCs in P1 and P2 stained with SM22α (green) and DAPI (blue). The negative control image represents the VSMC only stained with DAPI and the green fluorescence-conjugated secondary antibody but without the SM22α primary antibody. Scale bar=150 µm C, Western blot analysis for SM22α and desmin in the total protein extracts from cultured adventitial fibroblasts, aorta, P1 and P2 VSMCs, and heart tissue. Here the heart tissue is used as a positive control for desmin. The lower image in the panel C is the stain-free in-gel labeled total protein image to show the loading.
3.2.2 Effects of CSN8 hypomorphism on proliferation of cultured VSMCs

In order to mimic vascular injury in cellulo, we treated the cells with PDGF-BB. PDGF-BB is considered the most robust and primary chemoattractant for VSMCs, responsible for initiating the phenotypic changes in VSMC migration and proliferation. Previously, studies have demonstrated that the switch from contractile to synthetic VSMC phenotype is mediated by PDGF-BB; upon binding to its surface receptors, PDGF-BB can activate multiple signaling pathways such as PLC, AKT, ERK1/2, which can ultimately regulate gene expression and cellular function. Therefore, herein, we choose PDGF-BB as an in cellulo stimulant. Western blot analyses for the proliferation marker PCNA showed that the ability of VSMCs to proliferate was significantly increased under the stimulation of PDGF-BB and particularly, CSN8-hypo cells exhibited a more pronounced increase in the expression of PCNA (Figure 23A, 23B). Similar results were obtained from MTT assays which demonstrated a more significant increase in the cell proliferation in CSN8-hypo VSMCs vs. CTL VSMCs under PDGF-BB stimulation (Figure 23C). Further confirming our findings, cell-cycle analysis using flow cytometry showed a much higher population of CSN8-hypo cells in the G1 phase compared to the CTL upon stimulation with PDGF-BB (Figure 23D, 23E). At the same time, this correlated with significantly upregulated proportion of cells in S-phase in hypomorphic VSMCs in response to PDGF-BB. At 48h, the PDGF-BB induced increase in the S-phase population was alongside a significant increase in G2/M population as well (Figure 23F, 23G). Taken together our results indicated a significantly greater PDGF-BB induced VSMCs proliferation in CSN8-hypo VSMCs compared to control VSMCs, consistent with our in vivo findings. VSMCs at basal condition displayed no discernible difference in proliferation between CSN8-hypo and control groups.
Figure 23. VSMC proliferation was increased by CSN8 hypomorphism, in cellulo.
Cultured VSMCs were serum-starved for 24 hours and then treated with PDGF-BB. The cells were collected for further analyses 24 or 48 h after the treatment. A and B, Representative images (A) and pooled densitometry data (B) of immunoblot for PCNA. C, Quantification of untreated and treated (PDGF-BB) VSMCs assessed for the cell viability by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. D ~ G, Flow cytometry-based cell cycle analysis. Following the stimulation with PDGF-BB, VSMCs were detached by trypsinization and subjected to propidium iodide (PI) staining followed by flow cytometry. The percentage of cells in G0/G1 phase, S phase, and G2/M phase are compared. Representative histograms at 24 h (D) and 48 h (F) and the quantification of the percentage of cells in respective phases (F and G) at baseline and or PDGF-BB treatment. Mean±SEM; n=3; two-way ANOVA followed by Tukey’s tests (B, C, E, G).
3.2.3 The effect of CSN8 hypomorphism on stability of other CSN subunits, in cellulo

To test whether hypomorphism alters the stability of CSN5 in cultures, western blot analyses were performed. Similar to our *in vivo* findings, we observed that the reduction in the CSN8 proteins was sufficient to discernibly decrease the protein level of CSN5 (*Figure 24*). Interestingly, the expression of the CSN subunits were increased in response to the PDGF-BB stimulation in CTL VSMCs, but the increase was less pronounced in CSN8-hypo VSMCs, suggesting an essential role of CSN8 in the integrity and stability of the CSN holocomplex in VSMCs.
Figure 24. Expression of CSN5 is reduced in CSN8 hypomorphic VSMCs. Cultured VSMCs were serum-starved for 24 hrs, then treated with PDGF-BB or vehicle control for 24 or 48 hrs before collected for respective analyses. A ~ C, Representative images (A) and pooled densitometry data (B and C) of immunoblot for CSN5 (B), and CSN8 (C).

Mean±SEM; n=3 biological repeats; two-way ANOVA followed by Tukey’s tests.
3.2.4 Effect of CSN8 hypomorphism on the expression of p27

In response to PDGF-BB stimulation, the p27 level was decreased in both CSN8-hypo and CTL VSMCs; CSN8-hypo VSMCs nonetheless displayed greater decreases in total p27 than the CTL VSMCs (Figure 25A, 25B). Additionally, the effect of CSN8 hypomorphism on subcellular localization was assessed using nuclear fractionation. Subcellular fractionation revealed a strikingly decreased nuclear p27, increased cytoplasmic p27 and increased ratio of nuclear to cytoplasmic p27 in the CSN8-hypo VSMCs compared to the CTL (Figure 25C-25F), suggesting an increased nuclear export of p27 in CSN8-hypo VSMCs compared to CTL in response to PDGF-BB.
Figure 25. Nuclear export of p27 is more pronounced in CSN8 hypomorphic VSMCs.

Cultured VSMCs were serum-starved for 24 hrs, then treated with PDGF-BB or vehicle control for 24 or 48 hrs before collected for respective analyses. A ~ E, Representative images (A) and pooled densitometry data (B) of immunoblot for p27. C ~ F, Representative image (C) and pooled densitometry data (D-F) of Western blot analyses for cytosolic (Cyto) and nuclear (Nucl) p27. For western blot analyses, GAPDH was used as a loading control (L.C.). GAPDH and Histone H3 were probed as a marker and loading control for cytoplasmic and nuclear fractions, respectively. Mean±SEM; n=3 biological repeats; two-way ANOVA followed by Tukey’s tests.
3.2.5 Effect of CSN8 hypomorphism on the subcellular distribution of CSN5

The effect of CSN8-hypo on CSN5 distribution was assessed using nuclear fractionation followed by immunoblot for CSN5 in either regular denatured SDS-PAGE or native-PAGE. Nuclear fractionation for CSN5 demonstrated a significant increase in CSN5 in the cytoplasmic fraction and a decrease of CSN5 in the nuclear fraction of the CSN8-hypo VSMCs in comparison with the CTL (Figure 26A-26D). Further, native immunoblots confirmed an increased prevalence of CSN5-minicomplex in the cytoplasmic fraction of CSN8-hypo VSMCs (Figure 26E-26G). Corroborating our previous findings, the abundance of CSN5 in the holocomplex was increased upon PDGF-BB stimulation in CTL VSMCs. Together, these data demonstrate an increase of CSN5 minicomplex in the cytoplasm concurrently with a decrease of CSN holocomplex in the nuclei of CSN8-hypo VSMCs compared with CTL VSMCs, which correlates with increased nuclear export of p27 and subsequently increased VSMC proliferation.
Figure 26. Cytoplasmic CSN5 was increased in CSN8 hypomorphic VSMCs. Cultured VSMCs were serum-starved for 24 hrs, then treated with PDGF-BB or vehicle control for 24 hrs before collected for respective analyses. A ~ D, Representative image (A) and pooled densitometry data (B-D) of Western blot analyses for cytosolic (Cyto) and nuclear (Nucl) CSN5. GAPDH and Histone H3 were probed as a marker and loading control for cytoplasmic and nuclear fractions, respectively. E ~ G, Representative image (E) and pooled densitometry data (F and G) of Western blot analyses for cytosolic and nuclear native CSN5 protein complexes following native-gel electrophoresis. Mean±SEM; n=3 biological repeats; two-way ANOVA followed by Tukey’s tests.
3.2.6 Assessing the effect of pharmacological inhibition of nuclear export and CSN de neddy lase on VSMCs proliferation

Our \textit{in vivo} and \textit{in cellulo} findings prompted us to further determine a mechanistic involvement for the nuclear export of p27 by CSN5 minicomplexes in the proliferation-promoting property of CSN8 hypomorphism and further, also understand the effect of pharmacological inhibition of de neddylase function of CSN5 in cultured VSMCs. Hence, for this part of our study, we use leptomycin (LMB), a nuclear export inhibitor and CSN5i-3, CSN de neddylase inhibitor.

3.2.6.1 Verification of an effective dose of leptomycin in VSMCs

LMB is a potent and specific nuclear export inhibitor. The cellular target of LMB is CRM1 (chromosomal region maintenance 1), an export protein; LMB blocks the interaction of CRM1 with the nuclear export signal (NES) that functions to bring proteins from the nucleus to cytoplasm.\textsuperscript{194,195}

We first optimized the dose and verified the nuclear export blocking action of LMB in cultured VSMCs. Based on the previous literatures,\textsuperscript{196,197} we choose to test the action of LMB at a concentration of 5 ng/ml on p53 nuclear export.\textsuperscript{198} Nuclear fractionation followed by western blot for p53 in VSMCs treated with PDGF-BB showed that the nuclear export of p53 was remarkably inhibited upon the LMB treatment (\textbf{Figure 27}), indicating that the concentration of 5 ng/ml is discernibly effective in VSMCs.
Figure 27. Leptomycin (LMB) blocked the nuclear export of p53 in cultured VSMCs.

VSMCs were treated with PDGF-BB in the absence or presence of LMB (5 ng/ml) for 24 h before harvested for extraction of the cytoplasmic (Cyto) and nuclear (Nucl) fractions of proteins. The fractionated proteins were then subjected to Western blot analysis p53. GAPDH and Histone H3 were probed as the markers for cytosolic and nuclear fractions, respectively.
3.2.6.2 Optimizing the concentration of CSN5i-3 in VSMCs

CSN5i-3 is a potent and selective inhibitor of the deneddylase function of the CSN5. To verify the action of CSN5i-3 in VSMCs, we treated the cultured VSMCs with different concentrations of CSN5i-3. Western blot analyses for Cul1 revealed that even at the lowest concentration tested (100 nM), CSN5i-3 led to an accumulation of Cul1 in the neddylated state and caused a decrease in the steady state of Skp2 (Figure 28). Thus, we opted to treat the VSMCs with the 100nM of CSN5i-3 dosage in further experiments in cellulo.

![Western blot analysis](image)

**Figure 28. Verification of the actions of CSN5i-3 in cultured VSMCs.**

VSMCs in cultures were treated with the indicated concentrations of CSN5i-3 for 24 h, then harvested for protein extraction. The whole cell lysates were then used for Western blot analyses for neddylated and native Cul1, as well as total Skp2. CSN5i-3 at as low as 100 nM completely blocked Cul1 deneddylation and resulted in complete loss of Skp2.
3.2.6.3 Effects of the inhibition of nuclear export or CSN deneddylation on VSMCs proliferation

We examined the effect of LMB (5 ng/ml) and CSN5i-3 (100 nM) on PCNA and p27 protein expression. Treatment of VSMCs with LMB suppressed the PDGF-BB induced PCNA increases in both CSN8-hypo and control VSMCs. Interestingly, CSN5i-3 significantly suppressed the PDGF-BB induced PCNA increases in control VSMCs but not in CSN8-hypo cells (Figure 29A, 29B). LMB substantially diminished the difference in total p27 between the two genotypes; CSN5i-3 on the other hand increased the protein level of p27 in the CTL VSMCs but not CSN8-hypo VSMCs (Figure 30A, 30B). The increase in the p27 by CSN5i-3 correlates with the decreased PCNA in CTL VSMCs. These results indicate that blocking the nuclear to cytoplasmic shuttling of p27 by inhibition of nuclear export abolishes the proliferation-promoting property of CSN8 hypomorphism in VSMCs.
Figure 29. Nuclear export inhibitor leptomycin B (LMB) but not CSN deneddylyase inhibitor CSN5i-3 suppresses the exacerbation of VSMC proliferation by CSN8 hypomorphism.

VSMCs in cultures were treated with CSN5i-3 (100 nM) or LMB (5 ng/ml) in the absence or presence of PDGF-BB. Twenty-four hours after the treatment, cells were harvested for total protein extraction or nuclear fractionation. A and B, Representative images (A) and pooled densitometry data (B) of Western blot analyses for PCNA. Loading control used the stain-free total protein imaging immunoblot images. Mean±SEM; n=3 biological repeats; three-way ANOVA followed by Tukey’s tests.
Figure 30. The expression of p27 in VSMCs after the treatment with CSN5i-3 or LMB.

Twenty-four hours after the treatment of VSMCs with CSN5i-3 (100nM) or LMB (5ng/ml) in the absence or presence of PDGF-BB, the cultured cells were harvested for total protein extraction. Shown are representative images (A) and pooled densitometry data (B) of Western blot analyses for p27. Mean±SEM; n=3 biological repeats; three-way ANOVA followed by Tukey’s tests.
3.2.6.4 Effects of the inhibition of nuclear export or CSN deneddylase on p27 subcellular localization

Treatment with LMB resulted in a substantial nuclear accumulation of p27 in both CSN8-hypo and control VSMCs and diminished the difference in subcellular distribution of p27 between the two groups as shown by the nuclear fractionation followed by immunoblotting (Figure 31A, 31B). On the other hand, VSMCs treated with CSN5i-3, did not alter the subcellular distribution of p27 (Figure 32). These results suggest that the change in nuclear export is independent of the deneddylase function of the CSN.
Figure 31. Nuclear export inhibitor leptomycin B (LMB) suppresses the nuclear-exclusion of p27 by CSN8 hypomorphism.

VSMCs in cultures were treated with LMB (5 ng/ml) in the absence or presence of PDGF-BB. Twenty-four hours after the treatment, cells were harvested for total protein extraction or nuclear fractionation. A and B, Representative images (A) and the pooled densitometry data (B) of Western blot analyses for nuclear (Nucl) and cytosolic (Cyto) p27 in VSMCs following LMB treatment. GAPDH and Histone H3 are probed as loading controls and markers for the cytosolic and nuclear fractions, respectively. Mean±SEM; n=3 biological repeats; three-way ANOVA followed by Tukey’s tests.
Figure 32. CSN5i-3 does not affect the nuclear-export of p27.

Shown are representative images of Western blot analyses for nuclear and cytosolic p27 in VSMCs treated with CSN5i-3 for 24 hours in the presence or absence of PDGF-BB. GAPDH and Histone H3 (H3) are used as loading controls and markers for the cytosolic and nuclear fractions, respectively.
3.2.7 To define the mechanistic role of CSN5 in the promotion of proliferation by CSN8 hypomorphism

Our *in vivo* and *in cellulo* findings led us to hypothesize that enhanced nuclear export plays an important role in the promotion of VSMC proliferation by CSN8 hypomorphism. Hence, we took the step to understand the mechanistic role of CSN5 in this observed phenomenon. Previously, it has been shown that CSN5 contains the typical leucine-rich nuclear export signal (NES) sequence conserved among different species though which CRM1 binds to CSN5 in an LMB-sensitive manner. As a result, CSN5 acts as an adaptor between p27 and CRM1 to mediate the cytoplasmic translocation of p27 in proliferating fibroblasts.\textsuperscript{105} Additionally, CSN5 also contains the MPN-JAMM motif responsible for the deneddylation function. Thus, we tested whether NES and the MPN motif of CSN5 are required and is responsible for the increased nuclear export and thereby increased proliferation by CSN8 hypomorphism.

3.2.7.1 Transfection of plasmid DNA in VSMCs

We determined the transfection efficiency of the mammalian expression vector harboring the expression cassette for EYFP-conjugated wild type CSN5 (EYFP-CSN5) in cultured VSMCs using fluorescence microscopy, live-cell imaging and fixed cells stained with DAPI. The results showed that >90% of the VSMCs were efficiently transfected with the plasmid as indicated by the fluorescence from EYFP (Figure 33A, 33B). Further confirming our results, Western blot analyses for CSN5 and YFP demonstrated that the transfection of the plasmids gave rise to a tagged protein band of EYFP-CSN5 at ~65 kDa. The transfected VSMCs showed decreased level
of endogenous CSN5 (**Figure 33C**), indicating that the transgenic CSN5 replaces the endogenous CSN5 in the CSN.
Figure 33. Transfection efficiency of plasmids expressing EYFP or EYFP-CSN5 in VSMCs.

VSMCs were transfected with the plasmid expressing EYFP-CSN5 or EYFP only. After 72 h of transfection, some plated cells were live imaged (A), fixed and stained with DAPI (B) or harvested for proteins (C–E). A, Representative brightfield (left) and fluorescent (right) live-cell images of VSMCs, 72 h post-transfection with EYFP-CSN5. B, Representative images of VSMCs post transfection stained with DAPI (blue) and imaged in YFP fluorescent channel. C–E, Western blot images probed for CSN5 (C) and YFP (D and E) in VSMCs transfected with EYFP-CSN5 (C and D) or EYFP alone (E). The black arrows in C–E denote the observed band for transgenic proteins in transfected VSMCs; the red arrow in panel C denotes the endogenous CSN5.
3.2.7.2 Effects of CSN5-ΔNES on VSMC proliferation in cellulo

Transfection of CSN5-ΔNES significantly decreased the PDGF-BB induced proliferation in both CSN8-hypo and control VSMCs and abolished the difference in PDGF-BB-induced proliferation between the CSN8-hypo and control VSMCs as evidenced by the changes in PCNA protein levels (Figure 34A, 34B). The tendency of change in PCNA expression in VSMCs transfected with plasmid expressing EYFP alone (control) was comparable with the previously observed results in VSMCs at baseline and PDGF-BB stimulation. Double immunostaining with Ki-67 and SM22α demonstrated that transfection with CSN5-ΔNES did indeed suppresses PDGF-BB induced proliferation in both CSN8-hypo and control VSMCs (Figure 34C). To check the effect of nuclear-export disabled CSN5 on p27 subcellular distribution, nuclear and cytoplasmic fractionation followed by immunoblotting was performed. Transfecting CSN5-ΔNES significantly increased the nuclear p27 in both CSN8-hypo and control VSMCs and abolished the difference in subcellular distribution of p27 between the two groups (Figure 35). To conclusively differentiate between the mediating proliferating effect by CSN8 hypomorphism, Western blot analysis for cullin proteins were performed, which showed no discernible changes in cullin de neddylation following the transfection with CSN5-ΔNES (Figure 37). Together, these results suggest that the promotion of nuclear export and resultant increases in VSMC proliferation by CSN5 is independent of the change in the de neddylation function.
Figure 34. Overexpression of nuclear export-disabled CSN5 (CSN5-ΔNES) abolishes the hyperproliferation in CSN8 hypomorphic VSMCs.

VSMCs were transfected with plasmids expressing EYFP-fused CSN5-ΔNES. The plasmids expressing EYFP alone were used as the control. The VSMCs were then treated with PDGF-BB or vehicle control for 24 h before harvested. A and B, Representative images (A) of Western blot analyses for PCNA and CSN5 and pooled densitometry data of PCNA (B) in VSMCs treated as indicated. C, Representative confocal micrographs of immuno-staining for Ki-67 (red) and SM22α (green) in VSMCs treated as indicated. Nuclei were stained with DAPI.
(blue). Scale bar= 100 µm. For the Western blot analyses for PCNA, loading control (L.C.) used total protein stain-free imaging. Mean±SEM; n=3 biological repeats; three-way ANOVA followed by Tukey’s tests.
Figure 35. Overexpression of nuclear export-disabled CSN5 (CSN5-ΔNES) restored nuclear localization of p27.

VSMCs were transfected with plasmids expressing EYFP-fused CSN5-ΔNES. The plasmids expressing EYFP alone were used as the control. The VSMCs were then treated with PDGF-BB or vehicle control for 24 h before harvested. A and B, Representative images (A) and the pooled densitometry data (B) of Western blot analyses for nuclear (Nucl) and cytosolic (Cyto) p27 in VSMCs treated as indicated. GAPDH and Histone H3 are used as a loading control for cytosolic and nuclear protein, respectively. Mean±SEM; n=3 biological repeats; three-way ANOVA followed by Tukey’s tests.
Figure 36. Western blot analyses for neddylated cullin 1 (Cul1) and Cul2 proteins in cultured VSMCs transfected with CSN5-ΔNES. VSMCs were transfected with plasmids expressing EYFP-fused constitutive nuclear CSN5 (CSN5-ΔNES). The plasmid expressing EYFP alone was used as the control. After the transfection, VSMCs were stimulated with PDGF-BB or vehicle control for 24 h before harvested for analyses. Shown are the Western blot images probed for Cul1 and Cul2 showing that the transfection of VSMCs with CSN5-ΔNES does not affect the deneddylation function of CSN5.
3.2.7.3 The effect of deneddylase-dead CSN5 on the increased proliferation by CSN8 hypomorphism

Our earlier experiments using chemical inhibition of the CSN deneddylase activity have shown that the deneddylation function of the CSN is dispensable for the proliferation promoting effect by CSN8 hypomorphism. To further establish that conclusion using a genetic approach, we tested the effect of overexpression of a deneddylase-disabled CSN5 (CSN5-ΔMPN) in cultured VSMCs. Transfection with CSN5-ΔMPN revealed that the PDGF-induced proliferation of control VSMCs was significantly suppressed by CSN5-ΔMPN, but the exacerbation of PDGF-induced proliferation by CSN8 hypomorphism remained unaffected (Figure 37A, 37B). In addition, transfection with CSN5-ΔMPN did not affect the nuclear-export of p27 (Figure 38). Collectively, these experiments establish that CSN5-promoted nuclear export of p27 rather than CSN5-dependent deneddylation activity is responsible for the greater PDGF-BB induced proliferation in CSN8-hypo VSMCs.
Figure 37. Overexpression of a deneddylation-dead CSN5 (CSN5-ΔMPN) suppressed the hyperproliferation in control VSMCs but not in CSN8 hypomorphic VSMCs.

VSMCs were transfected with plasmids expressing EYFP-fused CSN5-ΔMPN. The plasmids expressing EYFP alone were used as the control. The VSMCs were then treated with PDGF-BB or vehicle control for 24 h before being harvested. A, Representative images of Western blot analyses for PCNA and CSN5. B, Pooled densitometry data of PCNA in VSMCs treated as indicated. For Western blot analyses for PCNA, loading control (L.C.) used total protein stain-free imaging. Mean±SEM; n=3 biological repeats; three-way ANOVA followed by Tukey’s tests.
Figure 38. Overexpression of deneddylase-dead CSN5 (CSN5-ΔMPN) does not affect the nuclear export of p27.

VSMCs were transfected with plasmids expressing EYFP-fused CSN5-ΔMPN. The plasmids expressing EYFP alone were used as the control. The VSMCs were then treated with PDGF-BB or vehicle control for 24 h before being harvested. Shown are representative images of Western blot analyses for nuclear and cytosolic p27 in VSMCs treated as indicated. For Western blot analyses for PCNA, loading control (L.C.) used total protein stain-free imaging. GAPDH and Histone H3 are used as a loading control for cytosolic and nuclear protein, respectively.
3.2.7.4 The effect of overexpressing wild type CSN5 on VSMC proliferation

Previous studies in align with our results have shown that downregulation of CSN5 has inhibitory effect on proliferation of cells. Moreover, studies have found that ectopic expression of CSN5 can rescue NIH3T3 cells from p27 induced cell cycle arrest.\textsuperscript{109} Hence, we sought to determine the impact of overexpression of wild type CSN5 (WT-CSN5) on VSMC proliferation. The expression of PCNA proteins was used as the indicator of proliferation. The protein level of PCNA was significantly increased in PDGF-treated CSN8-hypo and control VSMCs in response to overexpression of WT-CSN5 (\textbf{Figure 39A, 39B}).
Figure 39. Overexpression of wild type (WT) CSN5 promotes PDGF-triggered cell proliferation in control and hypomorphic VSMCs.

VSMCs in cultures were transfected with plasmids expressing EYFP fused full-length CSN5 (WT-CSN5) or plasmids expressing EYFP alone (Control). The cells were then treated with PDGF-BB or vehicle control for 24 h and then harvested for protein extraction. A, Representative images of Western blot analyses for PCNA and CSN5. B, Pooled densitometry data of PCNA. Mean±SEM; n=3; three-way ANOVA followed by Tukey’s tests.
DISCUSSION

The CSN is involved in a plethora of important biological processes such as photomorphogenesis, early development, apoptosis, cell cycle progression and check point control. As such their regulation has emerged as an attractive target for therapeutic manipulation. To that end, regulation of the cell cycle by the CSN and CSN subunits have been studied genetically in several model systems, but the (patho)physiological significance of CSN minicomplexes has not been established in animals and the role of the CSN in VSMC function remains obscure. Disorders of vascular function and VSMC proliferation contribute to a large subset of diseases including CVD. Likewise, NH is a common pathological response to vascular injury. NH not only participates in many forms of diseases but also remains a vulnerable point for endovascular interventions. Thus, an improved understanding of molecular mechanisms leading to neointimal thickening could pave a way to the identification of new targets for therapy.

The present study provides the first demonstration of the existence and pathophysiological significance of the CSN5 minicomplex in an animal model and delineates for the first time the mechanistic roles of the CSN in VSMC proliferation and NH. Here we uncover that vascular injury triggers the upregulation of the CSN in VSMCs in animals and the upregulation of at least CSN5 in the VSMCs of NH in humans. We have further established that both cullin deneddylation by the CSN and the nuclear export exerted by CSN5 minicomplexes are responsible for the promotion of VSMC proliferation and NH by CSN5. Both CSN5-SMKO and pharmacological inhibition of the CSN deneddylase by CSN5i-3 effectively suppress VSMC proliferation and NH whereas CSN8 hypomorphism exhibiting increased CSN5 mini-complexes exacerbates VSMC proliferation and NH (Figure 40). Thus, the present study provides novel
mechanistic insights into the CSN as a pivotal regulator of vascular remodeling, suggesting the CSN can be novel targets for prevention and treatment of NH.

Figure 40. Graphical summary of the main findings.
1. Upregulation of the CSN by vascular injury

Here we have discovered that the CSN expression is upregulated in neointimal VSMCs in both animal models and human tissues of PAH. Both the mRNA and the protein levels of all examined CSN subunits as well as the CSN holocomplex were significantly increased in LCCA after ligation and the increase correlated with the increased VSMCs proliferation in the neointima (Figures 2-6). Prior studies have revealed that increased expression of CSN/CSN subunits were more often found in the tumor tissues of multiple forms of cancer and the increase frequently corelates with poorer prognosis.\textsuperscript{199,201-203} Complementarily to our findings, Asare \textit{et al.} reported increases in CSN1, CSN5 and CSN8 expression in the endothelial layer of early human atherosclerotic plaques,\textsuperscript{204} whereas Liang \textit{et al.} documented a critical role for loss of CSN6 at cardiac desmosomes in the pathogenesis of arrhythmogenic right ventricular dysplasia/caridiomyopathy.\textsuperscript{205}

Moreover, our \textit{in cellulo} data also reveals that the CSN subunits were upregulated in VSMCs stimulated with PDGF-BB (Figure 24). Comparable with our findings, a time-dependent upregulation of CSN6 was observed in cultured pulmonary arterial SMCs upon PDGF stimulation.\textsuperscript{206} Taken together, these findings suggest a pathogenic role for the CSN or its subunits in vascular remodeling. It will be interesting for future studies to determine the upstream events controlling CSN expression in the pathogenesis of vascular diseases.

2. The requirement of the CSN deneddylase activity for neointimal hyperplasia

By virtue of the regulation of the activity of CRLs through cullin deneddylation by the CSN holocomplex, the CSN has been shown to be critical to cell proliferation. CSN5 is an obligatory subunit of the CSN holocomplex and harbors the catalytic site of the CSN \textit{bona fide} activity, deneddylation.\textsuperscript{109} In the present study, we show that ablation of \textit{Cops5}, the gene
encoding CSN5, in SMC can cause moderate vascular dysfunction as evident by increased resistance, decreased vessel blood flow, and decreased systolic and diastolic diameters in the LCCA of CSN5-SMKO mice; and probably due to the vascular malfunction, moderate cardiac malfunction was also observed in the CSN5-SMKO mice (Figures 8 and 9). These findings indicate that CSN5 in SMCs is required for the normal functioning of blood vessels and thereby of the heart.

CSN5-SMKO drastically increased neddylated Cul1 and Cul2 while decreased Skp2 in LCCA walls both at baseline and after LCCA ligation. This is consistent with the notion that CSN5 is indispensable for the cullin deneddylation activity of the CSN and this bona fide activity of the CSN is required for maintaining protein stability of the substrate receptors of CRLs. Further, genetic ablation of CSN5 in SMCs attenuates VSMC proliferation and NH in response to vascular injury, as reflected by that LCCA ligation triggered significantly less increases in PCNA, Ki-67-positive VSMCs, and intimal thickness in CSN5-SMKO mice than in the control mice (Figure 10, 12). Similar effects were achieved by pharmacological inhibition of the CSN by CSN5i-3 both in vivo (Figures 15) and in cellulo (Figure 29). We reason that suppression of VSMC proliferation by impaired deneddylation acts through inactivating CRLs. It was purported that inactivation of CRLs by impaired cullin deneddylation is because the sustained ubiquitination by the CRLs turns to destroy their own substrate receptor modules, resulting in loss of key components of CRLs and accumulation of the substrate proteins of the CRLs. As a key substrate receptor for the CDK inhibitor p27 in the CRL1^Skp2^ ubiquitin ligase, Skp2 is essential for the ubiquitin-dependent degradation of p27 and thereby cell cycle progression. Here we detected that Skp2 proteins in vascular wall were markedly reduced by CSN5-SMKO (Figure 10) or CSN5i-3 (Figure 28) and, reciprocally, total and nuclear p27
were significantly increased by CSN5-SMKO in LCCA wall (Figure 10, 11) or by CSN5i-3 in cultured PDGF-BB stimulated VSMCs (Figure 30), presenting a molecular link between loss of CSN deneddylase activity and the suppression of NH. Consistent with our findings, CSN5i-3 was reported to suppress cell proliferation and viability in tumor cell lines and inhibit growth of tumor xenografts in mice.83,188

3. Significant contributions of CSN5-mediated nuclear export to the promotion of neointimal hyperplasia

Taking advantage of the increase of CSN5 minicomplexes by CSN8 hypomorphism where the expression of other CSN subunits is decreased and deneddylation activity of the CSN is compromised and dissociated from other functions, we provide here the first in vivo evidence of the existence of CSN5 minicomplexes and have unraveled a deneddylation-independent mechanism that governs SMC proliferation by CSN5. Prior study showed that CSN8 hypomorphic MEFs displayed an increased ratio of CSN5 mini-complex to the CSN holocomplex.88 Likewise, our study here reveals an increase in the abundance of cytoplasmic CSN5 minicomplex by CSN8 hypomorphism in VSMCs, accompanied by increased nuclear-export of p27 (Figures 21, 26). Importantly, NH and VSMC proliferation in vivo and in cellulo were exacerbated by CSN8 hypomorphism (Figure 16,17,23). Our data further demonstrate that enhanced nuclear-export by free CSN5 or CSN5 minicomplexes, independent of cullin deneddylation, is responsible for the proliferation-promoting effect of CSN8 hypomorphism. First, despite a reduction in protein levels of CSN5 and CSN6 by CSN8 hypomorphism (Figure 25), which is consistent with the notion that ablation of any individual CSN subunits disrupts the CSN holocomplex and results in loss of the cullin deneddylation activity and destabilization of partner CSN subunits,88,177 cytoplasmic CSN5 mini-complexes were significantly increased by
CSN8-hypomorphism (Figure 22, 27). Second, neither pharmacological nor genetic inhibition of CSN deneddylation activity could rescue the reduced nuclear p27 and the increased proliferative phenotype in CSN8 hypomorphic VSMCs (Figures 29, 32, 37, 38). And lastly, both inhibition of nuclear export by LMB and genetically disabling the nuclear-export function of CSN5 restored nuclear p27 levels and attenuated the CSN8 hypomorphism-induced VSMC hyperproliferation (Figure 29, 31, 34, 35). Corroborating our findings, Tomoda et al. reported that CSN5 served as an adaptor for shuttling p27 from the nucleus to the cytoplasm of proliferating fibroblasts.105 Together, this also suggested that the deneddylation and the nuclear-export function of CSN5 can act independently of each other, probably dependent on the incorporation of CSN5 in either holocomplex or minicomplex/free form.

Notably, although the CSN subunits were upregulated after vascular injury and PDGF-BB stimulation in controls, CSN8 hypomorphism prevented this upregulation (Figure 24) but exacerbated NH and VSMC proliferation (Figures 16, 17, 23). Previously, CSN8 was shown to be necessary for the cell cycle re-entry in periphery T cells and MEFs and essential for postnatal hepatocyte survival and effective proliferation.88,140,210 Surprisingly, though CSN8 hypomorphism showed increased p27 and CSN5 in the cytoplasm, there was no difference observed in terms of proliferation in baseline, suggesting that an increase in CSN5 mini-complex is not enough to initiate cell proliferation but is capable of promoting the proliferation process by, for example, shortening the G1 phase.88

We here for the first time report the existence of CSN5 in holocomplex-independent forms in vessel but notably, the function of these minicomplexes becomes apparent in cell proliferation after the injury or PDGF-BB stimulation (Figure 22, 26). Further experimental support for our contention also comes from our findings that CSN5-SMKO mice displayed
decreased p27 nuclear-export and less severe NH after injury and that nuclear-export disabled CSN5 abolished the proliferation in control cells.

It was previously reported that in CML cells, oncogenic signal initiated by Bcr-Abl tyrosine kinase through Ras/MAPK and PI3K pathways regulates the CSN5-containing minicomplexes which apparently modulate the p27 level. Considering regulation of kinases is one of the many other functions by the CSN complex, it is therefore intriguing to conjecture that the regulation of the minicomplex in VSMCs may be a part of a downstream signaling mediated by kinases. Nevertheless, it will be interesting to determine in the future how the minicomplex is regulated in a pathophysiological scenario and whether nuclear export is an exclusive function of CSN5 minicomplex, or whether other subunits are involved.

As p27 gets translocated to the cytoplasm, initially it is ubiquitinated by the ubiquitin ligase complex KPC1/2, but later in the cell cycle, it is the second ubiquitin ligase complex, SCF$^{Skp2}$, that mediates the ubiquitination and degradation of p27 by the UPS. The CSN5 minicomplex suppresses p27 by nuclear-export while the CSN holocomplex inhibits p27 via Cul1 deeddlylation to support the ubiquitination of p27 by the SCF$^{Skp2}$ ubiquitin ligase complex. Hence, it is conceivable that CSN5 equilibrates between two distinct forms of CSN complexes which act either sequentially or in parallel to inhibit p27 and thereby propel the progression of the cell cycle.

4. Targeting CSN5 to treat neointima hyperplasia

NH is prominent in the pathology of primary PAH, a rare but devastating disease that lacks effective clinical treatment beyond lung transplantation. Restenosis due to NH remains a prevailing clinical problem and aberrant VSMC proliferation threatens almost every known vascular reconstructive procedure, bringing new cardiovascular risk to postoperative patients.
The mechanism underlying this proliferative process is not completely elucidated, hindering the search for effective countering measures. The present study demonstrates not only that genetic ablation of CSN5 in SMCs inhibits VSMC proliferation and NH in animals (Figure 12) but also that pharmacological inhibition of the CSN by CSN5i-3 exerts striking suppression on VSMC proliferation in cellulo (Figure 29) and on NH in animals (Figure 15), providing extremely promising support for targeting the CSN to treat NH-based disorders, such as PAH and restenosis. For collecting proof-of-principle evidence, present study only tested one effective regime of CSN5i-3 to suppress VSMC proliferation and NH after LCCA ligation, where the treatment is well tolerated although moderate cardiotoxicity was observed at the end of treatment regime (Figure 14). This is in agreement with the more severe phenotype caused by cardiomyocyte-restricted knockout of CSN8 in mice. Hence, it will be important to further test the effectiveness of lower degrees and shorter durations of pharmacological inhibition of the CSN for clinical translation. To this end, it will be extremely interesting to test the effect of local and controlled administration of the inhibitor(s) via, for example drug-eluting stents on post-operative restenosis.
CONCLUSIONS

Overall, the present study has collected multiple lines of unequivocal evidence that compellingly supports our novel hypothesis that the CSN promotes VSMC proliferation and NH through both deneddylation dependent and independent mechanisms.

In WT mice, vascular injury triggered an increase in the mRNA and protein levels of CSN subunits. The increase of the CSN subunits was observed alongside the increase in the abundance of the CSN subunits in the holocomplex. Correspondingly, in human lung tissue of PAH, CSN5 expression was increased in the neointimal SMCs of pulmonary artery compared to the SMCs in the control pulmonary artery. These findings in animal models and human tissues reveal that the CSN/CSN5 is increased VSMCs in response to vascular injury.

CSN5-SMKO mice exhibited impaired cullin deneddylation and nuclear export of p27 in vessel walls. By 3 months after tamoxifen induction, CSN5-SMKO mice showed discernible abnormalities in blood velocity, diastolic and systolic diameters, and cardiac functional parameters. More importantly, CSN5-SMKO suppressed NH and VSMC proliferation after vascular injury. Meanwhile, CSN8 hypomorphism in animals and cultured VSMCs displayed increased cytoplasmic CSN5 minicomplexes that were correlated with increased nuclear-export of p27 and hence, showed exacerbated NH and VSMC proliferation. This demonstrates the necessity of SMC CSN5 in vessels and an important regulatory role for the deneddylation as well as deneddylation-independent functions of CSN5 following vascular injury.

Pharmacological inhibition of nuclear-export as well as genetically disabling the nuclear-export of CSN5 abolished the PDGF-BB induced increases in proliferation and restored nuclear p27 in both CSN8-hypo and control VSMCs in cell cultures. On the other hand, pharmacological and genetic inhibition of CSN deneddylyase did not affect the nuclear exclusion of p27 but
suppressed the induced proliferation in control VSMCs. Importantly, the blockade of the
deneddlylation function did not influence the proliferation of CSN8-hypo VSMCs abiding by the
notion that deneddlylation function of the CSN requires all 8 CSN subunits assembled in the
holocomplex. These findings establish that the nuclear-export function of CSN5 rather than the
deneddlylation function is responsible for the greater induced proliferation in CSN8-hypo cells.
This also supports that the nuclear export and deneddlylation function of the CSN are
independent of each other.

In WT mice, consistent with the *in cellulo* and *in vivo* findings, pharmacological
inhibition of the deneddylase function suppressed NH and VSMC proliferation, exhibiting great
promise for the CSN inhibitor CSN5i-3 to be explored for the prevention of NH.

In summary, the present study compellingly demonstrates that the CSN plays a pivotal
regulator role in vascular remodeling after vascular injury, delineates that the CSN does so at
least in part through promoting VSMC proliferation and NH, which is in turn mediated at the
molecular level by both cullin deneddlylation and the CSN5 minicomplex exerted nuclear export
of cell cycle suppressors such as p27, and thereby identifies targeting the CSN as a potentially
novel strategy for NH prevention and treatment.
LIMITATIONS

This study does have limitations. Phenotypic switching of VSMCs, hallmark of NH is primarily characterized by the increase in the VSMC proliferation which occurs in concert with the decrease in contractile markers. We examined the role of the CSN in proliferation but did not explore the possibility of how CSN could affect the contractile proteins in this pathophysiological scenario.

To determine the baseline characteristics and the necessity of CSN5 in the vessel, we performed serial echocardiography and doppler ultrasound up to 4 months after tamoxifen induction of CSN5-SMKO when the mice survived well under baseline conditions but began to show moderate phenotypes. Further baseline characterization of the impact of CSN5-SMKO on the wellbeing is warranted as to help further and more completely decipher the physiological significance of SMC CSN5.

To provide only proof-of-principle evidence for deneddylase inhibition by CSN5i-3 on NH, we only tested the administration of 20 mg/kg/day dosage in response to vascular injury, which very likely limited our ability to explore the mediating effect of other dosage and administering site. An optimization of lower dosages at a local administration site could have had better outcomes in terms of prevention.
FUTURE DIRECTION

We have uncovered that the CSN subunits are upregulated in response to vascular injury and to PDGF-BB. Alteration in the homeostatic level of the CSN may imply a form of regulatory mechanism contributing to the pathological process. It will be interesting to determine in the future how the upstream signaling regulator such as PDGF-BB controls the expression of CSN genes.

The present study has demonstrated with strong evidence that both Cullin deneddylation and CSN5-mediated nuclear exclusion as an important underlying mechanism in vascular remodeling after injury. We compellingly showed that CSN5 in a cytoplasmic form as a minicomplex mediates the nuclear exclusion that is responsible for the increased proliferation by CSN8 hypomorphism. It will be interesting to determine whether the downregulation of other non-CSN5 subunits other than CSN8 would yield similar effects to CSN8 hypomorphism and whether formation of CSN5 minicomplex is required. Previously reported studies have shown that among the two forms of CSN5 that exist in a cell, the smaller one found in the cytoplasm contains either the free CSN5 or the CSN5 associated with only a subset of CSN components CSN4-CSN8 but not CSN1-CSN3. It will be interesting to understand if other CSN components in a CSN5-associated minicomplex have a role to play in the nuclear exclusion or if it is the act of free CSN5.

Cell proliferation and cell death are essential yet opposing cellular processes which are however linked by cell-cycle regulators that affect both processes. Previously it was reported that CSN8 hypomorphism mouse heart under stress condition impairs cardiac autophagy. Further, CSN5i-3 has been shown to suppress cell proliferation and induce apoptosis in human breast
cancer cells.\textsuperscript{188} It will be interesting to examine if the cell death is affected by CSN8 hypomorphism and the CSN deneddylase inhibitor in vessels.
APPENDIX I

Protocol of ligation of the left common carotid artery to induce NH

1. Place the mouse in an induction chamber until sedation (under 4.0% isoflurane).
2. Transfer the mouse to a station and position the snout fully within a nose cone connected to an anesthesia system to maintain a steady state sedation level (2% isoflurane with 100% O2).
3. Sterilize the neck region of the mouse by gently wiping it with 70% ethanol. Make an incision in the neck region of the mouse and locate the left carotid bifurcation.
4. Ligate the carotid artery with 6.0 silk suture approximal to the bifurcation.
5. Once the ligation is done, suture the neck incision and sterilize the incision area with a povidone-iodine swab stick.
6. Monitor the mice until recovery and use buprenorphine SR (1.0 mg/kg, subcutaneous injections) as an analgesic. Animals were then observed each day until tissue collection for pain or any health-related problems according to IACUC protocol.
7. After a certain time after ligation, sacrifice the mice and collect ligated LCCA section for further processing. The right common carotid artery (RCCA) was used as the uninjured intra-animal control vessel.
APPENDIX II

Isolation and Culture of Vascular Smooth Muscle Cells

I. Reagents required for the procedure

Prepare complete media by combining DMEM with 10% FBS and 1% penicillin/streptomycin and store at 4°C until use.

Prepare 70% ethanol by mixing 70 mL of 100% ethanol and 30 mL of distilled water and store at room temperature.

Prepare Fungizone by adding 10 μL of Fungizone to 10 mL of complete media and filter-sterilize using a sterile disposable vacuum filtration unit. Prepared Fungizone can be stored for a week at 4°C.

Prepare Collagenase Type II in complete media. Weigh and dissolve 14.2 mg of Collagenase Type II in 10 mL of complete media (final concentration 1.42 mg/mL) and filter-sterilize using sterile disposable vacuum filtration unit (0.45 μM filter). The volume of collagenase prepared can be adjusted according to the number of mice to be used. Prepared collagenase type II can also be stored at 4°C for a week.

Prior to euthanizing the mice, warm complete DMEM, collagenase type II, and Fungizone solutions to 37°C in a water bath.

II. Procedure steps

1. Euthanize mice with a continuous flow of carbon dioxide using a flow meter unit for 3–5 min at the flow rate of 2 L/min.
2. Weigh the mouse and secure it to a flat moveable surface in a supine position prior to viewing it under a dissecting microscope.

3. Wipe the abdomen and thorax regions with alcohol prep pads or 70% alcohol and open the skin to expose the abdomen and the thorax using surgical scissors.

4. Remove the lungs and expose the entire aorta until the renal bifurcation. Perfuse the aorta using a tuberculin syringe filled with sterile PBS to remove the remaining blood.

5. Performing in an efficient and rapid manner, the aorta is dissected out and placed in ~100 μL of Fungizone in a 6-well tissue culture plate and cleaned off of the surrounding fat and adventitial tissue with the help of fine-tipped forceps. Cut the aorta into 1- to 2-mm pieces using fine scissors and place into 5-mL tubes containing 0.1 mL of collagenase type II solution. Gently close the lid of the tube and place in a 37 °C incubator (with 5 % CO2) for 6 h.

6. After 6 h, remove the 5-mL tubes, and tap to suspend the cells. In the hood, add 3 mL of complete DMEM in the tube and gently mix the cell suspension.

7. Centrifuge at 300×g for 5 min at room temperature in a tabletop centrifuge to pellet the cells.

8. In the hood, remove the medium and wash the pellet using 3 mL of complete DMEM and centrifuge again at 300×g for 5 min.

9. Resuspend the resulting cell pellet in 0.5–1 mL of complete DMEM and plate cells in a 24-well plate.

10. Place cells in 37 °C incubator with 5 % carbon dioxide, undisturbed for 6 days without changing the media.

Subculturing of Murine Aortic VSMCs

11. When confluent, passage the cells to achieve confluency on the 6th day.
12. First, wash all the wells using 500 \( \mu \)L 1X sterile PBS. Incubate cells in 200 \( \mu \)L of trypsin with 0.25 % EDTA for 3–4 min and tap the plate gently to detach the cells. Do not mix vigorously.

13. Neutralize trypsin by adding 500 \( \mu \)L of complete DMEM. Pellet the cells by centrifuging at 800\( \times \)g for 10 min, remove the supernatant, and resuspend the cells in complete DMEM.

Figure 41. A flow chart for the isolation and culture of vascular smooth muscle cells (VSMCs) from mouse aorta.
APPENDIX III

Preparing proteins from carotid arteries and cultured VSMCs for SDS-PAGE

Carotid arteries collection

1. Anesthetize the animal. Open the neck region and take the carotid arteries out in cold PBS.
2. Gently remove the adventitial tissues surrounding the arteries and snap freeze in liquid nitrogen. Store the sample at -80°C until further use.

<table>
<thead>
<tr>
<th>1X Lysis Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3M Tris-HCL, pH 7.0</td>
</tr>
<tr>
<td>10% SDS</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Deionized water</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>0.5 ml</td>
</tr>
<tr>
<td>4.5 ml</td>
</tr>
<tr>
<td>3 ml</td>
</tr>
<tr>
<td>28.5 ml</td>
</tr>
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</table>

Total Protein Preparation

1. To prepare proteins, add 40 μl of 1X loading buffer per 0.8-1 mm of carotid arteries.
2. For efficient protein extraction, cut the carotid arteries into smaller pieces.
3. Extensively vortex the mixture of loading buffer and tissue/cells.
4. Boil the homogenates for 5 minutes.
5. Centrifuge the samples at 10000 rpm for 10 minutes at 4°C.
6. Separate the supernatant and the total protein fraction into a fresh tube.

Cultured VSMCs collection

1. Wash the cultured VSMCs twice with cold PBS.
2. For 60mm cell culture dishes, add 100 μl of 1X lysis buffer to the cells and scrape the cells off.
Protein Concentration Determination

Bicinchoninic acid (BCA) method is used to quantify protein concentration using Pierce BCA Protein Assay Kit (Catalog#23225, ThermoFisher scientific, Waltham, MA)

1. In a test tube, mix 1 μl of the samples in 39 μl of water.
2. Run parallel analysis of a series of known concentrations of bovine serum albumin for the development of the standard curve.
3. Add 200 μl of the mix solutions of reagent A and B (50:1 ratio) from the BCA kit to a 96 well plate with conical bottom.
4. Incubate the samples inside a 37°C incubator for 30 minutes.
5. Transfer the samples containing 96 well plate into a spectrophotometer cuvette and measure absorbance at 562 nm.
6. Use the absorbance to extrapolate the protein concentration of the sample from the standard curve.

Sample preparation for SDS-PAGE

1. Add loading buffer [2X times with b-mercaptoethanol (Catalog#M13148, Sigma, Livonia, MI)] to the aliquot of protein sample.
2. Incubate samples in 100°C water bath for 5 minutes. Cool the samples followed by short centrifugation to get the samples into the bottom of the tube before loading on to the gel.
APPENDIX IV

A Protocol for Western Blot Analysis

Gel casting Recipe

The formulation for 2 resolving gels.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
<th>14%</th>
<th>16%</th>
<th>18%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>9.4 mL</td>
<td>7.9 mL</td>
<td>6.6 mL</td>
<td>5.4 mL</td>
<td>4.2 mL</td>
<td>2.8 mL</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>5 mL</td>
<td>5 mL</td>
<td>5 mL</td>
<td>5 mL</td>
<td>5 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>30% Bis Acrylamide</td>
<td>5.4 mL</td>
<td>6.7 mL</td>
<td>8.0 mL</td>
<td>9.4 mL</td>
<td>10.6 mL</td>
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<tr>
<td>10% APS</td>
<td>100 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
<td>8.5 µL</td>
<td>8.5 µL</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
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</tbody>
</table>

Recipe for 2 Stacking gels:

<table>
<thead>
<tr>
<th>Reagent</th>
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</thead>
<tbody>
<tr>
<td>H2O</td>
<td>5.5 mL</td>
</tr>
<tr>
<td>1.0 M tris pH 6.8</td>
<td>1 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>80 µL</td>
</tr>
<tr>
<td>30% Bis Acrylamide</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>80 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>8.3 µL</td>
</tr>
</tbody>
</table>
PBS:

Dulbecco’s Phosphate Buffered Saline (9.5 g/L of H₂O)

PBS-T:

PBS + Tween 20 (detergent) (1mL Tween 20/L of H₂O)

10X Transfer Buffer (per Liter)

30.3 g Tris

144 g Glycine

Running Buffer

100 mL 10X transfer buffer

900 mL water

10 ml of 10% SDS (not in the common 10X mix)

Transfer Buffer (Keep chilled)

100 mL 10X transfer buffer

200 mL methanol
700 mL water

**Blocking Buffer**

2% BSA (2g BSA in 100mL PBS-T)

**1X Loading Buffer-no color dye**

0.5 mL 3 M Tris HCl

4.5 mL 10% SDS

3.0 mL Glycerol

28.5 mL H$_2$O

**3X Loading Buffer**

5 mL 3M Tris

3 mL 10% SDS

3 mL Glycerol

0.01% Bromophenol Blue

1.2 ml BME before use
Loading and Running Gels

1. Set up the casting frame with a short place, spacer plate, and combs to make the appropriate percentage of gel (low percentage for high molecular weight proteins and high percentage of gel for low molecular weight proteins).

2. Pour the gel in-between the plates up to 1cm below where the comb would be. Allow gel to polymerize at room temperature.

3. Prepare and place the stacking gel on top of the separating gel with an appropriately sized comb between the glass plates.

4. To start preparing for the gel running, carefully remove the comb after the gel is settled.

5. Remove the gel cassette sandwich from the casting stand and place it in the clamping frame of the running tank. Once placed inside the Mini tank, fill it with a running buffer.

6. Start to boil your protein samples for 5 minutes, and spin down at the end of boiling.

7. Load the first outside well with the ladders, Precision Plus Protein dual color standards (Catalog#1610374, BioRad, Hercules, CA) and load the remaining wells with the protein samples.

8. Plug in the color-coded leads into the power supply and run the gels for the appropriate amount of time at 100 volts.

9. Prepare the PVDF membrane and filter paper to the size of the gel. Soak the membrane in methanol for 5 minutes. Followed by soaking the membrane, filter paper, and filter pads in the transfer buffer.

10. When the loading buffer riches to the end of gel, remove the gel from the plates by carefully taking off the short plate and cutting along the edges of the gel, and rinsing the gel with water.
11. Stain-free technology was then used to take a total protein image of the gel.

12. Arrange the sandwich from bottom to top; lay the black side, filter-pad, filter paper, gel, membrane, filter-paper, filter-pad, and clear side, and roll out any bubbles with a pipet over the filter paper.

13. Close the pad in the black and white sandwich holders, gel to the (-), membrane to (+).

14. Slide the cassettes into the transfer box, add an ice pack, and put them in an ice-filled tub.

15. Fill the transfer tank with transfer buffer and add ~5 mL of 10% SDS sprinkled on top. Run at 100V for 140 minutes or 30V overnight.

16. Disconnect the leads from the power source and remove the membrane while keeping the membrane protein side up. Alternatively, use the ChemiDoc MP system to take stain-free blot images for total protein measurement.

17. Rinse the membrane once in PBS for 5 minutes and twice in PBS-T for 5 minutes each, followed by a 1-hour block at room temperature in a blocking buffer (2% BSA in PBS-T).

18. Incubate the membrane with the primary antibody at a proper dilution in the appropriate blocking overnight with gentle rocking.

19. Wash the membrane at least three times in PBS-T or TBS-T for 5 minutes wash.

20. Mix solution A and solution B of the ECL chemiluminescent reagents, SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Catalog#34578, ThermoFisher Scientific, Waltham, MA) at 1:1 ratio enough to cover the blot.

21. Place the PVDF membrane face on a clean glass plate and cover it with ECL mixtures.

22. Image lab 6.0 was utilized to perform densitometry and quantification of the western blot.
APPENDIX V

A Protocol for Immunofluorescence Staining

Sample Fixation

From cultured VSMCs

1. Remove the culture medium from the dishes and wash the VSMCs with PBS twice.
2. Fix the cells with 4% paraformaldehyde in PBS for 10 minutes and wash with PBS twice.
3. Allow the cells to be set in PBS until further processing.

From mouse carotid arteries

1. Remove the carotid arteries, wash in PBS, and place the tissue in the 4% paraformaldehyde in PBS for at least 12 hours.
2. Equilibrate the tissue in a gradient of sucrose.
3. Embed the carotid tissues in the Tissue-Tek O.C.T. Cut the tissue block with a 20°C cryostat.
   Place the 7-micron thick sections on charged glass slides for further use.

Fixed cells/Cryosections

1. Permeabilize the cells/cryosections with 0.1% Triton X-100 in PBS for 15 minutes at room temperature.
2. Quench the cells/cryosections with 1% glycine in PBS for 1 hour. Wash 2-3 times with PBS.
3. Block the cells/cryosections with 2% BSA in PBS for 1 hour.
4. Incubate the cells/cryosections with primary antibody at proper dilution (in 2% BSA) overnight at 4°C in a moist chamber. Wash at least three times with PBS to remove any unbound primary antibody.
5. Add a secondary antibody (in 2% BSA) and incubate in a moist chamber for 1 hour at room temperature. Wash at least 3 times in PBS.

6. Mount the cells/cryosections with an aqueous mounting medium (DAPI). Seal the edges of the coverslip with nail polish and store them in the -20°C freezer until ready to take the image.

Solutions

4% paraformaldehyde
Paraformaldehyde  4 g
PBS  80ml

Adjust pH to 7.4 with 1 M HCL
Adjust the final volume to 100 ml with PBS
Make fresh and store in aliquots at -20°C

30% Sucrose
Sucrose  30 g
Deionized water  100 ml

1% Glycine
Glycine  1 g
PBS  100 ml

1 % Triton X-100 in PBS
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<td>Triton X-100</td>
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<tr>
<td>PBS</td>
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<td>2% BSA in PBS</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
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<tr>
<td>PBS</td>
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</table>
APPENDIX VI

Native Gel Electrophoresis

Native polyacrylamide gel electrophoresis recipe

1 M Tris, pH 7.5

Dissolve 121.14 g Tris into 900 ml deionized H2O, adjust pH with concentrated hydrochloric acid to pH 7.5, fill up with deionized H2O to 1 L and autoclave. Can be stored at room temperature indefinitely.

M NaCl

Dissolve 292.2 g NaCl into 600 ml deionized H2O and add deionized H2O to 1L. Can be stored at room temperature indefinitely.

1 M MgCl2

Dissolve 95.21 g MgCl2 into 800 ml deionized H2O and add deionized H2O to 1L. Can be stored at 4°C indefinitely.

500 mM ATP in Tris, pH 7.0

ATP, disodium salt trihydrate

2 M Tris base
Dissolve ATP as much as possible in 15 ml of Tris base. Continue to add Tris base dropwise while stirring until ATP is completely dissolved. Determine the pH and adjust to 7.0 with additional Tris base or HCl until pH = 7.0. Bring the final volume to 25 ml using distilled H2O. Make aliquots and store them at -80°C.

Extraction buffer: 50 mM Tris-HCl, pH 7.5, 1mM ATP, 5 mM MgCl2, 1 mM DTT, 250 mM Sucrose

Acrylamide stock solution: 30% acrylamide, 0.8% bis-acrylamide

Separating gel buffer (TBE) (4X): 360 mM Tris, pH 8.3, 320 mM boric acid, 0.4 mM EDTA.

MAD (10X): 50 mM MgCl2, 10 mM ATP, 10 mM DTT.

Native gel loading buffer (4X): 200 mM Tris-HCl, pH 6.8, 60% (v/v) glycerol, 0.05% (w/v) bromophenol blue.

Electrophoresis buffer: 90 mM Tris, 80 mM boric acid, 0.1 mM EDTA, 5 mM MgCl2, 1mM ATP, and 1mM DTT.
Blotting buffer: 25 mM Tris, 192 mM glycine, 20% methanol, 0.1% sodium dodecyl sulfate (SDS)

Preparation of the gel

1. Set up the gel casting system.

2. Prepare the separating gel (4.0%) by mixing the following:

   a. TBE (4X) 3.75 ml
   b. Acrylamide stock 2.00 ml
   c. Sucrose (10%) 3.75 ml
   d. MAD (10X) 1.50 ml
   e. H2O 4.00 ml
   f. Ammonium persulfate (10%) 0.05 ml
   g. TEMED 10 μl

3. Mix carefully and fill in the solution to about 4 cm from the top of the glass plates. Carefully overlap it with distilled H2O.

4. Let the gel polymerize for at least 30 min.

5. Prepare a stacking gel (2.5%) by mixing the following:

   a. 1 M Tris-HCl, pH 6.8 0.50 ml
   b. Acrylamide stock 0.83 ml
c. H2O 8.70 ml
d. Ammonium persulfate (10%) 0.05 ml
e. TEMED 10 μl
f. 500 mM ATP 20 μl

6. Pour off the water over layering the gel, rinse with water, dry it, and fill in the stacking gel solution up to the top of the glass plates. Insert the well forming comb between the glass plates.

7. Let the stacking gel polymerize for at least 30 min.

8. Remove the comb and fill the slots with electrophoresis buffer.

Gel electrophoresis

VSMCs lysates or carotid arteries homogenates are plunged through a 29G gauge needle 8 times with an insulin syringe before centrifugation at 4°C for 30 min (15,000 × g).

1. Determine protein concentration with BCA reagents and dilute samples with 4× native gel loading buffer.

2. Load the samples on the gel, connect the power supply to the gel apparatus and adjust the current to 15 mA. Continue the electrophoresis for 8 hours at 4°C. Remove the gel from the glass plates either with a detection of proteasomal activity in the gel or by transfer of the proteins to a membrane for immunological detection.
Western blot analysis

Before transfer, the entire gel is soaked in blotting buffer for 10 minutes and transferred at 250 mA for 1.5 h. Once the transfer is complete, all of the usual techniques for Western blot apply.
APPENDIX VII

Protocol for RNA Isolation and Reverse Transcription PCR

I. Isolation of RNA from carotid arteries

1. Homogenize the carotid samples (performed on ice) in pre-cooled TRI reagent (Catalog#NC9330796, Molecular Research Center Inc., Cincinnati, OH) using a Bullet Blender homogenizer (Catalog#NA-BT5E, Stellar Scientific, Baltimore, MD). Use 1 ml of reagent for 50-100 mg of tissue. If RNA isolation is not carried out immediately after tissue collection, store the tissue sample in 1 ml of RNA Later at 4°C for up to a week.

2. Allow complete dissociation of nucleoprotein complexes by storing the homogenate in room temperature for 5 minutes.

3. Transfer the homogenate into a microcentrifuge tube and add 0.2 ml chloroform to the homogenate for every 1 ml of TRI reagent that was used in the beginning. Cap the tube tightly and shake vigorously for 15 seconds.

4. Store the mixture at room temperature for 2-15 minutes and centrifuge at 12,000 g for 15 minutes at 4°C.

5. Carefully transfer the clear upper aqueous phase into a fresh microcentrifuge tube.

6. Precipitate RNA from the aqueous phase by mixing with 0.5 ml isopropanol per 1 ml of TRI reagent used initially.

7. Store the mixture at room temperature for 5-10 minutes and centrifuge at 12,000 g for 8 minutes at 4°C.

8. Remove the supernatant and wash the RNA pellet with 1 ml of 75% ethanol.

9. Centrifuge the sample at 7,500 g for 5 minutes at 4°C and air dry the pellet for 3-5 minutes.

10. Store the RNA pellet in 100% ethanol or RNA/DNA-free water at -80°C until further use.
II. Reverse Transcription

1. Mix and briefly centrifuge each component before use.

2. Combine the following in a 0.5 ml tube to prepare RT-PCR kit

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Buffer</td>
<td>2 μl</td>
</tr>
<tr>
<td>25X dNTP</td>
<td>0.8 μl</td>
</tr>
<tr>
<td>10X primer</td>
<td>2 μl</td>
</tr>
<tr>
<td>Reverse Transcriptase</td>
<td>1 μl</td>
</tr>
<tr>
<td>DEPC water</td>
<td>4.2 μl</td>
</tr>
<tr>
<td>Total</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

3. Add 10ul RT-PCR kit and 10 μl of RNA sample in each new PCR tube.

4. Mix gently and collect by brief centrifugation. Incubate in the PCR machine using the following program:
   a. 10 minutes at 25°C
   b. 120 minutes at 37°C
   c. 85°C for 5 minutes

5. Collect the mixture by brief centrifugation.

6. The cDNA synthesis reaction can be stored at -20°C or used for further experiments.
APPENDIX VIII

A Protocol for Immunohistochemistry

I. Deparaffinization of paraffin sections

2. Deparaffinize sections in xylene, 3×5 min.

3. Hydrate with 100% ethanol, 2×2 min.

4. Hydrate with 95% ethanol, 2×2 min.

5. Rinse in distilled water.

6. Follow procedure for pretreatment as required.

II. Antigen retrieval

Most formalin-fixed tissue requires an antigen retrieval step before immunohistochemical staining can proceed. The paraffin slides were brought to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at a sub-boiling temperature for 15 minutes. Cool slides on the bench top for 30 minutes.

III. Staining Procedure

1. Rinse sections in PBS-Triton X-100 (0.025%) for 2×2 min.

2. Serum Blocking: incubate sections with 3-4 drops of RTU normal goat serum for 30 minutes to block non-specific binding of immunoglobulin.

3. Primary Antibody: incubate sections with primary antibody (mouse/rabbit IgG) at appropriate dilution in antibody dilution buffer for 1-2 hours at room temperature or overnight at 4 °C. Rinse in PBS.
4. Peroxidase Blocking: incubate sections in 0.3% hydrogen peroxide in PBS for 10 minutes at room temperature. Rinse in PBS.

5. Detection: incubate sections with 3-4 drops of RTU polymeric peroxidase anti-mouse/rabbit secondary antibody for 30 minutes at room temperature.

6. Rinse in PBS for 3×2min.

7. Chromogen/Substrate: incubate sections with 3 drops of DAB solution for 2-8 minutes.
   Monitor signal development under a microscope. DAB solution is made by a mixture of 25 µl of DAB stock solution with 1ml of DAB buffer.

8. Rinse in distilled water for 2×2 min.

   Rinse in tape water 2×2 min.

10. Dehydrate through 75% ethanol for 2 min, 95% ethanol for 2 min, and 100% ethanol for 2x3min. Clear in xylene for 2×5min.

11. Coverslip with mounting medium, Permount (Catalog#SP15-100, ThermoFisher Scientific, Waltham, MA).
APPENDIX IX

Protocol for Tamoxifen Administration in mice

1. Dissolve 40mg tamoxifen (Catalog#HY-70062; MedChem Express, Monmouth, NJ) in 400 μl 100% ethanol. For a complete solubilized tamoxifen, you need an extensive vortexing at high speed.

2. Add tamoxifen/ethanol solution to 3.6 ml of autoclaved sunflower seed oil at a final concentration of 10 μg/μl. Oil should be autoclaved and cooled down to room temperature. Always prepare the mix fresh and save the tamoxifen/oil emulsion in a sterile glass vial (absolutely do NOT use a plastic container; tamoxifen will stick to plastic). Old oil will not only lose solubility but also will cause animal peritoneal inflammation and granulomas after injection.

3. Sonicate the glass vial of tamoxifen/oil emulsion in a float for 30 mins in an ultrasonic cleaner bath.

4. Wrap up the glass vial using aluminum foil and save at room temp. up to 1 month. It can be saved in -20° at least for 3 months, but it should be sonicated before each use and avoid freezing and thaw cycles.

5. Use 100ul tamoxifen/oil mix (1 mg tamoxifen/mouse) for each intraperitoneal injection (IP) to each adult mouse (8-12 weeks old, ~20 grams). Each animal is IP 2 rounds of 5 days with 2 days break between (i.e., first round IP from Mon. to Fri. each day for 5 days, break at weekends, start another round IP from next Mon to Fri for another 5 days; total 10 injections).

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6. After the last IP, the animal should wait for at least 1 week to wash off the side effects of exogenous tamoxifen and for 2 weeks to start experiments.
1. After weighing the mouse, place and anesthetize the mouse in an induction chamber for approximately 1 minute until the sedation (under 4.0% isoflurane).

2. Place the mouse on a warm pad in the supine position and maintain the body temperature around 37°C. Remove hair from the mouse’s chest using hair removal cream. Once the hair is removed, wipe the area with wet gauze to ensure all hair is removed.

3. Keep the mouse under light anesthesia with isoflurane (1%~1.5%) in room air supplemented with 100% oxygen through a nose cone. Limbs are taped to the ECG leads for electrocardiogram monitoring. Heart rates are maintained at 400-500 beats per minute (maintain anesthesia for proper heart rate).

4. Place an ample amount of warmed ultrasound gel on the shaved chest.

5. Lower the probe (40 MHz) to the gel until it makes contact, and make sure all areas of the probe are covered with gel and the image is free of bubble interference.

6. Place the probe along the long-axis of LV and record a video image of the heart in the long-axis in B-mode. Ensure that the heart is level from the aortic inflow to the apex and that the probe is adjusted to mid-heart. Drop an M-mode line, transition the computer to M-mode, and record an M-mode video at mid heart.

7. Rotate the probe clockwise to 90° and record a video of the LV short-axis view in B-mode. When in B-mode, optimize the cardiac image for wall clarity and the presence of papillary muscle.

8. Once the imaging is complete, remove the residual ultrasound gel on the chest, and return the mouse to the cage for recovery.
9. After collection, videos were reviewed and measured using Vevo LAB software at an offline location.

10. Short-axis videos were reviewed and analyzed using the LV-Trace function. LV endocardial borders were traced manually, and the measurements were averaged over a minimum of 3 cardiac cycles. Further, heart rate was also manually calculated to confirm the accuracy of ECG heart rate.

11. Utilizing the algorithm developed in Vevo LAB software 5.6.0, calculate the parameters such as: heart rate (HR), end-diastolic left ventricular (LV) internal diameter (LVID; d), fractional shortening (FS), ejection fraction (EF), stroke volume (SV), cardiac output (CO), end-diastolic LV posterior wall thickness (LVPW;d), LV mass.
APPENDIX XI

Protocol for Vascular Doppler Ultrasound Evaluation Using Vevo 3100

1. After weighing the mouse, place and anesthetize the mouse in an induction chamber for approximately 1 minute until the sedation (under 4.0% isoflurane).

2. Place the mouse on a warm pad in the supine position and maintain the body temperature around 37°C. Remove hair from the mouse’s chest using hair removal cream. Once the hair is removed, wipe the area with wet gauze to ensure all hair is removed.

3. Keep the mouse under light anesthesia with isoflurane (1%~1.5%) in room air supplemented with 100% oxygen through a nose cone. Limbs are taped to the ECG leads for electrocardiogram monitoring. Hearts rates are maintained at 400-500 beats per minute (maintain anesthesia for proper heart rate).

4. Place an ample amount of warmed ultrasound gel on the neck region.

5. Lower the probe (40 MHz) to the gel until it makes contact, and make sure all areas of the probe are covered with gel and the image is free of bubble interference.

6. Place the probe along the long-axis of the left common carotid artery (LCCA) and record a video image of the vessel in the long-axis in B-mode. The systolic and diastolic diameter was obtained from the B-mode imaging keeping the carotid artery as horizontal as possible.

7. For the blood flow measurements, the machine was switched to pulsed wave doppler mode, and aliasing was used to identify the region of peak flow. Color-flow doppler was employed to help locate the arteries. The transducer angle and orientation were adjusted as necessary to obtain an angle between 45°- 50° throughout the experiment, to ultimately obtain a long-axis video of blood flow and blood velocity, directing the probe parallel to the blood flow.
8. Once the imaging is complete, remove the residual ultrasound gel on the chest, and return the
   mouse to the cage for recovery.

9. Doppler spectral trace for blood velocity was calculated using the on-board software on the
   Visual Sonics system, Vevo LAB 5.6.0.

10. Long-axis videos were reviewed and analyzed using the Spectral Trace function, and the
    measurements were averaged over a minimum of 3 cardiac cycles.

11. The software was used for calculating parameters such as end-diastolic velocity (EDV), peak
    systolic velocity (PSV), velocity-time interval (VTI), pulsatility index (PI), and resistivity
    index (RI).
APPENDIX XII

Protocol for Nuclear and Cytosolic Fractionation

Cytoplasmic Extract Preparation

For Cell Samples

1. The cells with washed with PBS twice. Add 1 ml of fresh PBS per 20 cm² area and scrape cells into a 15 ml conical tube.
2. Centrifuge the cells for 5 minutes at 1000 rpm and discard the supernatant.
3. Dilute 10X Pre-Extraction Buffer (NE1) with distilled water at a 1:10 dilution (1X). Add DTT Solution and 1000X Protease Inhibitor Cocktail (PIC) to ice-cold diluted NE1 (1X) at a 1:1000 dilution. Re-suspend cell pellet in 100 µl of diluted NE1 (1X) per 10⁶ cells and transfer to a micro-centrifuge vial. Incubate on ice for 10 minutes.
4. Vortex vigorously for 10 seconds then centrifuge the preparation for 1 minute at 12,000 rpm in a desktop centrifuge (about 11,000xg). Carefully remove the cytoplasmic extract from the nuclear pellet.

For Tissue Samples

1. Weigh the tissue and cut it into small pieces.
2. Place the cut pieces in a clean homogenizer. Dilute NE1 with distilled water at a 1:10 dilution(1X). Per gram of tissue, add 5 ml of diluted NE1 (1X) containing 5 µl of DTT and homogenize tissue pieces (50-60 strokes).
3. Incubate on ice for 15 minutes, then centrifuge for 10 minutes at 12,000 rpm at 4°C.
4. Remove the supernatant.
Nuclear Extract Preparation

1. Add DTT Solution and PIC to Extraction Buffer (NE2) at a 1:1000 dilution. Add 2 volumes (based on pellet size) of NE2 containing DTT and PIC to nuclear pellet (about 10 µl per 106 cells or per 2 mg of tissue).

2. Incubate the extract on ice for 15 minutes with a vortex (5 seconds) every 3 minutes. At high speed. The extract (especially tissue extract) can be further sonicated for 3 x 10 seconds to increase nuclear protein extraction.

3. Centrifuge the suspension for 10 minutes at 14,000 rpm at 4°C and transfer the supernatant into a new micro-centrifuge vial. Measure the protein concentration of the nuclear extract.

4. Use immediately or aliquot and freeze the supernatant at -80°C until further use. Avoid multiple freeze/thaw cycles
APPENDIX XIII

Protocol for Amplification of Plasmid

Preparation of LB Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

Swirl to mix all the components mentioned above.

1. Cover the flask with aluminum foil and autoclave.
2. Meanwhile, prepare a water bath at 60°C with sufficient water to submerge ~75% of the flask.
3. Once autoclaved, leave the LB agar mixture in the water bath, and slowly let the temperature decrease.
4. Once the temperature of the flask subdues, mix your diluted stock antibiotic, kanamycin (working concentration 50 μg/ml). Make sure the temperature of the flask does not exceed 60°C, which ensures that the antibody does not break in high temperature. Swirl to ensure even distribution of the antibiotic.
5. Pour the prepared culture media into plates (avoid bubbles) and allow roughly 30 minutes for the plate to solidify.
6. Streak the bacterial strain and culture overnight at 37°C.
7. Check for single colony.

Preparation of LB Broth
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>6 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>3 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>6 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>600 ml</td>
</tr>
</tbody>
</table>

Autoclave the flask containing all the above components. Sterile broth can be stored at room temperature for about a month.

8. When ready to grow culture, add liquid LB to tube (with loose cap) and add kanamycin at appropriate working concentration.

9. Using a sterile pipette, select a single colony from LB agar plate.

10. Drop the tip into liquid LB and swirl.

11. Incubate the bacterial culture at 37°C for ~8 hours/overnight in a shaking incubator.

12. Check for growth, characterized by cloudy haze in the media.

13. Purify the plasmid from the cultured broth using appropriate plasmid purification kits.
APPENDIX XIV

TA Cloning

Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng (20 fmoles) of pCR®2.1 vector:

\[ x \text{ ng PCR product} = (y \text{ bp PCR product}) \times (50 \text{ ng pCR®2.1 vector}) \]

(Size in bp of the pCR®2.1 vector: ~3900)

where \( x \) ng is the amount of PCR product of \( y \) base pairs to be ligated for a 1:1 (vector: insert) molar ratio.

1. Centrifuge one vial of pCR®2.1 to collect all the liquid in the bottom of the vial.
2. Determine the volume of PCR sample needed to reach the required amount of PCR product.
   
   Use sterile water to dilute the PCR sample if necessary.
3. Set up the 10 µL ligation reaction as follows:

<table>
<thead>
<tr>
<th>Fresh PCR product</th>
<th>x µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X T4 DNA Ligase Reaction Buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>pCR®2.1 vector (25 ng/µL)</td>
<td>2 µL</td>
</tr>
<tr>
<td>Water to a total volume of 9 µL</td>
<td>Final volume 10 µL</td>
</tr>
<tr>
<td>ExpressLink™ T4 DNA Ligase (5 units)</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

4. Incubate the ligation reaction at room temperature for a minimum of 15 minutes. Ligation reaction can be stored at -20°C until ready for transformation.

Transformation into competent cells

5. Centrifuge the vials containing the ligation reactions briefly and place them on ice.
6. Thaw, on ice, one 50 µL vial of frozen One Shot® Competent Cells for each transformation.
7. Pipet 2 µL of each ligation reaction directly into the vial of competent cells and mix by stirring gently with the pipette tip.

8. Incubate the vials on ice for 30 minutes. Store the remaining ligation mixtures at −20°C.

9. Heat shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the vials to ice.

10. Add 250 µL of room temperature S.O.C. medium to each vial.

11. Shake the vials horizontally at 37°C for 1 hour at 225 rpm in a shaking incubator.

12. Spread 10–200 µL from each transformation vial on LB agar plates containing X-Gal and 50 µg/mL of kanamycin

13. Incubate plates overnight at 37°C. Transfer plates to 4°C for 2–3 hours to allow for proper color development.

Analyze transformant

14. Pick at least 10 white colonies for plasmid isolation and restriction analysis.

15. Grow colonies overnight in 2–5 mL LB broth containing 50 µg/mL kanamycin.

16. Isolate and analyze the plasmid by restriction mapping or sequencing for orientation of the insert.
APPENDIX XV

Protocol for Restriction Digestion

<table>
<thead>
<tr>
<th>DNA</th>
<th>1ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X NEB Buffer 3.1</td>
<td>5 μl (1X)</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1 μl (20units)</td>
</tr>
<tr>
<td>BamHI</td>
<td>1 μl (20units)</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>Up to 30 μl</td>
</tr>
</tbody>
</table>

1. Mix all components mentioned above gently by pipetting.

2. Incubate the tube at 37°C for 1 hour or overnight.

3. Run the samples in a freshly prepared 0.8%-1% agarose gel and visualize the digestion.
REFERENCES


34. Lui KO, Huang Y. PHB2 Governs Metabolism and Phenotypic Switching of VSMCs in Vascular Remodeling. *Circ Res*. 2022;131:825-827. doi: 10.1161/circresaha.122.321985


<table>
<thead>
<tr>
<th>ID</th>
<th>Reference</th>
</tr>
</thead>
</table>


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