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Review Article

The COP9 signalosome and vascular function: intriguing possibilities?

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Abstract: Disorders of vascular function contribute importantly to cardiovascular disease which represents a substantial cause of morbidity and mortality worldwide. An emerging paradigm in the study of cardiovascular diseases is that protein ubiquitination and turnover represent key pathological mechanisms. Our understanding of these processes in the vasculature is growing but remains incomplete. Since protein ubiquitination and turnover can represent a terminal event in the life of a given protein, entry into these pathways must be highly regulated. However, at present understanding of these regulatory mechanisms, particularly in the vasculature, is fragmentary. The COP9 (constitutive photomorphogenic mutant 9) signalosome (CSN) is a heteromeric protein complex implicated in the control of protein degradation. The CSN participates critically in the control of Cullin Ring Ligases (CRLs), at least in part via the detachment of a small protein, Nedd8 (deneddylation). CRLs are one of the largest groups of ubiquitin ligases, which represent the most selective control point for protein ubiquitination. Thus, the CSN by virtue of its ability to control the CRLs ubiquitin ligase activity is ideally positioned to effect selective modulation of protein turnover. This review surveys currently available data regarding the potential role of the CSN in control of vascular function. Data potentially linking the CSN to control of regulatory proteins involved in vascular smooth muscle proliferation and to vascular smooth muscle contraction are presented with the intent of providing potentially intriguing possibilities for future investigation.

Keywords: The COP9 signalosome, cullin-RING ligases, ubiquitination, vascular smooth muscle proliferation, vascular smooth muscle contraction

Introduction

Cardiovascular diseases such as atherosclerosis, hypertension, heart failure, ischemic heart disease, myocardial infarction, stroke, and peripheral artery disease represent a leading cause of morbidity and mortality globally[1]. Of the approximately 53 million deaths recorded worldwide in 2010, fully one third are attributed to cardiovascular diseases [1]. The economic impact of this disease burden is staggering. It is estimated that the economic costs of these diseases amounted to $320 billion in 2011 for the United States alone [2]. Moreover, while the incidence of some adverse cardiovascular outcomes is decreasing others are increasing. Analyses of National Health and Nutrition Examination Survey (NHANES) data suggest that hypertension prevalence is increasing [3, 4]. Recent American Heart Association predictions suggest that hypertension prevalence will increase by 10% by 2030 [2, 5] by which time ~40% of the US population will suffer from this disease. Given the high prevalence of cardiovascular diseases increased understanding of the pathological mechanisms underlying these diseases is critical to revealing new targets for therapy. New approaches to treat cardiovascular disease could have substantial impact on worldwide health.

Ultimately, cell function and dysfunction hinge on cellular proteins. The amount and function of cellular proteins depend on a dynamic balance between protein synthesis and degradation. It is well recognized that degradation of cellular proteins by the ubiquitin proteasome system (UPS) or macroautophagy is critical to cell signaling and cell function [6, 7]. Recent work from our laboratories showed that these processes

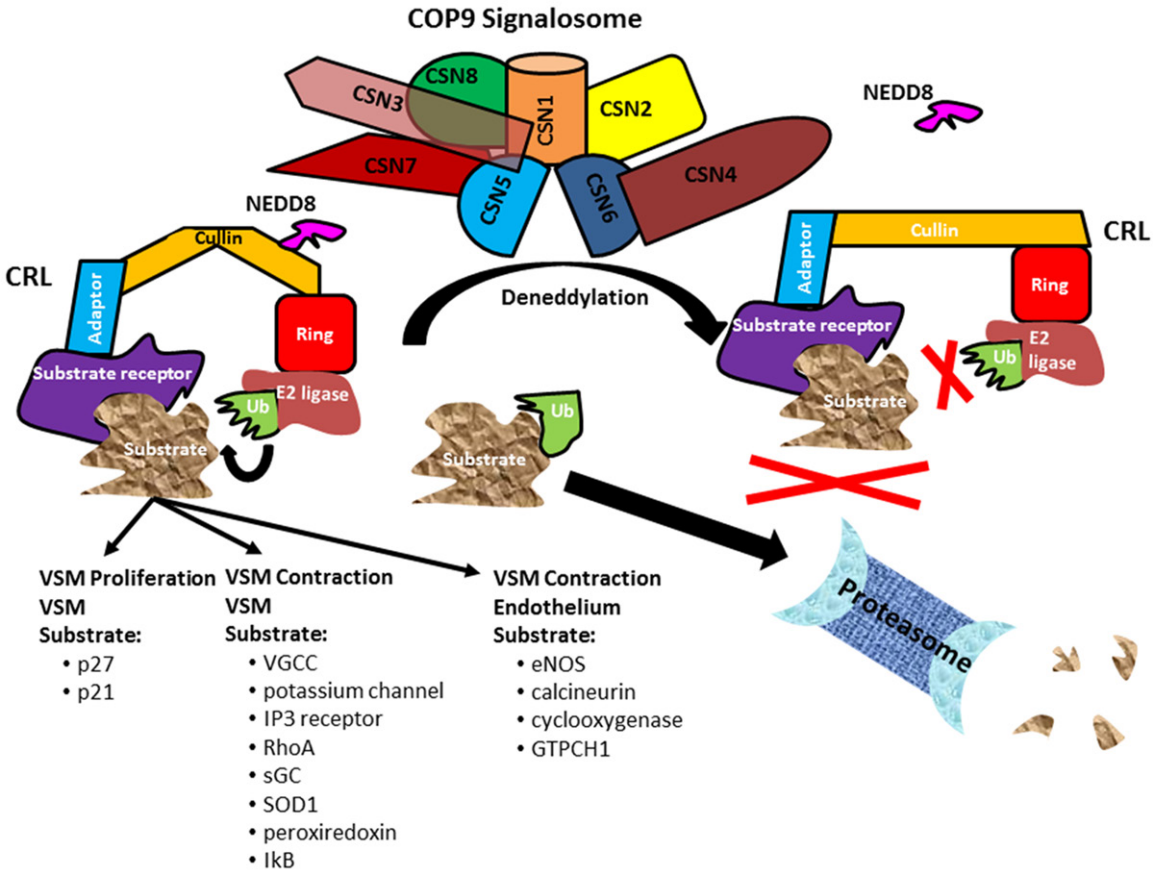

Figure 1. A summary of potential CSN targets in vasculature. The CSN is a heteromeric complex consisting of 8 subunits, CSN1~CSN8. The CSN is responsible for cullin deneddylation, with the deneddylase activity residing in CSN5. Neddylation/deneddylation is a key mechanism for controlling the activity of cullin RING ligases (CRLs). Attachment of NEDD8 (neddylation) renders the CRL able to ubiquitinate substrates to direct them to the proteasome for degradation. One of the major actions of the CSN is to remove NEDD8 (deneddylate) and inactivate CRLs. The deneddylated form of CRLs prevents the ubiquitination and proteasomal degradation of targeted substrates. The substrate recognition receptor of the CRL allows selective targeting of substrates. Thus by controlling CRLs the CSN may provide selective control of protein turnover in vascular smooth muscle (VSM). Key target substrates that may be under CSN control and participate in VSM proliferation include the cyclin dependent kinase inhibitors, p27 and p21. The CSN may also be involved in the control of vascular contraction by modulating the ubiquitination of substrates such as the voltage gated calcium channel (VGCC), potassium channel, IP3 receptor, RhoA, soluble guanylate cyclase (sGC), super oxide dismutase (SOD1), peroxiredoxin, or IkB. In the endothelium the CSN may control ubiquitination of endothelial nitric oxide synthase (eNOS), calcineurin, cyclooxygenase or GTP cyclohydrolase 1 (GTPCH1) to modulate the production and release of endothelium dependent vasodilators or constrictors.

are involved in both cardiac and vascular disease [8, 9]. Since UPS protein processing can be a terminal event in the life of a given protein, entry into these pathways must be highly regulated. However, at present, understanding of these regulatory mechanisms is fragmentary. Ubiquitin E3 ligases, the substrate recognition components of the UPS, represent a major mechanism conferring specificity to proteasomal protein clearance. As such their regulation is an attractive target for therapeutic manipulation. The COP9 signalosome (CSN) represents a key mechanism for regulating a major subset of E3 ligases, the cullin-RING ligases (CRLs). The role of the CSN in regulating cardiac function is discussed in a companion paper in this issue [10]. This paper presents a brief overview of the potential for the CSN to participate in vascular function. Vascular dysregulation leading to the major cardiovascular diseases is represented largely by either VSM cell proliferation (e.g. neointimal growth in restenosis) or VSM contraction (e.g. elevated vascular resistance in hypertension). Accordingly, this review will focus on the potential role of the CSN in VSM cell proliferation and contraction.

Overview of the ubiquitin proteasome system, cullin Ring ligases and the COP9 signalosome

Since the UPS, CRLs and CSN systems are described in detail in a companion paper in this issue, this section will present only a brief overview. Readers interested in greater detail are directed to the companion paper [10]. Recent proteomic studies have revealed that ubiquitination is a major form of posttranslational modification in the cell [11], similar to or perhaps even more common than phosphorylation [12], which underscores the significance of ubiquitination. Ubiquitination, especially polyubiquitination with lys48-linkage, often targets the substrate for degradation by the 26S proteasome. Lys-48 ubiquitination targeting for proteasomal degradation represents the most studied function of ubiquitination, and is, in fact, responsible for the degradation of most cellular proteins [13]. Beyond targeting proteins for proteasomal degradation, ubiquitination can also serve as a signal to regulate a variety of other cellular processes, depending on the number of ubiquitins involved and the nature of ubiquitin chain linkage [14-17]. In all cases, ubiquitination attaches a small protein, ubiquitin, via an isopeptide bond to a lysine residue of the target protein or the preceding ubiquitin. This is accomplished by a series of stepwise reactions catalyzed by the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). The E3 ligase is responsible for substrate recognition and facilitates the transfer of an activated ubiquitin from E2 to the substrate target; hence, the E3 ligase determines which substrate protein to be ubiquitinated, conferring specificity of ubiquitination [18]. Consequently, ubiquitin E3 ligases are rightfully one of the most critical points in the UPS to be targeted for regulating the degradation of a target protein, which represents an emerging and potentially powerful strategy for therapeutic development [19].

The ubiquitin E3 ligases can be grouped based on certain common structural features. A ubiquitin E3 ligase usually carries a HECT (Homologous to E6AP Carboxyl Terminus) [20], RING (Really Interesting New Gene) [21], or U-box [22] domain. The RING domain containing group encompasses the largest number of ligases [22]. A large number of RING type ubiquitin ligases function in the form of protein complexes using a cullin (CUL) protein as the backbone, known as cullin-RING ligases (CRLs) [23, 24]. The prototype of CRLs is the SCF (SKP1-CUL1-F-box) complex, in which CUL1 serves as the scaffold. CUL1 uses its carboxyl terminal to bind to a RING protein (RBX1) which recruits ubiquitin-charged E2 and uses its amino-terminal to bind a substrate receptor module consisting of SKP1 and variable F-box proteins. The F-box protein serves as the substrate receptor to directly recruit its specific substrate targets [25] (**Figure 1**). There are at least 7 cullins found in mammalian cells (CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, and CUL7); each of them can form a subfamily of CRLs. Thus, based on the specific cullin used, CRLs are further classified into CRL1 (SCF), CRL2, CRL3, CRL4A, CRL4B, CRL5, and CRL7 subfamilies [24]. All subfamilies of CRLs share the same basic architecture but use different substrate receptor modules (for a detailed description of CRL subfamilies, please refer to the companion article by Wang and Martin in this Issue of *Am J Cardiovasc Dis* [10] and references therein).

A major mechanism controlling CRL function is the process of neddylation. For CRLs to perform ubiquitin ligation, their cullin backbone must be covalently modified with a ubiquitin-like protein NEDD8 (Neural precursor cell Expressed Developmentally Downregulated protein 8) via a ubiquitination-like process known as neddylation (for a detailed description of the biochemistry of neddylation and enzymes involved, please refer to another article by Kandala et al. in this Issue of *Am J Cardiovasc Dis* [26] and references therein) [27, 28]. Conversely, removal of NEDD8 from the neddylated cullin via a process termed deneddylation inactivates the CRL. Cullin deneddylation not only deactivates the E3 ligase function but also allows the CRL to be partially disassembled. The components are then recycled to form a new CRL with a different substrate receptor module to target other substrates in the cell [24]. Thus cullin deneddylation is a mechanism regulating the activity of CRLs and also provides a degree of molecular flexibility and efficiency to this system.

Cullin deneddylation is catalyzed by the COP9 (COnstitutively Photomorphogenic mutant 9) signalosome [29-31] (**Figure 1**). The COP9 signalosome (CSN) was initially discovered in a model plant *Arabidopsis* [32, 33]. The CSN is a protein complex conserved from yeast to

humans and apparently evolved in parallel to the UPS. The CSN is composed of 8 unique proteins termed CSN1 through CSN8, considered a paralogue of the lid complex of the 19S proteasome as both display a similar electron microscopic morphology and the same domain structure of their subunits [34-36]. The catalytic activity of CSN deneddylase resides in subunit CSN5 but cullin deneddylation by the CSN requires all 8 subunits to form a holocomplex [37]; hence, ablation of any of the CSN subunits leads to loss of cullin deneddylation. Therefore, the CSN is central to regulation of CRL-dependent ubiquitination. The (patho) physiological significance of the CSN in mammalian system has just begun to be deciphered.

Vascular structure

The vascular wall is comprised of the tunica intima made up of endothelial cells, the tunica media which consists primarily of vascular smooth muscle (VSM) cells and the tunica adventitia which contains a mixture of cells including fibroblasts and immune cells. The major function of these structures is to provide contractile ability and compliance to blood vessels to modulate vascular resistance, pressure waves and blood pressure. However, the vascular wall is extremely plastic and responds to both physiological (e.g. pressure) or pathophysiological (e.g. oxidized lipoproteins) stimuli with changes in vascular proliferation, vascular function (contraction/relaxation), or both. Direct investigation of the CSN on blood vessel structure and function is sparse. Nevertheless, indirect evidence suggests that the CSN modulates each of these aspects of blood vessel biology.

Vascular smooth muscle proliferation

Under physiological conditions, VSM cells are more or less quiescent exhibiting relatively low rates of proliferation. However, unlike cardiac cells, VSM cells are not terminally differentiated and under the appropriate stimuli can dedifferentiate to alter their phenotype in subtle or extreme fashion [38]. At its most profound, this process termed “phenotypic switching” results in transformation of VSM cells from a predominantly contractile phenotype to a predominantly proliferative/secretory phenotype that has been implicated in the pathophysiology of atherosclerosis, neointima formation in stent restenosis, pulmonary hypertension, systemic hypertension and cancer [38, 39].

Phenotypic switching of VSM cells requires reentry into the cell cycle. Normally, VSM cells exist in the G0 phase of the cycle [40, 41], however under the appropriate stimuli VSM cells can enter the G1 phase and progress through the cell cycle. An important mechanism regulating this process is inhibition of cyclin dependent kinase(s) by cyclin dependent kinase inhibitors (CKDI) [40-42]. An important group of CDKI is the KIP/CIP family consisting of p21, p27 and p57. While these proteins share a similar N terminal structure, the remainder of their structure is sufficiently different that each may serve different functions [42]. Moreover, different mechanisms may control these CDKIs at different stages of the cell cycle. For example, p27 is elevated during the G0 phase to effectively inhibit the cell cycle and maintain cells in a quiescent state. At the G0-G1 transition, current evidence suggests that p27 degradation is promoted via a ubiquitination complex termed the Kip1 ubiquitination-promoting complex (KPC) [43]. However, at the S/G2 interface, p27 levels are regulated by an E3 ligase complex, SCF^{Skp2} [44]. p57 is regulated post-transcriptionally in a manner similar to p27 [45]. Conversely, p21 is maintained at low levels during G0 but is increased transcriptionally later in G1 as a counter-regulatory mechanism [46, 47].

These regulatory mechanisms have been shown to have important effects on vascular pathophysiology. Vascular injury was associated with decreased p27 expression and increased proliferative markers in VSM cells in dogs and mice [48, 49]. Moreover, VSM cell proliferation in response to vascular injury was increased in p27 null mice [50]. On the other hand, overexpression of p27 in blood vessels attenuated balloon injury-induced VSM cell proliferation in rats, rabbits and dogs [47, 51-53]. Similarly, drug treatments that increased p21 expression were found to attenuate VSM cell proliferation in response to injury in the carotid artery of rats [54, 55] while overexpression of p21 reduced VSM cell proliferation in response to balloon injury in the pig [56]. Thus, the role of CDKI in VSM cell proliferation for arterial repair following mechanical injury is relatively well established. p27 is also implicated as a regulatory factor in the vascular proliferative response in disease. Closely related to the increased VSM

cell proliferation observed with mechanical injury is the increase in VSM cell proliferation associated with the atherosclerotic process. p27 was identified as a critical regulatory factor in this setting. Double transgenic mice lacking apoE and p27 showed increased atherosclerotic burden compared to apoE knock out alone, suggesting that loss of p27 accelerated the atherosclerotic process [57]. Qualitatively similar results were obtained in p21 null mice [58]. Direct comparison of the effects of p27 or p21 knockout suggested that the magnitude was substantially less with p21 knockout than that seen in p27 null mice [57]. Conversely, treatment that reduced plaque burden in apoE null mice was associated with induction of p27 expression and reduced intraplaque VSM cell proliferation [59]. Likewise, adenovirus mediated overexpression of p21 inhibited atheroma formation in apoE null mice [60]. Thus, CKDI appear to play a critical role in the control of focal VSM proliferation in response to injury or atherosclerosis.

These control mechanisms have also been implicated in more diffuse vascular disease models. Pulmonary hypertension is a debilitating disease characterized by excessive proliferation of VSM cells in the pulmonary arteries to the point of vascular occlusion [61]. Chronic exposure to hypoxia and endothelin 1 are potent triggers of pulmonary hypertension. Exposure to hypoxia or endothelin 1 was associated with increased proliferation of VSM cells and a decrease in p27 expression [62, 63]. On the other hand, treatments that upregulated p27 expression were found to reduce pulmonary artery VSM cell proliferation [63, 64]. VSM cell proliferation associated with systemic hypertension may also involve changes in p27. Angiotensin II infusion to produce hypertension in rats increased proliferation indices in mesenteric arterial VSM cells and caused a reduction in p27 [65]. Similarly, wild type mice made hypertensive with angiotensin exhibited increased aortic medial thickness and aortic VSM cell proliferation (PCNA staining), effects that were exaggerated in p27 null mice [66]. In the spontaneously hypertensive rat, a genetic model of hypertension, agonist-induced VSM cell proliferation was exaggerated compared to normotensive Wistar Kyoto rats [67, 68]. The enhanced VSM cell proliferative response was associated with a reduction in p27 expression in spontaneously hypertensive compared to Wistar Kyoto rats [67]. In addition drug treatment that reduced the VSM cell proliferative response triggered an increase in p21 expression [68]. Thus CKDI appear to play a central role in the VSM cell proliferation associated with pulmonary or systemic hypertension. Collectively these data suggest that CDKIs represent an important control mechanism for modulating VSM cell proliferation in a number of cardiovascular diseases.

The COP9 signalosome and vascular smooth muscle proliferation

Direct study of the role of the CSN in control of VSM cell proliferation is limited. In non-vascular cells there are data showing that the CSN is a critical regulator of cell proliferation. Perhaps the best established link between the CSN and cell cycle regulation is the recognition that p27 degradation is regulated by the CSN. Initial evidence to support this possibility was provided by studies showing that CSN5 (formerly Jab1) bound p27 and that overexpression of CSN5 resulted in accelerated degradation of p27 and increased cell proliferation [69]. Subsequent work suggested that CSN5 contributed to p27 degradation by also enhancing nuclear to cytoplasmic export thus enhancing delivery to the UPS [70]. Moreover, ectopic expression of CSN5 could rescue NIH3T3 cells from p27-induced cell cycle arrest [69] showing that these interactions were important functionally. Similarly, overexpression of CSN6 was found to release NIH3T3 cell from p57 induced G1 arrest [71]. Conversely, knockdown of CSN4 or CSN5 reduced S phase entry in HeLa or HEK293T cells [72]. Similar findings were obtained in thymocytes where selective CSN5 knockdown arrested the cell cycle in the S/G2/M phase [73]. These data provided a picture wherein CSN function was a positive regulator of the cell cycle. Indeed impairment of CSN function by deleting various CSN subunits (CSN2, CSN3) proved to be embryonically lethal in mice at least in part due to defective cell proliferation [74, 75].

However, further study has shown that the picture is not nearly so straightforward. Complicating interpretation is the fact that CSN5, the most frequently targeted CSN subunit (presumably because of its catalytic activity), has functions independent of the CSN complex [76, 77]. In addition, in contrast to some earlier work, it has been reported that siRNA induced reduc-

tion in CSN3 increased proliferation in mouse fibroblasts [78]. Subsequently, it was shown that conditional silencing of CSN5 or CSN8 in the same cell system resulted in opposite effects on proliferation of HeLa cells. Reduction in CSN5 expression caused a marked attenuation of cell proliferation consistent with earlier studies, whereas CSN8 reduction was associated with accelerated growth [79]. A similar increase in proliferation was observed in mouse embryonic fibroblasts harvested from CSN8 hypomorphic mice which exhibit a reduction in CSN8 expression and CSN function [79]. On the other hand, conditional knockdown of CNS8 in postnatal hepatocytes resulted in impaired hepatocyte proliferation [80]. Thus, perhaps fittingly for a complex as intricate as the CSN, more recent work suggests that the CSN may both promote and inhibit the cell cycle. The net effect of CSN disruption may depend on the CSN subunit affected and the tissue/cell type in which the deficit occurs. Nevertheless in aggregate, these findings indicate that the CSN participates critically in the control of the cell cycle. Accordingly, the CSN has the potential to be involved in conditions which exhibit alterations in VSM cell proliferation.

Given the strong data tying CSN function to regulation of the cell cycle, it is curious that direct study (knockdown, overexpression) of CSN in VSM proliferation is lacking. Nevertheless, indirect evidence ties the CSN to the control of VSM proliferation via regulation of the CKDIs. One of the important mechanisms regulating the CKDIs is UPS-mediated degradation [81]. Substantial evidence supports the view that CKDI degradation during the cell cycle is controlled at least in part by SCF complexes by targeting of the CKDIs for ubiquitination and degradation [82]. SCF complexes represent a subfamily of CRLs that contain four primary components: Skp1 (an adaptor protein), Cullin1, Roc1/Rbx1 (a RING finger protein), and an F-box protein which represents the substrate recognition site [83]. SCF complexes containing the F-box protein Skp2 (SCF^{Skp2}) have the CKDIs as one class of its targets. Convincing data show that SCF^{Skp2} contributes to regulation of VSM proliferation by controlling the turnover of CKDIs. In fact, Skp2 was shown to be required for p27 ubiquitination and degradation [84]. While p27 is constitutively expressed in quiescent VSM, Skp2 expression is quite low [85, 86]. Overexpression of Skp2 in rat aortic smooth muscle cells triggered a reduction in p27 and an increase in proliferative indices in isolated rat aortic VSM cells (thymidine incorporation) or rat aorta organ culture (BrDU incorporation) [87]. Moreover, physiologic stimuli such as balloon injury that promote VSM proliferation trigger an increase in Skp2 and a concomitant reduction p27 [86, 88]. A similar finding was reported for pulmonary artery VSM cells that were stimulated to proliferate with endothelin [62]. Conversely, siRNA suppression of Skp2 or expression of a dominant negative form of Skp2 in rat thoracic aorta VSM cells resulted in an increase in p27 expression and reduced proliferation [86, 89, 90]. In vivo relevance of these findings was further strengthened by the demonstration that Skp2 null mice exhibited reduced neointima formation in response to balloon injury [88]. On the other hand, AMPK null mice which exhibited an increase in Skp2 expression developed more severe neointimal VSM growth in response to wire injury of the carotid artery [90]. AMPK activation was shown to inhibit pulmonary artery VSM cell proliferation by preventing endothelin mediated increases in Skp2 expression [62]. In the same vein, treatment of rats with hypoxic pulmonary hypertension that reduced pulmonary artery thickness was reported to prevent hypoxic induction of Skp2 expression [91]. While the preponderance of data has addressed the role of SCF^{Skp2} control of VSM proliferation via interactions with p27, there are data showing that other CDKIs may also be involved. Overexpression of Skp2 triggered p21 degradation in rat [92] and in mouse [93] aortic VSM cells.

Collectively these findings suggest that SCF^{Skp2} mediated degradation of CDKI plays a critical role in the control of VSM cell proliferation. Given that SCF^{Skp2} E3 ligase complex is cullin based it is logical to predict that the CSN is involved in controlling VSM cell proliferation under various conditions by modulating the degradation of CDKI such as p27 and p21 (**Figure 1**). Nevertheless direct further investigation is needed to directly link the CSN to VSM cell proliferation, to establish the precise role of CSN subunits, and to decipher the molecular mechanisms by which the CSN regulates SCF^{Skp2} complex function in VSM.

Vascular smooth muscle contraction

Vascular tone

Vascular smooth muscle is responsible for the dynamic maintenance of blood vessel diameter, a primary determinant of vascular resistance. Vascular tone plays a major role in maintaining peripheral vascular resistance and systemic blood pressure to provide a constant pressure for tissues to regulate blood flow in proportion to their metabolic demand. In addition, VSM in regional vascular beds (organs) is also subject to a myriad of local influences that help determine regional vascular resistance and blood flow distribution, again mostly in proportion to the local tissue demands. Unlike skeletal and cardiac muscle whose contractions are predominantly episodic, VSM exists in a state of perpetual contraction, so called vascular tone that is highly modulated by a number of control mechanisms.

Vascular contraction mechanisms

As in other muscle, an increase in intracellular calcium is the primary stimulus for VSM contraction. Increases in intracellular calcium are mediated via calcium entry from the extracellular space or from intracellular stores in the sarcoplasmic reticulum [94-96]. One important control point for VSM contraction is calcium entry via the L type voltage gated calcium channel which is regulated in large part via membrane voltage. Plasmalemmal potassium channels are a major determinant of VSM membrane voltage [97, 98]. Four types of potassium channel have been implicated in control of VSM tone: the voltage dependent potassium channel (K_v), the inward rectifying potassium channel (K_{ir}), the ATP dependent potassium channel (K_{ATP}) and the calcium activated potassium channel (K_{Ca}) [97-99]. The K_{Ca} is further classified into small (SK_{Ca}), intermediate (IK_{Ca}) and large conductance (BK_{Ca}), with the BK_{Ca} recognized as a major contributor to vascular tone [100, 101]. Calcium release from the sarcoplasmic reticulum is mediated by inositol 1,4, 5-trisphosphate (IP_3) released from the plasma membrane acting on IP_3 receptors present in the sarcoplasmic reticulum [102]. Changes in IP_3 receptor expression were recently shown to play a critical role in modulating vascular tone [103].

Subsequent to an elevation in intracellular calcium, the contractile mechanisms differ substantially from the heart and skeletal muscle. Calcium binds to calmodulin and the calcium calmodulin complex stimulates myosin light chain kinase to phosphorylate myosin light chain which then stimulates force generation via the canonical actin myosin interaction [94, 104]. Thus, myosin light chain phosphorylation is the proximal event triggering VSM contraction. A key regulatory feature of VSM is that myosin light chain is also dephosphorylated by myosin light phosphatase [95, 96, 104]. Therefore, the magnitude of the VSM contractile response to calcium is determined by the balance of myosin light chain phosphorylation and dephosphorylation. Accordingly, myosin light chain kinase and myosin light chain phosphatase represent key control points for vascular tone.

Signaling mechanisms that impact these core processes provide further fine tuning of vascular tone. One important such mechanism is provided by the phosphorylation status of myosin light chain phosphatase. Myosin light chain phosphatase consists of a catalytic subunit (PP1c), a regulatory subunit (MYPT1) and a 20 kDa subunit of unknown function [95, 96, 104]. Phosphorylation of MYPT1 by Rho kinase is an inhibitory signal that reduces myosin light chain phosphatase activity [104, 105]. A second mode of control is through activation of protein kinase C which also inhibits myosin light chain phosphatase. This inhibitory input is mediated by a small protein CPI17 that inhibits the PP1c subunit to reduce myosin light chain phosphatase activity [95, 104]. Both these pathways ultimately result in an increase in myosin light chain phosphorylation and vascular tone for any given level of intracellular calcium rise, a process called “calcium sensitization”. Moreover both pathways are modulated by factors that affect the increased vascular tone in hypertension [105-108]. The reverse process of calcium desensitization can also occur. In this situation myosin light chain phosphatase is targeted by vasodilator mechanisms that activate protein kinase G (PKG) or protein kinase A (PKA) signaling. PKA or PKG phosphorylate MYPT1 at sites that impair the ability of Rho kinase or PKC to inhibit myosin light chain phosphatase [109]. Thus, there are several control points at which the CSN may modulate VSM contraction.

The COP9 signalosome and vascular contraction

The role of the CSN in modulating vascular contraction has received very little direct research attention. Nevertheless, there are indirect clues that the CSN is well positioned to interact with several VSM control points and thus impact control of vascular tone and blood pressure regulation. An increase in intracellular calcium is clearly a requisite event for VSM contraction. While not studied directly in VSM, there is evidence to suggest that the CSN is involved in the control of L type voltage gated calcium channels. The L type calcium channel is a multimeric transmembrane protein of which the $\alpha 1_C$ ($Ca_v 1.2$) subunit confers most of the primary functional properties [110]. The $\alpha 1_C$ subunit is a predominant form of the pore forming unit expressed in cardiac and VSM cells [111]. Studies in the rat showed that CSN5 coimmunoprecipitates with $\alpha 1_C$ subunit consistent with an interaction between these two proteins [112]. Moreover, this interaction appeared to be functionally relevant since siRNA knockdown of CSN5 increased calcium currents significantly [112]. This increase in calcium current occurred without significant changes in calcium channel kinetics suggesting an effect on voltage gated calcium channel density [112]. Thus, CNS5 may be involved in controlling voltage gated calcium channel turnover, at least in cardiac cells. The similarities in voltage gated calcium channel structure between cardiac and vascular muscle suggests a potentially similar role in the vasculature. However, this remains to be tested experimentally.

Other data, albeit circumstantial, may implicate the CSN in the control of VSM potassium channels. Expression of the β1 subunit of the BK_{Ca} potassium channel was reduced in aortic VSM harvested from diabetic rats and mice or human coronary artery VSM cells exposed to high glucose [113, 114]. Since there were no changes in BK_{Ca} transcript levels and BK_{Ca} channel protein expression was increased by MG-132 [114], this change in expression was linked to proteasomal degradation. Under diabetic conditions, increased expression of the F-box proteins, atrogin-1 (FBXO32) and FBXO9 were associated with concomitant reductions in BK_{Ca} channel expression [113]. Moreover, siRNA reduction of atrogin-1 was paralleled by an increase in BK_{Ca} β1 subunit expression [114]. Furthermore, drug treatment that reduced atrogin-1 expression was associated with an increase in BK_{Ca} expression and vasodilation in response to NS-1619, a specific activator of BK_{Ca} [113]. Since atrogin-1 and FBXO9 are substrate recognition subunits for SCF ligase complexes, it seems plausible that BK_{Ca} channel proteins may be regulated by the CSN. This possibility requires experimental validation. It is interesting to note that in the case of voltage gated calcium channels CSN activity may inhibit the channel thus producing a vasodilator response. In contrast, at least in the diabetic state, CSN activity would be predicted to enhance the degradation of BK_{Ca} potassium channels, which would result in vasoconstriction. Thus, while there are clues that CSN may be involved in controlling VSM ion channel function, there is a need for direct experimentation to unravel the precise effects.

The CSN may also be implicated in the control of calcium release from intracellular stores. It has been recognized for some time that stimuli which increase the production of IP_3 trigger a reduction in IP_3 receptor protein in the endoplasmic reticulum. Since this effect was blocked by proteasome inhibitors, IP_3 receptor downregulation appears to be mediated via the UPS [115]. More recently a similar phenomenon has been reported for VSM [116]. Rat aortic VSM cells treated with either hydrogen peroxide or angiotensin II exhibited a sustained increase in IP_3 that was associated with an increase in IP_3 receptor ubiquitination and a concomitant reduction in IP_3 receptor protein content. Cotreatment with a proteasome inhibitor (MG132) prevented the decrease IP_3 receptor content. Importantly, this study also demonstrated that these effects were functionally relevant since the reduction in IP_3 receptor expression was associated with reduced IP_3 mediated calcium release and decreased agonist-induced contraction in isolated VSM [116]. The mechanisms regulating IP_3 receptor degradation have not been fully elucidated. RING ligases were implicated in IP_3 receptor turnover [117]. However, it is not known at this point whether CRL E3 ligases are responsible for targeting IP_3 receptors for proteasomal degradation. If this proves to be the case, the CSN may be involved in modulating vascular contraction by influencing agonist stimulated intracellular calcium release.

The CSN may also control cellular pathways that modulate myosin light chain phosphorylation via myosin light chain phosphatase. A variety of agonists that induce VSM contraction are known to do so, at least in part, via activation of the RhoA-RhoKinase (ROCK) pathway [118, 119], which as outlined above potentiates vascular contraction. The application of MLN4924, an inhibitor of neddylation, triggers an increase in Rho A protein levels in Hela cells [120]. Since the CSN is involved in controlling CRLs via deneddylation, this finding suggests that the CSN may be involved in controlling RhoA-ROCK function via modulation of CRL activity. Indeed, siRNA deletion of cullin-3 also increased Rho A protein levels. The increase in Rho A protein was concomitant with decreased Rho A polyubiquitination and an extended the half life of Rho A [121], consistent with the possibility that a cullin-3 based ubiquitin ligase is involved in the cellular clearance of Rho A. Cullin-3 knockdown was also reported to increase the active form of Rho A, Rho A-GTP [121]. An important step in activating Rho A is the exchange of GDP for GTP which is mediated by the Rho guanine exchange factors (Rho GEFs) [122]. siRNA knockdown of KLHL20, a BTB protein that functions as the substrate recognition unit for a cullin-3 based E3 ligase, increased PDZ-RhoGEF and Rho A activation [123]. Thus, CRL3 ligases appear to play a critical role modulating activation of the Rho A pathway and, by inference, the CSN is implicated as well. While these data were not obtained in VSM cells, recent work suggests that these findings have relevance for control of VSM contraction. Consistent with work described above, siRNA knockdown of cullin-3 in rat aortic VSM cells increased Rho A protein [124]. Treatment of rat aortic rings with MLN4924 to inhibit cullin activation also increased Rho A protein levels and amplified the vasoconstrictor response to G protein coupled receptor agonists such as phenylephrine and endothelin [124]. Perhaps most importantly, this work also showed that subcutaneous administration of MLN4924 over the course of 3 days lead to a significant increase in mean arterial blood pressure (~20 mmHg) in conscious mice [124]. Collectively these data are consistent with the idea that one mechanism by which the CSN may control vascular tone is via regulating the activity of cullin based E3 ligases that are responsible for modulating agonist activated vasoconstrictor pathways such as RhoA-ROCK. Validation of this idea will require studies that directly manipulate CSN function while monitoring cardiovascular parameters such as blood pressure, cardiac output and total peripheral resistance.

In summary, there may be multiple points of interaction between the CSN and mechanisms controlling vascular smooth muscle contraction (**Figure 1**). The CSN may control the trigger for contraction, calcium flux, via effects on voltage gated calcium channels, potassium channels or IP3 receptors. Additionally, the CSN might also interact with intracellular signaling pathways such as RhoA-ROCK that impact sensitivity of the contractile machinery to calcium or to vasoactive agents released from the endothelium (described below). The current findings linking the CSN to control of vascular smooth muscle contraction are limited but sufficiently intriguing to spur more investigation in the area.

Endothelial control of vascular function

The endothelium makes up only a small proportion of the blood vessel wall, a single cell lining the vascular lumen. Nevertheless, the vascular endothelium plays a pivotal role in cardiovascular homeostasis as a pleiotropic endocrine/paracrine organ involved in functions ranging from control of vascular tone to secretion of factors involved in remodeling the extracellular matrix [125]. One major function of the endothelium is control of VSM tone via the release of endothelium derived relaxing factors. The best characterized of these factors is nitric oxide (NO). As a diffusible gas, NO is not stored but is synthesized on demand by the actions of nitric oxide synthase (NOS), of which three isoforms exist: endothelial nitric oxide synthase (eNOS; NOS-3), neuronal nitric oxide synthase (nNOS; NOS-1) or inducible nitric oxide synthase (iNOS; NOS-2). The canonical pathway for NO medicated dilation is activation of soluble guanylate cyclase and protein kinase G [125, 126]. NO may also activate BK_{Ca} [126]. In addition, the endothelium releases prostacyclin, a vasodilator arachidonic acid metabolite that generates cAMP by activating adenylate cyclase and ultimately stimulates protein kinase A [127]. A third group of endothelial dilators, the endothelial hyperpolarizing factors (EDHF) is less well characterized but triggers VSM hyperpolarization and thus relaxation via the opening of SK_{Ca}, and IK_{Ca} potassium channels [125, 127].

The endothelium is also a source for endothelial derived contracting substances. Endothelin and arachidonic acid metabolites such as thromboxane or 20-hydroxyeicosatetraeonic (20-HETE) represent the best studied of these factors [128-130]. Endothelin has been characterized as the most potent vasoconstrictor agent known [128, 131]. Endothelin acts on ETA, ETB and ETC receptors, the balance of which determines the ultimate effect of this peptide paracrine factor [128, 129]. Thromboxane is a prostanoid vasoconstrictor agent generated by cyclooxygenase metabolism of arachidonic acid to endoperoxides which are subsequently acted upon by thromboxane synthase [130]. 20-HETE is also generated by cyclooxygenase metabolism of arachidonic acid, however in this pathway the resultant endoperoxides are processed by specific cytochrome P450 isoforms [129, 130]. Thus, cyclooxygenase metabolism of arachidonic acid is a major source of endothelial derived contracting substances. Lastly, reactive oxygen species (ROS) can be an important endothelial derived factor that impacts VSM function. Important sources for ROS include NADPHoxidase, xanthine oxidase, mitochondria and NOS in its "uncoupled" form [132-135]. Various conditions can lead to NOS uncoupling including persistent oxidative stress, lack of the substrate L-arginine or deficiency in essential cofactors such as tetrahydrobyopterin (BH_4) [133, 135]. In addition a number of systems are in place to counterbalance ROS production by scavenging these reactive molecules. These systems include superoxide dismutase (SOD), catalase and the peroxiredoxins [133, 136]. Ultimately, the equilibrium amongst these factors determines the prevailing level of ROS, which in turn can trigger VSM contraction or proliferation [133-136]. In summary, the endothelium is a source for a number of factors that can influence VSM function. While a great deal of research effort and information is now available characterizing the endothelial control of VSM at various levels, the role of the CSN in this aspect of vascular function remains largely unexplored.

The COP9 signalosome and endothelial control of vascular function

As a key mechanism for endothelial modulation of vascular function, NOS is modulated by a variety of mechanisms including posttranslational mechanisms such as phosphorylation and protein-protein interactions [125, 126]. Increasing evidence suggests that ubiquitination and proteasomal processing may represent yet another mechanism for posttranslational control of NOS function. A number of studies have shown that iNOS is regulated by ubiquitination and proteasomal degradation [137, 138]. Comparatively fewer studies have studied the relationship between eNOS and the ubiquitin proteasome pathway. Nevertheless it has been reported that treatment with the proteasome inhibitor lactacystin increased eNOS protein levels in COS-7 cells transfected to express the protein [139]. Lactacystin was similarly found to increase eNOS in bovine aortic endothelial cells in culture [140]. Graded inhibition of proteasomal function with MG-132 resulted in graded increases in eNOS expression in human umbilical vein endothelial cells [141]. A similar effect was reported in bovine pulmonary artery endothelial cells [142]. In some cases eNOS mRNA was increased after proteasome inhibition [142] whereas in others there were no changes in eNOS mRNA [141]. Thus, whether the effects of proteasome inhibition on eNOS expression represent a transcriptional or posttranslational effect remains an open question. Nevertheless, these data are at least consistent with the possibility that eNOS is regulated by proteasomal degradation. The E3 ligases involved in mediating eNOS degradation remain to be determined. While carboxyl terminus of Hsp70-interacting protein (CHIP) was found to interact with eNOS, it reportedly does not direct eNOS to the proteasome for degradation [139]. The cullin based E3 ligase Elongin B/C-Cullin-5-SPRY domain- and SOCS box-containing protein (ECS (SPSB)) was reported to interact with iNOS but not with eNOS [143]. On the other hand, resveratrol, an activator of the sirtuin pathway, was found to increase eNOS expression in rat pulmonary vessels [144]. Resveratrol is reported to increase cullin 5 expression in cancer cells [145], suggesting the possibility the eNOS expression may be susceptible to CSN based regulation.

Aside from direct control by eNOS protein turnover, eNOS function is also regulated via a number of other posttranslational mechanisms. Several observations are consistent with the possibility that the CSN could modulate eNOS activity at this level. It is relatively well established that eNOS activity is controlled in part

via phosphorylation/dephosphorylation at either excitatory (e.g. serine 1177) or inhibitory (e.g. threonine 495) sites [146]. It was reported that calcineurin (protein phosphatase 3) mediated dephosphorylation of serine 116 leads to an increase in agonist stimulated eNOS activity [147, 148]. Interestingly, an SCF complex, $SCF^{atrogin-1}$ promoted ubiquitination of calcineurin, and atrogin-1 overexpression reduced calcineurin levels in cardiac cells [149]. Similarly, BH_4 is a necessary co-factor for nitric oxide synthase activity [146]. BH_4 synthesis was decreased in hypertensive or diabetic mice due to increased proteasomal degradation of GTP cyclohydrolase 1 (GTPCH1), the enzyme responsible for BH_4 synthesis [150-152]. Treatment with a PPAR gamma agonist, GW501516, increased BH_4, eNOS activity and nitric oxide availability in BH_4 deficient mice, at least in part due to an increase in GTPCH1 activity [153]. Since transgenic mice expressing a dominant negative form of PPAR gamma exhibited reduced neddylation of cullin 3, collectively these data are consistent with the possibility that GTPCH1 degradation is mediated through a cullin based E3 ligase. Lastly, soluble guanylate cyclase (sGC) is a major downstream target of nitric oxide and is thought to mediate many of the vascular actions of nitric oxide. Studies in HEK cells showed that sGC subunits undergo polyubiquitination and proteasomal degradation [154]. Recent data in cancer cells suggest that the CSN is involved in modulating proteasomal degradation of sGC. siRNA knockdown of CSN4 or CSN5 resulted in decreased sGC protein, an effect that was reversed by treatment with a proteasome inhibitor [155]. As a whole these findings suggest that in addition to controlling eNOS protein, the CSN may regulate endothelial function by modulating the turnover of regulatory factors that control eNOS activity or downstream targets that mediate its effects.

Cyclooxygenase metabolites of arachidonic acid represent another pathway by which the endothelium regulates vascular smooth muscle. Studies in human megakaryocytic cells showed that both cyclooxygenase 1 and prostaglandin synthase D were ubiquitinated and degraded by the proteasome [156]. Similarly cyclooxygenase 2 was found to be a substrate for proteasomal degradation [157, 158]. In a study using CaCo cells, which constitutively express COX2, it was found that immunoprecipitation of CSN7 pulled down COX2. Moreover, in Hela cells the reverse immunoprecipitation of COX2 pulled down CSN5 [157]. Importantly, this study also revealed that COX2 along with the CSN subunits formed complexes with cullin 1 and cullin 4 as well as with the RING protein ROC1 [157]. These data suggest that COX2 physically interacts with a CSN/CRL complex. Collectively these findings are consistent with the possibility that the CSN is involved in modulating proteasomal degradation of cyclooxygenase. Since the activity of this enzyme is critical for the formation of prostacyclin, a vasodilator, and of thromboxane, a vasoconstrictor, the net effect of CSN on endothelial vasoactive pathways involving cyclooxygenase is unclear at this time, but does warrant further study.

ROS play an important role in VSM regulation both as a signaling molecule generated by a variety of agonists and by virtue of their ability to scavenge NO. Relatively little data are currently available regarding the impact of the CSN on ROS formation or scavenging. Direct evidence of CSN8/CSN involvement in redox regulation was recently provided when Su and colleagues reported that an increased amount of oxidized proteins was detected in mouse hearts with adult-onset cardiomyocyte-restricted CSN8 knockout [159]. The greatest amount of evidence to link the CSN to control of ROS is less direct and comes from study of the NF-Kappa B (NFκB) pathway. NFκB becomes activated when phosphorylation of its cognate inhibitor IκB is triggered by agonist stimulation. IκB is then acted upon by $SCF^{\beta\text{-}TrCP}$, a cullin based E3 ligase that targets IκB for proteasomal degradation [160-162]. The CSN is implicated in this control mechanism. Immunoprecipitation experiments demonstrated that CSN2 associated with IκBα in TNFα stimulated Hela cells [161]. Other work revealed that the CSN interacts with other components of the NFκB activation pathway. Co-immunoprecipitation experiments showed that CSN3 physically interacts with IKK_g, a component of the IKK_α-IKK_β-IKK_g-complex responsible for phosphorylating IκBα [163]. A subsequent study showed that IKK_β was complexed with CSN3 and $SCF^{\beta\text{-}TrCP}$, the E3 ligase that ubiquitinates IκBα, suggesting that an assembled CSN/CRL complex associated with the NFκB control machinery [160]. The functional impact of this association has also been demonstrated. siRNA knockdown of CSN2 was associated with decreased IκBα protein and increased NFκB

translocation into the nucleus [161]. Similarly, siRNA knockdown of CSN5 was reported to increase basal NFκB activity in 293T cells [160, 164]. CSN3 oligo antisense treatment also resulted in an increase, albeit slight, in basal NFκB activity [163]. Some work has shown that CSN knockdown also increased TNFα stimulated NFκB activation [163], whereas other work failed to detect such an effect [160, 161]. Nevertheless, on the whole these data suggest that the CSN acts as a negative modulator of NFκB activation. The precise mechanism by which the CSN attenuates NFκB activation remains an open question since some authors suggest a primary role for the deubiquitinating activity associated with the CSN [161], while other data suggest that the deneddylating activity is critical [160]. At least in cancer cells, inhibition of neddylation with ML4924 was associated with an increase in NFκB activation and downstream target protein expression [165].

A complex and as yet not fully understood interaction occurs between ROS and NFkB. ROS are a stimulus for activation of NFκB. Amongst the many downstream effects of NFκB activation this pathway can, under the appropriate conditions, trigger an increase in the expression of antioxidant enzymes such as super oxide dismutase or catalase [166] to act as a negative feedback loop. Yet other evidence suggests that activation of NFκB results in increased expression of ROS generating enzymes such as NADPH oxidase [166, 167], a positive feedback loop implicated in inflammatory processes. The ultimate outcome of NFκB activation may depend on the composition of the NFκB dimer formed or the cell type [166]. Accordingly, it is difficult to predict the precise effect that CSN regulation of NFκB activation will have on ROS generation and scavenging. Further study is required to identify the precise nature of this control under various conditions and in specific cell types.

In addition to affecting transcription of pro-oxidant or antioxidant proteins, indirect evidence suggests that the CSN may also be linked to clearance of oxidant-redox proteins themselves. Proteomic analysis of control and Cullin 4B null HEK293 cells revealed upregulation of protein for superoxide dismutase 1 and for peroxiredoxin III [168] both of which are involved in scavenging ROS. Peroxiredoxin III was chosen for follow up. Since there were no changes in peroxiredoxin III mRNA expression, these changes were ascribed to reduced degradation of the protein. Consistent with this possibility, ubiquitination of peroxiredoxin III was reduced and cycloheximide chase experiments revealed a markedly increased half-life for peroxiredoxin III in the Cullin 4B null cells. The effects were functionally significant since ROS indices were markedly reduced in the Cullin 4B knockdown cells [168].

As a whole, these findings suggest that the CSN may modulate endothelial function by interacting with multiple control points (**Figure 1**). The CSN may control eNOS function either by regulating turnover of eNOS itself or of proteins that regulate its activity such as calcineurin or GTPCH1. The CSN may also affect endothelial vasoactive factors by regulating COX degradation. Lastly, the CSN may be involved in controlling ROS generation or scavenging which will greatly impact endothelial control of VSM. While suggestive, currently available data are less than conclusive. Accordingly, much more work is necessary in this area to establish the precise role of the CSN in endothelial function.

Concluding remarks and perspectives

Cardiovascular diseases represent a leading cause of morbidity and mortality worldwide. An emerging paradigm in understanding cardiovascular regulation and dysregulation is the recognition that protein modification via ubiquitination can play a critical role in modifying cellular function. A bottleneck in moving this concept forward to therapeutic advantage has been deciphering ways to selectively manipulate protein ubiquitination. The CSN represents a control mechanism for a large group of E3 ligases, the CRLs, which represent the substrate recognition component of the ubiquitination system and are thus the most selective control point. The role of the CSN in cardiovascular control is just beginning to be explored. Perhaps the best characterized interaction is with VSM proliferation. There is sound evidence linking SCF complexes (CRL1s) to degradation of CKDI proteins and control of proliferation in VSM. Current data do not paint an entirely clear picture however, since the effects of CSN manipulation on VSM cell proliferation appear to be dependent on the specific cell type or CSN subunit manipulated. Nevertheless, further exploration of these mechanisms may pro-

vide a new avenue to target diseases characterized by excessive VSM proliferation such as neointimal hyperplasia after balloon angioplasty or vascular wall hypertrophy in hypertensive vascular remodeling.

The data linking the CSN to VSM contractile function are less well developed. However, current findings suggest that the CSN may be involved in controlling the turnover of voltage gated calcium and potassium channels and the IP3 receptor. These proteins represent mechanisms controlling calcium flux in VSM. In addition, available data also imply that the CSN may regulate intracellular signaling mechanisms such as the RhoA-Rho kinase pathway which modulate calcium sensitivity in VSM. Lastly, the CSN may interact with endothelial mechanisms modulating vascular tone. This may occur through turnover of eNOS protein itself. Alternatively, eNOS regulatory mechanisms such phosphorylation or downstream targets such as sGC may be subject to CSN control. Collectively these data are consistent with the possibility that the CSN regulates vascular tone at several levels (**Figure 1**). Accordingly, further understanding of these interactions could reveal targets for development of vasoactive agents for the treatment conditions such as hypertension, Raynaud's disease or coronary artery spasm.

Admittedly, current evidence linking the CSN to vascular control remains largely circumstantial. However, the available data provide intriguing clues for further investigation. The CSN is an intricate control mechanism that is itself not as yet fully understood. However, within that complexity lays the promise of the requisite specificity of protein ubiquitination regulation that will be necessary for development of novel therapeutics to treat vascular disease.

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