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Syndecan2 Controls Vascular Smooth Muscle Development And Cytoskeletal Dynamics

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Syndecan2 Controls Vascular Smooth Muscle Development and Cytoskeletal Dynamics

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by
Deepak Shankar Atri

2015
Syndecan2 Controls Vascular Smooth Muscle Development and Cytoskeletal Dynamics

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Syndecan2 is a member of the Syndecan family of transmembrane proteoglycans, expressed broadly in mammals. Syndecan2, along with Syndecan4, is expressed in the vasculature of mouse and man. Global deletion of Syndecan2 in mice does not affect viability and fertility of mice. However, it does result in defects in vascular smooth muscle cell coverage of the dermal vasculature of the mouse embryo.

We here report that lineage specific deletion of Syndecan2 in vascular smooth muscle results in delayed development of vascular smooth muscle coverage in the developing retinal vasculature. Postnatally, mice that lack Sdc2 in vascular smooth muscle mice exhibit gaps in smooth muscle coverage and reduced extent in coverage of retinal arterioles. Furthermore, smooth muscle cells have a disorganized and dysplastic appearance in the arterial wall in all tissues examined.

In the adult mouse, Syndecan2 deletion also results in hypotension in the systemic circuit without an accompanying decrease in vasopressor responsiveness. Pulmonary pressures in mice that lack Sdc2 in smooth muscle are equal to those of their wild-type littermates in normoxia. After exposure to chronic hypoxia however, mice lacking Syndecan2 in their smooth muscle are protected from the development of hypoxia-induced pulmonary arterial hypertension.

In vitro, vascular smooth muscle cells lacking Syndcan2 exhibit defects in expression of Notch3 and its downstream targets. They also exhibit numerous abnormalities in cytoskeletal dynamics. These defects may be rescued by re-expressing the intracellular domain of Syndecan2, are dependent on its PDZ-binding domain and likely are due to PDZ-dependent Sdc2-Syntenin1 complex formation.

Taken together, these findings assert that Sdc2 is a key regulator in the development and differentiation of vascular smooth muscle. Furthermore, it is a therapeutic target that might slow the progression of pulmonary arterial hypertension.
All that I am, I owe to my loving parents, Chitra and Srinivas, and older brother, Prashant.

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**Introduction**

The vasculature defines the barrier between the circulation and the interstitium. Endothelial cells and their supporting cells, which vary depending on the size and nature of the vessel, play a key role in the formation of this barrier. The arterial circulation, which includes elastic arteries, muscular arteries and arterioles, has a wall comprised of three layers: the tunica intima, tunica media and tunica adventitia. These layers are composed, respectively, of endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and elastic tissue, and fibroblasts. Smaller blood vessels lack a distinct VSMC layer, but still have support cells known as pericytes. Together VSMCs and pericytes are referred to as mural cells.¹ Mural cells are critical to the regulation of vascular tone and permeability, and thus, to the regulation of blood pressure in both physiologic and pathologic states. Furthermore, they have a pronounced importance in atherosclerosis, the microvascular complications of diabetes mellitus, and numerous states other states of vascular pathology. Due to their exquisite complexity, the factors that govern the differentiation and development of mural cells, as well as the factors that determine the VSMC fate, are poorly understood.

*Differentiation and Development of Vascular Smooth Muscle Cells*

As the vascular wall has diverse properties throughout the circulation and may be altered by environmental factors, there is considerable variety in functional state and molecular markers displayed by mural cells. Extrinsic cues are important determinants of these functional states and molecular profiles. Among these cues
are physical factors, such as vessel wall stress and matrix adhesion, and biological factors such as mitogens, inflammatory mediators and direct cell-to-cell signaling.\textsuperscript{2,3}

Certain key cell signaling pathways have been identified as participants in the regulation of these phenotypic states. They include Angiopoietin, Transforming Growth Factor-β (TGF-β), Platelet-derived growth factor (PDGF) and Notch signaling pathways.\textsuperscript{1}

In the broadest sense, VSMCs are defined by the expression of a range of proteins that confer contractile functionality to the cells of the arterial wall. However, there are no known proteins that identify all VSMCs and exclude all other cells with contractile properties that are not VSMCs.\textsuperscript{3} For example, the first of the VSMC proteins to be expressed in development is α-smooth muscle actin (αSMA). This protein, however, is also expressed in skeletal and cardiac myocytes and is not specific to VSMCs.\textsuperscript{3} The expression of the genetic profile associated with VSMC contractile functionality is controlled, at least in part, by the transcription factors Serum Response Factor (SRF) and Myocardin: examples of such genes are Calponin1 (Cnn1) and smooth muscle myosin heavy chain (SM-MHC). These proteins are more specific to VSMCs than αSMA and are acquired later in the process of differentiation.\textsuperscript{2-4}

The contractile state of differentiation for VSMCs, however, is not a terminal event in the life of these cells. VSMCs can alternate between contractile or synthetic states, which reflect specific differences in cell physiology and behavior.\textsuperscript{5} The contractile state reflects a quiescent state of cell physiology, in which VSMCs express contractile genes and associated functionality with limited proliferative behavior,
whereas the synthetic state reflects the converse set of cell behaviors. While the factors controlling the transition between these two states have not been fully defined, key regulators include the protein TET2. In the absence of TET2, Myocardin and SRF are downregulated whereas KLF4, a transcription factor involved in proliferation and stem cell-like behavior, is upregulated. Together, these alterations in transcription factor expression effect a switch of cell behavior to the synthetic phenotype. In animal models, the synthetic phenotype in vascular smooth muscle, produced by knockdown of TET2, contributes to an exaggerated inflammatory response in models of vascular injury. Contrastingly, TET2 overexpression leads to mitigation of the pathologic response. Thus, it is evident that control of the contractile and synthetic phenotypes in VSMCs has an important role in development and disease.

The process of studying VSMCs in vitro adds an additional complexity. Increased time in culture conditions causes de-differentiation and loss of key markers of VSMC identity. For example, VSMCs that are well-differentiated, and contractile, express SM-MHC, Cnn1 and αSMA. However, with increasing passage number and the absence of cues that governed their phenotype in vivo, cells undergo de-differentiation with sequential loss of protein expression in the reverse order from their acquisition during differentiation: expression of SM-MHC and Cnn1 may be lost despite persistent expression of αSMA. Thus, to ensure studies of VSMCs in vitro will have physiologic relevance, specific care must be taken in order to validate expression of contractile smooth muscle genes.
Apart from state of differentiation or contractile and synthetic variability, VSMCs display distinct molecular expression patterns that are dependent on vessel identity. For example, arterial smooth muscle cells express the proteins Smoothelin and Notch3 whereas venous smooth muscle cells do not express these proteins.\textsuperscript{6-8} Additionally, NG2 appears to be a marker of arteriolar VMSCs that is not expressed by VSMCs in venules.\textsuperscript{9} Taken together, the great variation in gene expression pattern, and consequent variability in cellular behavior, makes the study of VSMC differentiation a complex line of investigation.

\textit{The Syndecan Family}

The Syndecans, numbered 1 through 4, are a family of transmembrane proteoglycans with numerous and poorly-understood signaling functions. There is considerable sequence homology between the four family members. That similarity is greatest between the pairs Syndecan1 and Syndecan3 or Syndecan2 and Syndecan4, respectively (Supplemental Figure 0). All four Syndecans are expressed in the vasculature,\textsuperscript{10} although Syndecan2 and Syndecan4 are expressed more strongly in endothelial and vascular smooth muscle cells.\textsuperscript{11} The function of Sdc4 in the vasculature has been well-characterized by our group and the Syndecan family of molecules remains of considerable interest to our work.\textsuperscript{12-17}

Each member of the family has considerable glycosylation in its extracellular domain that allows interaction with the extracellular matrix, heparin-binding growth factors, such as fibroblast growth factors (FGFs) or vascular endothelial growth factors (VEGFs), or the circulation itself in the case of the endothelial cell.\textsuperscript{11}
Glycosylation moieties are either Heparan Sulfate (HS) or Chondroitin Sulfate (CS) in nature: the identity of the glycosylating chain is contingent upon the initial conjugation of $N$-acetylglucosamine (for HS chains) or $N$-Acetylgalactosamine (for CS chains) to the serine or threonine residue in the extracellular domain.\textsuperscript{11} Chains are further modified by sulfation and epimerization of individual sugar residues.\textsuperscript{11} The identity of the HS and CS chains is significant because it alters the binding profile of the Syndecan to the various extracellular molecules.\textsuperscript{11}

The transmembrane domains of all Syndecans are nearly identical in sequence and are important for Syndecan-Syndecan dimerization, a high affinity and specificity process that is known to be critical for their signaling activity.\textsuperscript{11,18} Clustering of Syndecans has proven, in certain cases, sufficient to effect signaling mediated by their intracellular domains.\textsuperscript{12,18} Thus, the molecular regions responsible for their homo- or hetero-dimerization are critical for functionality and well-conserved.

Syndecans’ intracellular domains, exhibit considerable sequence homology, which is reflected by canonical binding sites for interacting proteins.\textsuperscript{11} The intracellular domains are divided into three segments: the conserved domain 1 ($C_1$), variable domain (V) and conserved domain 2 ($C_2$) (Supplemental Figure 0). The variable domain, as its name suggests, differs widely between family members and is responsible for the individualized functions of the Syndecans. For example, our group has shown that the V domain of Sdc4 is responsible for activating PKC\textalpha\ in a calcium-independent manner and inducing cytoskeletal changes in endothelial
The C2 domain contains a PDZ-binding domain that is known to bind many PDZ domain-containing partners such as synectin, synbindin, and syntenin.

In general, the family of molecules has broad modulatory effects in cell signaling and cytoskeletal biology that are mechanistically understudied, but have proven importance in studies of epithelial malignancy as well as vascular development and remodeling. For example Sdc4 in endothelial cells serves as a key mediator of FGFR1 signaling and intracellular signaling cascades, is involved trafficking of integrins that regulate cell adhesion, and has an atheroprotective role in vascular flow sensing. In VSMCs, Syndecans have been implicated in pathologic states such as the formation of neointima and the response to inflammation both in animal models of vascular pathology and in vitro.

*Syndecan2 functions broadly as a regulator of the cytoskeleton and modulator of signaling cascades*

Syndecan2 is a long-known but little-studied proteoglycan with diverse cytoskeletal and signaling effects that have been examined primarily in the context of cancer cell biology. The peptide backbone of the protein weighs approximately 20 kiloDaltons, but due to SDS-resistant dimerization and heavy glycosylation, the proteoglycan is observed to weigh between one hundred and two hundred-seventy kiloDaltons on Western Blot (Supplemental Figure 0). The mouse isoform of the polypeptide chain is 202 residues in length, including an eighteen-residue signal peptide. Of the remaining 184-residue chain, the carboxy-terminal twenty-nine residues constitute the intracellular domain. The extracellular domain has three
serine residues which comprise its glycosylation sites that are conjugated by HS or CS chains as noted above.26

Syndecan2 is well-known to have marked effects in the regulation of cytoskeletal dynamics. It is known to alter cell structure through cytoskeletal GTPases such as CDC4227, Rho28 and Rac129 and through changing integrin behavior.30 These cytoskeletal effects have been studied in early development30,31 and development of the nervous system32,33, but more notably in epithelial cell behavior and metastatic potential of malignant cells in the breast28, skin34,35 lung36,37, colon29, and pancreas38.

In the field of vascular development, however, there are very few studies that pertain to either the developmental or pathologic role of Sdc2. Early embryonic lethality in the setting of impaired vascular sprouting in a zebrafish model of Sdc2 knockdown suggested that Sdc2 possesses a critical role in the developing vasculature.39 In the mouse, Sdc2 is expressed strongly in both endothelium and vascular smooth muscle (unpublished data from our group). In endothelial cells, it seems that there are two pathways by which Sdc2 modulates the process of angiogenesis. Primarily, the absence of full-length Sdc2 in endothelial cells abrogates angiogenic behaviors.40 Alternatively, the shedding of the extracellular domain of Sdc2 impairs angiogenesis via a pathway that is dependent on CD14841, a membrane-associated phosphatase that is known to associate with Sdc2.42 Ultimately, in vivo, global deletion of Sdc2, as well endothelial-specific deletion of Sdc2, results in developmental and pathologic angiogenic defects, as well as a

Thus far, there has been only one study, performed in vitro, that addressed the role of Sdc2 in the physiology of VSMCs. In this study, a link was found between Sdc2 and the signaling of a key regulator of VSMC identity, Notch3. It was observed that, without Sdc2, the transcriptional effects of a Notch3-stimulating signal were abrogated and that there is a physical association between Notch3 and Sdc2. The biological significance of this interaction and its underlying mechanism remain unaddressed, due in large part to the prior absence of a global or lineage-specific mouse knockout model for Sdc2.

*Notch3 Signaling in Vascular Development and Disease*

The Notch signaling pathway is an evolutionarily well-conserved pathway with manifold effects in developmental biology. Schematically, Notch signaling is achieved by direct cell-to-cell interaction between membrane-bound ligands and membrane-bound receptors on adjacent cells. Ligand-presenting cells express a range of ligands that includes Delta-like Ligands (DLL) 1, 3 and 4 and Jagged (Jag) 1 or 2. The ligands bind membrane-bound Notch receptors (Notch1, 2, 3, or 4). Conformational changes in the receptor upon ligand binding induce a two-step cleavage event that culminates in the release of the intracellular domain (Notch ICD) by the membrane-associated protease γ-secretase. The released Notch ICD is translocated to the nucleus and associates with a larger transcriptional complex containing the proteins CSL and MAML to effect gene expression. Notch signaling
is well-known to have a broad and significant role in development and disease states in the vasculature as it controls arterial and venous identity, tip- and stalk-cell identity as well as vascular smooth muscle development.\textsuperscript{44,45}

Notch3 is a Notch family receptor whose expression in the vasculature is limited to VSMCs.\textsuperscript{8} Notch3 is a clinically-significant molecule because it is associated with two notable disease states. Mutations in Notch3 are causative in the disease known as Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)\textsuperscript{46}, although there remains considerable ambiguity as to the precise cause of the disease.\textsuperscript{47} Additionally, Notch3 hyperactivity contributes to the development of pulmonary arterial hypertension.\textsuperscript{48}

CADASIL is a disease of the vasculature with an estimated prevalence of four in one hundred thousand individuals and is inherited in an autosomal dominant fashion. The disease makes its first appearance in the fourth decade of life and claims the life of the afflicted in the sixth decade from neurologic complications.\textsuperscript{49,50} Clinical features of the disease are broad, ranging from hemiplegic migraine to frank stroke with a stuttering course that results in vascular dementia.\textsuperscript{50-52} VSMCs are affected in the vasculature of all tissues, and diagnosis may be achieved by skin biopsy, but the clinical consequences of this dysfunction are observed only in the central nervous system given its low tolerance to vascular insult.\textsuperscript{53,54}

On the pathologic level, the histologic hallmark of CADASIL is the presence of small-vessel infarcts with deposition of perivascular plaques known as granular osmiophilic material (GOM) that are visualized using electron microscopy.\textsuperscript{53-55} GOM
is composed, at least in part, of aggregates of the extracellular domain of Notch3 protein.\textsuperscript{56}

On the molecular level, canonical mutations in Notch3 protein seem to be causative in patients with CADASIL. All somatic mutations of the gene encoding Notch3 appear to add or eliminate a single cysteine residue in the extracellular domain of the protein: more than one hundred-fifty such mutations have been described.\textsuperscript{53} It is not surprising, given the numerous possible mutations which cause CADASIL, that there is a broad spectrum of clinical pictures in the afflicted.\textsuperscript{50}

To further complicate attempts to understand the pathophysiology of the disease, there is no good animal model which clinically or pathologically recapitulates CADASIL.\textsuperscript{57,58} Mice lacking Notch3, as well as mice which express transgenic Notch3 protein with human mutations of CADASIL, do not faithfully mimic the disease state.\textsuperscript{57-59}

Dysregulation of Notch3 signaling in the form of hyperactivation also contributes to a disease state by causing pulmonary vascular changes that result in pulmonary arterial hypertension (PAH).\textsuperscript{48} PAH is defined as the presence of elevated resting mean pulmonary arterial pressures (≥25mmHg) without abnormal elevation in pulmonary arterial wedge pressures (≤15mmHg).\textsuperscript{60} The prevalence of the disease is likely underestimated, but thought to be between fifteen and fifty per million and is greatest among young adult women.\textsuperscript{61} While the exact causes of the disease remain unknown and may differ between patients, several factors are known to contribute to its pathogenesis. They include mutations in elements of the TGF-β signaling pathway, abnormality in the signaling of small molecules such as
nitric oxide, serotonin, prostacyclin and thromboxane, protein signals such as endothelin-1 and VEGF, environmental factors such as hypoxia and drug exposure or comorbidities such as collagen vascular disease, HIV infection or blood cell abnormalities.62

The common pathologic findings between all cases of pulmonary arterial hypertension include vascular smooth muscle cell hyperplasia leading to hypertrophy in the pulmonary circulation and vascular pruning. Together, these lead to a reduction in cross-sectional area of the vasculature and limitation of the surface area for gas diffusion. Pulmonary arterial hypertension has a five-year survival from time of diagnosis of roughly fifty percent.61 The few available therapies that exist for pulmonary arterial hypertension target the signaling of prostaglandins, nitric oxide and endothelin. Importantly, none of these therapies slow disease progression: they provide only symptom benefit.61,62

Excessive activity of Notch3 in the pulmonary vasculature is responsible for the development of pulmonary arterial hypertension in both patients and mouse models of the disease.48 Notch3 and Hes5, a transcription factor that is induced by Notch3 transcriptional activity, are over-expressed in patient samples of pulmonary arterial hypertension and their degree of expression correlates with the severity of PAH exhibited in patients. Mice lacking Notch3 as well as mice that were treated with chemical inhibitors of Notch signaling were protected from pulmonary arterial hypertension induced by either hypoxia or pharmacologic means.48 Furthermore, these mice exhibited protection from vascular hypertrophic changes and pruning, as well as secondary cardiac remodeling. Notch3 hyperactivity increased proliferation
of VSMCs and hypertrophy of vascular smooth muscle in the pulmonary arteries, whereas genetic or pharmacologic blockade of Notch3 caused apoptosis in VSMCs.\textsuperscript{48}

The understanding of Notch3 signaling has been assisted by the existence of a Notch3 global knockout mouse (Notch3\textsuperscript{\textminus/\textminus}). Notch3\textsuperscript{\textminus/\textminus} mice are viable, but exhibit enlarged arteries with disorganized medial layers that progress to an atrophic-appearing state before adulthood. However, despite these rather marked abnormalities, mice up to one year of age show no evidence of end-organ damage secondary to vascular insult.\textsuperscript{8} Notch3\textsuperscript{\textminus/\textminus} mice also display an age-dependent permeability defect: young adult mice exhibit extravasation of serum proteins at sites of VSMC apoptosis.\textsuperscript{63} However, these leakage defects may occur secondary to endothelial changes in the absence of VSMC coverage.\textsuperscript{63} Regardless, neither white-matter degeneration nor deposition of GOM has ever been observed in a Notch3\textsuperscript{\textminus/\textminus} mouse, suggesting that CADASIL is not simply due to loss-of-function in Notch3. Furthermore, Notch3\textsuperscript{\textminus/\textminus} mice exhibited normal blood pressures and normal responsiveness to the effects of vasopressors\textsuperscript{8}, which is notably defective in arteries from CADASIL patients.\textsuperscript{64-66} \textit{In vitro}, it was shown that Notch3 activation has significant effects on the shape of VSMCs, however there remains no mechanistic explanation for how it mediates cytoskeletal changes.\textsuperscript{8}

The absence of Sdc2 is thought to be important because it compromises the signaling activity of Notch3. That activity may only be achieved in presence of a ligand for the Notch3 receptor. Thus in the examination of the role of Sdc2, it is important to consider the identity of a Notch3-ligand in the signaling event in question. It is likely that the Notch ligand Jagged1 serves as the physiologic ligand of
Notch3 in VSMCs and assists in their differentiation and maturation from the unspecified mural cell lineage: mice with a lineage-specific knockout of Jagged1 in endothelial cells suffer embryonic lethality due to cardiac defects and impaired vascular smooth muscle development. Notch3 expression is increased in mural cells in the presence of endothelial cells expressing Jaged1 and increases in Notch3 expression are dependent on basal levels of Notch3 expression. Thus, activation of Notch3 by endothelial Jagged1 produces an increase in the Notch3-ICD-mediated transcription of Notch3 and its likely ligand Jag1 in VSMCs. Taken together, these findings suggest that Notch3 serves as a feed-forward transcriptional mediator that contributes to the arterial VSMC phenotype.

Among the transcriptional targets of Notch3, are two key regulators of VSMC physiology: Platelet-Derived Growth Factor Receptor β (PDGFRβ) and EphrinB2. Both of these genes are down-regulated in VSMCs lacking Notch3. PDGFRβ is the receptor tyrosine kinase that binds the ligand PDGF-BB, a key cytokine for the differentiation, recruitment and growth of VSMCs and pericytes. The absence of PDGF-BB or PDGFRβ causes perinatal lethality due to diffuse vascular abnormalities that are associated to failure of mural cell recruitment.

EphrinB2 is a receptor in the Ephrin/Eph pathway, another pathway that is dependent on direct cell-to-cell interaction. It has been well-characterized in the endothelium as a marker of arterial endothelial fate. Limited work on EphrinB2 in VSMCs has demonstrated its significance in that cell type, as a lineage-specific smooth muscle cell knockout of EphrinB2 results in postnatal lethality with diffuse visceral and cutaneous hemorrhage. On the cellular level, the absence of EphrinB2
results in considerable abnormalities of cytoskeletal dynamics, including defective focal adhesion formation and impaired cell spreading.\textsuperscript{75} These abnormalities are due, at least in part, to effects of EphrinB2 on the control of PDGFRβ signaling, as VSMCs lacking EphrinB2 exhibit enhanced internalization and degradation of PDGFRβ in response to ligand.\textsuperscript{76} The absence of EphrinB2 also abrogated the effects of PDGF-BB on the induction of Rac1 activity that is necessary for cell spreading and migration.\textsuperscript{76}

The phenotypes exhibited by animal models of Notch3 or Notch3-target abnormalities and the human Notch3-associated disease states indicate a critical role for Notch3 signaling in the development and maintenance of vascular smooth muscle. Further insight into the signaling of Notch3 would enhance our understandings of developmental and pathologic processes. Given that Sdc2 is known to associate physically with Notch3 and modulate Notch3 signaling, insight into Sdc2 biology might serve as an avenue to better understand biology or treat the disease states wherein Notch3 is implicated.
Statement of Purpose

Previous work from our group and others addressed the function of Syndecan4 in blood endothelial cells. Syndecan2 and Syndecan4, as noted above, are the Syndecan family members that are most highly expressed in the vasculature. Due to the homology between Sdc2 and Sdc4, our lab generated a mouse with a Syndecan2 constitutive global deletion (Sdc2\(^{-/-}\)) and a mouse with Sdc2 flanked by LoxP sites (Sdc2\(^{fl/fl}\)) to generate lineage specific knockouts with various Cre mice (John Rhodes in Simons’ Lab).

Sdc2\(^{-/-}\) mice exhibit endothelial phenotypes such as retarded developmental angiogenesis, impairment in pathological arteriogenesis and delayed wound healing (Yingdi Wang, Jiasheng Zhang) which are due to defects in signaling of VEGF-A through VEGFR2 in endothelial cells (Federico Corti). Developmentally, it was also observed that there was abnormal vascular smooth muscle coverage in the embryonic dermal vasculature with sporadic gaps in arterial smooth muscle (Yingdi Wang). Mice that lack Sdc4, however, exhibit no defects in vascular smooth muscle coverage despite the fact that Sdc4 is expressed in VSMCs.

From this preliminary data, and the previous findings that Sdc2 functions to augment the signaling of Notch3 in VSMCs, we set out to characterize the function of Sdc2 in the development of vascular smooth muscle \textit{in vivo} and to assess the downstream effects of Sdc2 on cell signaling and cytoskeletal dynamics \textit{in vitro}. We had in our laboratory the previously unpublished Sdc2\(^{-/-}\) mouse and Sdc2\(^{fl/fl}\) mouse, which enabled us to understand the physiologic roles of Sdc2. Our \textit{in vitro} studies were also empowered by making use of VSMCs cultured from Sdc2\(^{-/-}\) mice, an
approach superior to the use of siRNA for genetic knockdown. Using molecular mutants and chimeras expressed with adeno-associated viruses, we sought to identify the domain-dependence of Sdc2 signaling functions that are distinct from those of Sdc4.

Specific Aims:

1. To generate a conditional knockout mouse line which lacks Sdc2 in vascular smooth muscle (Sdc2\textsuperscript{SMKO})
   a. To characterize vascular smooth muscle development in Sdc2\textsuperscript{SMKO} mice
   b. To evaluate the effects of Sdc2 deletion in vascular smooth muscle in pathologic conditions

2. To define and characterize cellular phenotypes in vascular smooth muscle cells which lack Sdc2

3. To determine the protein domains of Sdc2, which are not present in Sdc4, that are responsible for the distinct functions of the Sdc2 with re-expression of molecular mutants in Sdc2\textsuperscript{-/-} VSMCs.
Materials and Methods

Except where noted, all methods described were performed by the author.

Animal Breeding and Genotyping

Mice globally lacking Sdc2 (Sdc2<sup>-/-</sup>) and Sdc2-floxed mice (Sdc2<sup>fl/fl</sup>) were generated for the Simons laboratory using embryonic stem cells obtained from the Knockout Mouse Project (KOMP) on a background of C57BL/6N (John Rhodes). Mice bearing a transgene for cyclic recombinase (Cre) under the promoter of the gene encoding Sm22α, a vascular smooth muscle-specific gene, were obtained from Jackson Laboratories via the laboratory of Anne Eichmann in the Cardiovascular Research Center (tg; Sm22α-Cre). These mice were on a background of C57BL/6J.

Mice with a lineage-specific deletion of Sdc2 in vascular smooth muscle cells (Sdc2<sup>SMKO</sup>) were generated from crosses between Sdc2<sup>fl/fl</sup> mice and tg; Sm22α-Cre mice. The F1 generation produced mice with the genotype Sdc2<sup>fl/+</sup>, (tg; Sm22α-Cre), which were then mated with Sdc2<sup>fl/fl</sup> mice to produce progeny which included mice of the genotype Sdc2<sup>fl/fl</sup>; (tg; Sm22α-Cre), or Sdc2<sup>SMKO</sup>.

Automated genotyping was performed for Cre, wild-type and floxed Sdc2 alleles with the use of services provided by the Transnetyx Corporation.

Immunohistochemistry of Mouse Tissues

Histologic samples were obtained by dissection of tissues with intracardiac perfusion from Sdc2<sup>SMKO</sup> and control mice. Samples were fixed in paraformaldehyde
(4%, Electron Microscopy Sciences) overnight followed by sinking in 30% sucrose solution in PBS and freezing in OCT medium before sectioning at 25-50μm on a cryostat (Leica).

Retinas of postnatal mice were prepared as previously described. Briefly, eyes were excised from mice at neonatal and adult stages and immersed in 4% PFA for 15 minutes. Eyes were then washed in PBS and retinas were dissected and subsequently immersed in methanol overnight before staining.

Retinas and other tissues were blocked for one hour at room temperature before immersion in primary antibody solution overnight at 4°C. Samples were then washed four times in 1X PBS for fifteen minutes per wash before immersion in secondary antibody for four hours at room temperature. Samples were then washed as before and mounted with ProLong Gold AntiFade mounting media with DAPI (Life Technologies; Molecular Probes) on glass slides under glass coverslips (VWR).

For immunohistochemistry, samples were blocked with 1% BSA (Sigma) and 0.3% Triton X-100 (Sigma) diluted in PBS (VWR). This solution was also used for the dilution of primary and secondary antibodies. The list of primary antibodies is included below (Table 1). Secondary antibodies conjugated with AlexaFluor fluorophores (Life Technologies) were diluted 1:300. Fluorophore-conjugated IsolectinB4, a marker of endothelial cells, was diluted at 1:100 (Life Technologies).

High-power images were obtained with a spinning disk confocal microscope (PerkinElmer) with Volocity Software. Low power images were taken with a Nikon 80i upright fluorescence microscope. Image measurements were taken using ImageJ (NIH).
Catheterization-based experiments and Echocardiography

Experimental design of catheterization experiments was conducted by the author. Technical aspects of these experiments were performed Jennifer Hu (laboratory of Hyung Chun).

Age-matched adult male mice of at least twenty grams body weight were used for systemic catheterization experiments. Briefly, mice were anesthetized with isoflurane via nasal cannula through the course of the experiment and immobilized with adhesive tape in the supine position. The common carotid artery was catheterized with a small animal research catheter/pressure transducer (Millar) and the systemic pressure was recorded for five minutes. Calculations of mean arterial pressure were performed using LabChart Pro Software.

In order to assess the contractile function of vascular smooth muscle and its effects on mean arterial pressure, phenylephrine was administered at 50 μg/kg in less than 100μl total volume via a catheter placed in the internal jugular vein while the carotid artery pressure catheter remained in place. Measurement of systemic pressure continued for at least five minutes after infusion of the vasopressor. Analysis of mean arterial pressure was performed as before with averaging of pressure over one respiratory cycle at thirty-second intervals.

For experiments requiring hypoxia, mice were housed in original cages in an atmosphere containing 10% oxygen as previously described by our collaborators. Mice were catheterized after three weeks of hypoxia exposure. Right-heart catheterization was performed via the internal jugular vein into the right ventricle
to measure right ventricular systolic pressure (RVSP). After obtaining RVSP traces for five minutes, the catheter was extracted and the mouse was sacrificed with heart and lungs being extracted for histology and protein samples. As before, LabChart Pro Software was used for analysis. RVSPs were calculated from systolic and diastolic pressures, assuming that RVDP is equal to zero, at the middle of the respiratory cycle.

Mouse echocardiography was conducted in collaboration with Yale Translational Research and Imaging Center (Y-TRIC) director Prof. Albert Sinusas and Alda Bregasi. Briefly, mice were transported from our vivarium to the Y-TRIC by YARC for transthoracic echocardiography and returned to our vivarium on the same day. Mice were anesthetized with isoflurane by nasal cannula during echocardiography. Raw data was conveyed to the authors.

*All in vivo experimentation was conducted in accordance with protocols approved by Yale University Institutional Animal Care and Use Committee Protocol #11231 to Professor Simons.*

**Cell Culture**

Cells were isolated as previously described. Briefly, aortas were extracted from adult mice and stripped of adipose tissue before immersion in an elastase- and collagenase-based solution. Adventitial tissue was then stripped from the aortas and they were minced and subjected to further digestion in a higher-strength elastase solution. Cells were then triturated through a glass Pasteur pipette and plated in growth medium.
Growth medium consisted of DMEM with 10% fetal bovine serum with non-essential amino acids and penicillin/streptomycin solution. Cells were plated on standard tissue-culture treated surfaces (Corning). Cells were passaged by washing with PBS solution and incubation with 0.25% trypsin for at least five minutes (all from Life Technologies). For immunocytochemistry, cells were plated on coverslips (VWR) coated with 0.1% gelatin (Sigma).

Tranfection with siRNA (Origene) was performed on cells which were 80% confluent in a 6-well plate. Growth medium was removed and OptiMEM (GIBCO) was added. Five microliters of both siRNA and Lipofectamine RNAiMax (Life Technologies) were each diluted in 150μl of OptiMEM (Life Technologies). The two solutions were mixed and incubated for twenty minutes before being added to cells. Six hours later, growth medium was added and twenty-four hours later all media was exchanged. Two days after medium exchange, cells were harvested for protein and RNA for analysis or fixed for immunocytochemistry. Sequences for siRNA are listed below as provided by the manufacturer (Table 2).

Western Blotting and Quantitative PCR

For Western Blotting, cells were lysed and scraped using RIPA buffer (Boston Bioproducts) with PhosStop phosphatase inhibitor and CompleteMini protease inhibitors (Roche Biosciences). Concentrations of protein cell lysates were measured by spectrophotometry (BioRad) and normalized with additional lysis buffer before mixing with 6x LaemmlI’s Dye in reducing conditions. Proteins were separated on 4%-20% tris-glycine polyacrylamide gels (BioRad) before transfer to PVDF membranes (Immobilon). Blocking of membranes was performed with 5%
milk powder (American Bioanalytical) in TBS-Tween (Boston Bioproducts). Primary antibodies for Western blotting were diluted in 5% BSA in TBS-T. Membranes were immersed overnight after one hour of blocking. After washing with TBS-T, secondary antibodies (Vector Biosciences) were added after being diluted with 5% milk. Primary antibodies that were used for Western Blotting are listed below (Table 1).

Total RNA was extracted from cells using RNAeasy Mini kit (Qiagen). cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad) and loaded into reactions with SYBR Green Supermix (BioRad) before analysis on the BioRad CFX96 RealTime System. Primers were designed by the author using PrimerBlast software from the National Center for Biotechnology Information and are listed below (Table 3).

**Cell Biology Experiments**

Assays of cell adhesion were conducted as previously described. Briefly, 96-well tissue culture plates were coated in solutions containing Collagen I, Fibronectin or Laminin (Sigma) and blocked with heat-inactivated BSA solution before plating thirty-thousand cells per well of wild-type or Sdc2−/− VSMCs. After two hours of incubation, cells were either washed once with PBS and fixed or fixed without disruption so that a ratio between wells would represent fractional adhesion. Cells were then stained with crystal violet stain for one hour before subsequent washing and solubilization in 10% acetic acid. Absorbance of wells at 570nm was then read with a plate-reader, as solubilized stain would correlate with
the quantity of cells in each well. Measurements of cell proliferation and migration were conducted using the Roche xCELLigence system, which measures the electrical resistance of cells, bound to a plate as a surrogate for the quantity of cells in the plate (for proliferation) or the quantity of cells bound (for migration).\textsuperscript{80}

GTPase activation assays for Rac1 and RhoA activity were conducted using an ELISA-based method to assay G-Protein activity (Cytoskeleton Inc.).\textsuperscript{81} Briefly, the assay is an ELISA that is dependent on the PAK1 binding domain that is able to bind only the active form of Rac1, which is GTP-bound. The plate is blocked before lysates of equal concentration are added and incubated. GTP-bound cytoskeletal GTPases are bound to the plate and then bound by the addition of primary and secondary antibodies. Secondary antibodies are conjugated to horseradish peroxidase, which is used as a detection method in a luminometer after incubation with a chemiluminescent substrate.

\textit{Cloning and Adenovirus Construction}

Plasmids with full-length sequences for Sdc2 and Sdc4 were previously available in the lab. These constructs were appended with a 5' influenza hemagglutinin (HA) tag (Federico Corti). By overlap extension PCR, chimeras were generated which fused the extracellular and transmembrane domains of Sdc4 to the intracellular domain of Sdc2 (Sdc2\textsuperscript{ΔECTM}) and the extracellular and transmembrane domains of Sdc2 fused to the intracellular domain of Sdc4 (Sdc2\textsuperscript{ΔICD}).\textsuperscript{82} Additionally, by site-directed mutagenesis, individual mutations were made to Sdc2 such that it's C\textsubscript{1}, C\textsubscript{2} and V domains were mutated to the sequence of Sdc4 (Sdc2\textsuperscript{ΔC1}, Sdc2\textsuperscript{ΔC2},
Sdc2\textsuperscript{ΔV}) and lastly to delete the PDZ-binding domain of Sdc2 (Sdc2\textsuperscript{PDZ-}) by either truncating the carboxy-terminal alanine or four carboxy-terminal amino acids.

Full-length Sdc2, Sdc4 and all mutants were then packaged into an adeno-associated viral vector under a CMV-minimal promoter that was transfected into HEK293A cells for packaging of adeno-associated viruses (Life Technologies). Adeno-associated viruses were used to infect VSMCs such that expression, as measured by Western blotting for the HA-tag, was comparable. Adeno-associated virus containing Green Fluorescent Protein was used as control.

### Table 1. Primary Antibodies

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---|---|---|---|---|---
HA | Mouse | Covance | mms-101p | ICC | 1:300
| | | | | WB | 1:1000

Table 2. siRNA Sequences

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Table 3. QPCR Primers

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Table 12.11

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Table 13.1

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Results

Generation of Sdc2<sup>SMKO</sup> Mice

In order to evaluate the consequences of the loss of Sdc2 expression might have on vascular smooth muscle physiology, we crossed our previously-generated Sdc2<sup>fl/fl</sup> mice of C57/BL6 background with mice expressing Cre recombinase under the Sm22α (transgelin) promoter (Sm22α-Cre) in order to generate Sdc2<sup>fl/fl; tg(Sm22α-Cre)</sup> mice (Sdc2<sup>SMKO</sup>). Sdc2<sup>SMKO</sup> mice were delivered in expected Mendelian ratios, were fertile and exhibited no gross abnormalities up to six months of age. These mice exhibited deficiency of Sdc2 in the smooth muscle of the carotid arterial system as early as embryonic day 18.5 (E18.5) (Supplemental Figure 1). Histologic study of brains from mice at two weeks and six months of age revealed no evidence of histologic abnormality. In particular, there was no evidence of small vessel infarction and leukoencephalopathy, which are the pathologic hallmarks associated with CADASIL.

The retinal vasculature of Sdc2<sup>SMKO</sup> mice exhibits reduced and abnormal development of vascular smooth muscle in the postnatal period

A common model to observe developmental angiogenesis and arteriogenesis is the neonatal mouse retina. The retina in the mouse is avascular at birth and exhibits a stereotyped pattern of vascular ingrowth from the central artery of the retina thereafter. Thus, a histologic whole-mount of the retina obtained during the first four weeks of life provides a way to assess vascular growth and patterning. Retinas were examined at postnatal days 5 and 12 (P5, P12) in order to evaluate
vascular smooth muscle investment of the developing vasculature in Sdc2^{SMKO} mice and their wild type littermates. P12 retinas were also examined in mice ROSA26^{mTmG/mTmG}; tg(Sm22α-Cre) reporter mice to assess the recombination effectiveness of Cre in our system (Supplemental Figure 2).

At P5, retinas exhibited aberrant vascular smooth muscle cell coverage. Gaps in SMC coverage, which are normally visualized at the distal boundaries of arteriolar VSMC coverage, were seen in retinal arterioles near to their origins from the central artery of the retina (Figure 1A, D).

In order to more-closely examine the changes in VSMC structure evident by fluorescence microscopy, we collaborated with the laboratory of Christer Betsholtz at the Karolinska Institute, to perform electron microscopy on the retinal arteries of Sdc2^{SMKO} mice. Preliminary results of these images reveal that gaps in coverage are actually due to the absence of mural cell coverage rather than the presence of a cell, which does not stain immunohistochemically for smooth muscle actin. We are awaiting further results from follow-up samples.

At P12 retinal arterioles exhibited no gaps in VSMC coverage comparable to those observed at P5 (Figure 1B, E). However, retinal arterioles exhibited a decreased radial extent of vascular smooth muscle coverage when measured from the center of the retina (1941 ± 69μm in wild-type compared with 1764 ± 42μm in Sdc2^{SKMO}, p<0.05, Figure 1C). This was accompanied by a reduction in the distance between the center of the retina and the first major branch point of each arteriole beyond which further branches also exhibit VSMC coverage (1433 ± 79μm in wild-type compared with 890 ± 88μm in Sdc2^{SMKO}, p<0.0001, Figure 1F). Inspection of
retinal arterioles at P12 also demonstrated that retinal arterioles of Sdc2^{SMKO} mice were dilated relative to their wild-type littermates (15.5 ± 0.26μm in wild-type compared with 16.2 ± 0.19μm in Sdc2^{SMKO} mice, p<0.05, Figure 1G, H, I).

*Sdc2^{SMKO} mice exhibit abnormal vascular smooth muscle coverage at P14 in all tissues examined*

In order to determine whether smooth muscle coverage abnormalities existed in other tissues, we examined the cerebral (Figure 2A, B, E, F), cardiac (Figure 2C, G) and tail (Figure 2D, H) vasculatures of Sdc2^{SMKO} mice at P14. Examination of smooth muscle coverage of arteries on cross section revealed a disorganized appearance to the arterial wall. Vascular smooth muscle layers in arteries of Sdc2^{SMKO} mice seemed dysplastic in nature. Cells were poorly aligned relative to well-organized smooth muscle layers of comparable arteries in wild type mice. VMSCs in arteries of Sdc2^{SMKO} mice exhibited irregular size and shape.

*Sdc2^{SMKO} mice exhibit reduced systemic blood pressure but no abnormality in vasopressor response*

To evaluate whether there were physiologic correlates to arterial smooth muscle disorder and arteriolar dilatation observed in Sdc2^{SMKO} mice, carotid artery catheterization was performed in order to measure systemic blood pressures in adult mice. At baseline, Sdc2^{SMKO} mice exhibited reduction in mean arterial pressure compared with wild-type mice (94.36 ± 11.32mmHg in wild-type; 75.46 ± 9.93mmHg in Sdc2^{SMKO} mice, p<0.05, Figure 3A).
We hypothesized that reductions in resting blood pressure in Sdc2<sup>SMKO</sup> mice might be caused by changes in vascular tone or contractile dysfunction. In order to test these possibilities phenylephrine, a pure $\alpha_1$ adrenergic agonist was infused into the internal jugular vein while the pressure catheter remained in place within the carotid artery. Mean arterial pressures were calculated at the pressure peak and at thirty-second intervals thereafter. There were no differences in mean arterial pressure between groups at the highest pressure reached after phenylephrine infusion or at each thirty-second interval over the five following minutes (Figure 3B), suggesting no abnormality in contractile function of VSMCs or in the sensitivity of VSMCs to contractile stimuli.

In order to determine whether differences in baseline systemic mean arterial pressures were due to cardiac abnormalities, we performed transthoracic echocardiography on wild-type and Sdc2<sup>SMKO</sup> mice. There were no differences in any of the parameters measured by echocardiography between wild-type and Sdc2<sup>SMKO</sup> mice (Supplemental Figure 3), suggesting that the difference in baseline systemic mean arterial pressures were non-cardiac in origin.

*Sdc2<sup>SMKO</sup> mice are protected against the development of hypoxia-induced pulmonary arterial hypertension*

Hyperactivation of the vascular smooth muscle cell-specific Notch receptor, Notch3, has been shown to cause pulmonary arterial hypertension. In order to evaluate whether Sdc2 might cooperate with Notch3 signaling, we exposed our Sdc2<sup>SMKO</sup> mice to an atmosphere containing ten percent oxygen for three weeks,
which is known to induce pulmonary arterial hypertension in mice through a Notch3-dependent mechanism, and then performed right-heart catheterization. While there was no difference in baseline right ventricular systolic pressure (RVSP) between wild-type and Sdc2\textsuperscript{SMKO} mice before exposure to hypoxia (25.50 ± 0.85mmHg in wild-type mice compared with 25.00 ± 1.43mmHg in Sdc2\textsuperscript{SMKO} p=0.77, \textbf{Supplemental Figure 4A}), we found that Sdc2\textsuperscript{SMKO} mice had markedly lower RVSP than wild-type mice after three weeks of hypoxia (41.26 ± 1.24mmHg in wild-type mice compared with 34.56 ± 0.52mmHg in Sdc2\textsuperscript{SMKO} mice, p<0.001, \textbf{Figure 4A}). This difference was preserved when RVSP values were controlled for gender (\textbf{Figure 4B}).

\textit{Deficiency of Sdc2 results in reduction of Notch3 target expression}

Primary VSMCs expressing αSMA and SM-MHC may be efficiently and specifically cultured from the aortas of mice (\textbf{Supplemental Figure 5}). Western blots of protein lysed from low-passage number cells reveal a reduction in full-length Notch3 protein and an even greater decrease in the Notch3 ICD in Sdc2\textsuperscript{-/-} VSMCs compared with wild-type VSMCs (\textbf{Figure 5}). This was accompanied by a reduction in Notch3 downstream targets PDGFRβ and EphrinB2 (\textbf{Figure 5}) and corresponding reductions in the transcriptional level of these genes as measured by qPCR (\textbf{Figure 6}). There were no differences in the protein level of αSMA or SM-MHC (\textbf{Figure 5}). Quantitative PCR also showed no reduction in canonical markers of VSMC differentiation such as Serum-responsive factor (SRF) and myocardin, as well as no change in the mRNA levels of the Notch ligand Jagged1 (\textbf{Figure 6}).
Notch1 and Notch-pathway transcription factor HeyL transcript levels were increased considerably in the absence of Sdc2 (Figure 6).

We hypothesized that an abnormal reduction in the Notch3-ICD, which results from the absence of Sdc2, would cause a reduction in Notch3 target gene expression. In order to verify that the protein expression changes are parallel between cells lacking Sdc2 and cells lacking Notch3, we assayed protein expression of known Notch3 transcriptional targets that were reduced on the protein level in the absence of Sdc2. After Notch3 knockdown, we observed reduction in PDGFRβ and EphrinB2 protein compared with cells transfected with control siRNA (Figure 7), suggesting parallel signaling pathways between Sdc2 and Notch3.

**Re-expression of the Sdc2 intracellular domain rescues Notch3-target defects**

In light of the fact that Sdc4−/− mice have no abnormalities in vascular smooth muscle coverage, and that there is considerable sequence homology between Sdc2 and Sdc4, we generated Sdc2 and Sdc4 chimeras to determine which domains of Sdc2 might be necessary and sufficient to rescue abnormalities in Notch3 signaling seen in Sdc2 mutants (Supplemental Figure 6). Expression of GFP and HA-Sdc4 were insufficient to rescue expression of Notch3, PDGFRβ or EphrinB2 in Sdc2−/− VSMC. Expression of HA-Sdc2 as well as HA-Sdc2ΔECTM were sufficient to rescue abnormalities of Sdc2−/− cells. Neither HA-Sdc2ΔICD or HA-Sdc2PDZ− were sufficient to rescue these abnormalities (Figure 8). These data show that an intracellular domain of Sdc2 and the intact Sdc2 PDZ-binding site are necessary and sufficient for rescue of Notch3 signaling in Sdc2−/− SMC. We aim to use further available chimeras
(Supplemental Figure 6) in order to identify the individual residues responsible for the Notch3-associated signaling of Sdc2.

*Sdc2 does not alter proliferation rate, but contributes to cell migration*

We determined that there was no difference in proliferation rate after an initial difference within the first six hours of the experiment likely due to differences in cell adhesion (Figure 9A). In migration studies using the same device, we normalized the number of migrated cells at 24 hours to the number of cells migrated after 96 hours. Given that initial populations were equal and that proliferation rates were the same, the relative reduction in migrated VSMCs in the Sdc2\(^{-/-}\) group indicates a migratory delay in these cells compared with control cells (Figure 9B), consistent with findings observed in the retina of Sdc2\(^{SMKO}\) mice.

*Sdc2 affects cytoskeletal dynamics in VSMCs*

Due to *in vivo* observations of abnormal cellular structure, we sought to determine if there were cytoskeletal abnormalities in VSMCs lacking Sdc2. Compared with the uniform distribution by their wild-type counterparts, VSMCs isolated from the aortas of Sdc2\(^{-/-}\) exhibit an abnormal cellular distribution of focal adhesions, stained by vinculin, which were localized primarily in the periphery of the cell (Figure 10A, C). Filamentous actin is comparably redistributed: in wild-type cells filamentous actin, stained by phalloidin, is organized in stress fibers that traverse the middle of the cell, but in Sdc2\(^{-/-}\) cells it is redistributed to the periphery of the cell (Figure 10B, D).
Further analyses of cytoskeletal function exhibited abnormalities. Measurement of Rac1 activity by ELISA for Rac1-GTP revealed a 30% rise in GTP-bound Rac1 relative to wild-type cells cultured in basal conditions (Figure 11A), but there was no change in the activity of RhoA (Figure 11C). In studies of cell adhesion, there were no changes in fibronectin adhesion of Sdc2−/− VSMCs compared with wild-type VSMCs (WT: 54.0 ± 3.7%, Sdc2−/−: 58.0 ± 6.1%, p=.37). However, Sdc2−/− exhibited a trend toward increased adherence to collagen I (WT: 9.8 ± 5.0%, Sdc2−/−: 25.1±9.0%, p = 0.062) and laminin (WT: 4.5 ± 1.8%, Sdc2−/−: 25.6 ± 14.2%, p = 0.063) (Figure 11B). Quantitative PCR also showed concordant increases in mRNA levels of integrin α4, α6 and β4 without change in the expression of integrin β1 (Figure 11D).

Re-expression of the Sdc2 intracellular domain rescues Sdc2−/− cytoskeletal abnormalities

In order to assay the domain-dependence of the effects of Sdc2 on cytoskeletal changes, we re-expressed mutants of Sdc2 into VSMCs cultured from Sdc2−/− mice (Supplemental Figure 6). Expression of GFP (data not shown) and HA-Sdc4 (Figure 12D, E, F) were insufficient to rescue abnormal distributions of focal adhesions and cellular actin. Expression of HA-Sdc2 (Figure 12A, B, C) was sufficient to rescue these abnormalities, as was expression of HA-Sdc2ΔECTM (Figure 12G, H, I). Expression of HA-Sdc2PDZ− (Figure 12J, K, L) was insufficient to rescue these cytoskeletal abnormalities. Taken together, these data suggest the necessity of the PDZ-binding domain and one other portion of the intracellular domain of Sdc2.
that is not present in Sdc4, as this latter protein may not rescue Sdc2−/− VSMC cytoskeletal defects.

*Syntenin1 binds Sdc2 by its PDZ-binding domain and its knockdown induces cytoskeletal changes in VSMCs*

Syntenin1 is a known binding partner of Syndecans and is known to have effects on cytoskeletal dynamics. We found, by re-expressing HA-Sdc2 and HA-Sdc2PDZ− in Sdc2−/− VSMCs, that Syntenin1 may be co-immunoprecipitated with Sdc2 in VSMCs and that the interaction between Sdc2 and Syntenin1 is dependent on the PDZ-binding domain (Figure 13).

Furthermore, when Syntenin1 is knocked-down in wild type VSMCs by siRNA transfection (Figure 14), there is a comparable redistribution of filamentous actin and focal adhesions to those changes seen in Sdc2−/− VSMCs (Figure 15C-H). Importantly, knockdown of Syntenin1 had no effect on the expression of PDGFRβ (Figure 14), suggesting that Notch3-pathway abnormalities and changes in the cytoskeleton observed in Sdc2−/− VSMCs likely occur through different pathways.
Discussion

Syndecans are a poorly-understood family of signaling molecules with diverse effects in the vasculature. Our work with Sdc2−/− and Sdc2SMKO mice represents one of the first explorations into the function of Sdc2 in a mammalian system and are the first studies of Sdc2 in the mammalian vasculature in vivo. There is great breadth to the effects of Sdc2 in the vasculature, including changes in cell biology, alterations in physiologic parameters and protection from a disease state. We further identified domain-dependence of these effects and interacting proteins. Taken together, these findings identify Sdc2 as a significant proteoglycan in vascular biology with functions distinct from those attributed to its well-characterized homolog Sdc4.

Syndecan2 and Notch3 Signaling

Sdc2 has previously been shown to have important consequences for Notch3 signaling in vitro, but studies of Sdc2 have been wanting for significance attributed by physiologic effects in vivo. We demonstrated that Sdc2 has an important role in the development of vascular smooth muscle coverage with a lineage specific knockout of the gene in smooth muscle. We further validated prior in vitro findings that the absence of Sdc2 abrogates the transcriptional effect of Notch signaling, as we observed reductions in Notch3ICD protein with concordant decreases in mRNA and protein of Notch3 targets. We further observed rises in Notch1 expression in the absence of Sdc2: this suggests parallel signaling between Sdc2 and Notch3, as Notch3 knockdown has been shown to cause a rise in Notch1 in pericytes (Natalie
Kofler in Simons’ lab, unpublished data). It is unclear why there is not also a reduction in the expression of Jagged1, as it is a known Notch3 transcriptional target. This suggests that Jagged1 is regulated by a Sdc2-Notch3 independent mechanism or that the modest changes caused by reductions in Sdc2 are insufficient to change Jagged1 mRNA levels.

However, there are significant distinctions between Sdc2−/− and Notch3−/− mice. Sdc2SMKO mice display delayed VSMC ingrowth and changes in retinal branching pattern, suggesting that Sdc2 has importance in two critical processes outside of its functions related to Notch3: cell migration and control of vascular guidance. Additionally, Notch3−/− mice have not been noted to exhibit any changes in systemic blood pressure, whereas Sdc2−/− mice are notably hypotensive, albeit without significant consequence in the absence of exogenous stressors. Furthermore, we observed no age-associated neurovascular defects in Sdc2−/− mice comparable to those observed in Notch3−/− mice, suggesting that Sdc2SMKO mice more closely resemble Notch3−/− mice and that neither of these models bear resemblance to a CADASIL phenotype.

Sdc4−/− mice have no abnormalities in VSMC coverage despite the fact that Sdc4 is expressed in VSMCs at levels comparable to Sdc2. This fact suggests that any differences in VSMC phenotype are due to the minimal sequence differences between Sdc4 and Sdc4. We therefore generated chimeras between Sdc4 and Sdc2 that exchanged domains or individual residues between Sdc2 and Sdc4. We were thereby able to determine that the functions of Sdc2 that are required to augment Notch3 signaling reside within the intracellular domain. This is not surprising for
two reasons. First, the extracellular domain of the peptide backbones for Sdc2 and Sdc4 are heavily shrouded by HS and CS chains. Hence, any specificity for molecular association with the Sdc2 protein backbone in the extracellular domain is likely abrogated. Second, it seems that the functions of Sdc2 are separable between intracellular and extracellular domains, as the shed components of extracellular domains seem to interfere with angiogenesis whereas the full-length protein seems to contribute to angiogenesis and even more fundamental stages of vascular development.

What remains to be determined is the degree to which the phenotypes, both cellular and physiologic, can be explained specifically by cytoskeletal changes observed in Sdc2−/− VSMCs and not by changes in expression of other Notch3 target genes. Mechanistically, there must be other effects of Sdc2 apart from its control of Notch3 signaling, as Sdc2SMKO mice do not recapitulate the phenotype of Notch3−/− mice. EphrinB2SMKO animals also demonstrate aberrant VSMC coverage with gaps and abnormal contact between VSMCs, resembling changes in Sdc2SMKO VSMCs, and comparable reductions in PDGFRβ in vitro. However, these similarities in phenotype do not mandate that abnormalities in Sdc2SMKO mice are a mitigated form of the phenotypes in EphrinB2SMKO mice, as it is unclear whether the 50% reduction of EphrinB2 observed in Sdc2−/− VSMCs is sufficient to induce these abnormalities.

The existence of a second pathway by which Sdc2 effects its changes is likely, given our findings that demonstrate physical association of Sdc2 with Syntenin1. Syntenin1 knockdown changes the VSMC cytoskeleton with respect to focal adhesions and cellular actin, as observed with knockout of Sdc2. Importantly,
knockdown of Syntenin1 did not cause changes in expression of PDGFRβ: a protein that we have found to be reduced in the absence of Sdc2 and Notch3. Taken together, these findings suggest the existence of two distinct pathways of Sdc2 function in VSMC: one that is dependent on Notch3 signaling and one that is likely dependent on Syntenin1.

Other phenotypes, however, remain unexplained by either Notch3 signaling or cytoskeletal changes: control of arterial branching and control of lumen size. Syndecans are not known to contribute to either of these processes and vascular smooth muscle cells are not known to contribute to arterial branching. Mechanistic insight into the contribution of Sdc2 to these processes would elaborate on a novel function of this class of signaling molecules and the role of VSMCs in vessel guidance.

*Sdc2 is a therapeutic target in pulmonary arterial hypertension and systemic hypertension*

Notch3 has been identified as therapeutic target in pulmonary arterial hypertension. Pharmacologic inhibitors of Notch signaling, which generally target γ-secretase, are not used to treat patients with pulmonary arterial hypertension because of excessive toxicity that takes root in both the broad expression of Notch receptors and their common dependence on the expression of γ-secretase for release of the Notch ICD. The activity of γ-secretase is also not limited to the processing of Notch receptors, leading to many off-target effects. Thus, any avenue
to block pathologic Notch receptor signaling in a more targeted manner might present an exciting therapeutic opportunity.

The work presented above identifies Sdc2 as a therapeutic target in pulmonary arterial hypertension and presents such an avenue. Deletion of Sdc2 limited to VSMCs is protective from pulmonary arterial hypertension induced by hypoxia. Furthermore, mice that lack Sdc2 in VSMC, as well as mice globally lacking Sdc2, develop without any gross abnormality. In combination, these two facts suggest that the targeting of Sdc2 may be protective in pulmonary arterial hypertension and also may not have significant unintended effects. In addition, through our study of molecular mutants and their ability to rescue Notch3 signaling, we have isolated the requisite functions of Sdc2 to aid in Notch3 signaling to the PDZ-binding domain, and likely also to the V domain, of the Sdc2 intracellular domain. Thus, we have identified a narrow peptide sequence which may become a therapeutic target.

Further work is needed to assess pulmonary development in Sdc2−/− and Sdc2SMKO mice at baseline and after treatment with hypoxia in order to determine the effects of Sdc2 in the lung and to more rigorously establish that the mechanism of protection is the abrogation of Notch signaling. Also, Sdc2−/− must be studied more broadly to ensure that targeting Sdc2 specifically may not have more chronic effects unrelated to the expression of Sdc2 in the vasculature.

Additionally, work is needed to identify binding partners of Sdc2 that would effect Notch3 transcriptional changes or to determine if the interaction that has been observed between Sdc2 and Notch343 is direct (either in cis or trans) or with a
Notch3 ligand. Such a partner would have a PDZ domain, but also a domain that binds the V region of Sdc2 and not the V region of Sdc4. Regardless, blockade of Sdc2 with a small molecule that targets its intracellular domain or antibody that targets its extracellular domain would serve as a promising therapeutic for pulmonary arterial hypertension. It is granted that the latter approach might pose a greater challenge to antibody-mediated therapy due to its HS and CS chains.

The use of Sdc2 in VSMCs as a therapeutic target in hypertension also needs to be explored. There is a biologically significant reduction in systemic pressure in the absence of Sdc2SMKO that is seemingly not secondary to overt defects in the contractile apparatus of VSMCs or due to cardiac function. Possibilities for this change include alterations in muscularization of the peripheral resistance arterioles that are thought to control resting tone, alterations in stretch-sensing leading to defects in autoregulation or a change in the renal vasculature with subsequent changes in fluid status and neurohumoral mediators of pressure control. It is further unclear how arteries with stark abnormalities in vessel wall architecture and associated changes in resting blood pressure do not exhibit a contractile deficit when challenged with a vasopressor.

Possible insensitivity to PDGF-BB, due to reductions in PDGFRβ, and inhibited migration in Sdc2−/− VSMCs might also present important therapeutic avenues in other disease states involving vascular smooth muscle. For instance, the formation of neointima in atherosclerosis and the development of stent restenosis are known to involve PDGF signaling and the migration of VSMCs into the tunica intima. Work with the Sdc2SMKO mouse in other disease models, such as the ApoE−/−
mouse or arterial wire injury models, will inform us about the therapeutic potential of Sdc2 blockade in these states.

*S Syndecan2 in Cytoskeletal Dynamics*

Syndecans are already well-known to participate in cytoskeletal dynamics due to their interactions with integrins in focal adhesions and the activity of cytoskeletal GTPases. We report for the first time that Sdc2 leads to several changes in cytoskeletal behavior, which contribute to developmental and pathologic phenotypes in a lineage-specific knockout mouse.

The abnormalities that we observe in Sdc2⁻/⁻ VSMCs include changes in actin stress fibers, the distribution of focal adhesions, the activation of the cytoskeletal GTPase Rac1 and transcription of integrins. These abnormalities culminate in observable changes in the biology of the cell, such as hyperadherence to extracellular matrix, and, early gaps in vascular smooth muscle coverage of the retinal arteriolar wall and changes observed in disorganization of the arterial wall. Finally, cellular hyperadherence might also contribute to the reduction in baseline systemic and hypoxically-induced pulmonary arterial pressures. These are putatively due to distal migration and muscularization of the normally muscle-impoverished small vasculature. Hyperadherence and impaired migration might reduce these pressures by limiting the muscular coverage of the pressure-determining small vasculature.

It is likely that Syntenin1 serves as the binding partner that mediates the cytoskeletal roles of Sdc2 in VSMC. Deletion of Syntenin1 produces cytoskeletal changes comparable to those changes observed in VSMC lacking Sdc2. Syntenin1
(also known as Syndecan binding protein 1; Sdcbp1) is already known to bind Syndecans and has broad effects as a mediator of their functions. It is certainly possible that other Syndecan-binding proteins such as Syntenin2 and Synectin, or any PDZ domain-containing proteins might serve as mediators of these cytoskeletal changes. However, Syntenin2 has less significant effects on cytoskeletal changes and Synectin is likely not the mediator of these functions, as Synectin<sup>SMKO</sup> mice do not demonstrate comparable developmental or cell biological effects. As stated, it is likely that the cytoskeletal functions mediated by Sdc2 are distinct from its Notch3-associated effects, as PDGFRβ is unchanged in a knockdown of Syntenin1. Further examination of Syntenin<sup>−/−</sup> mice to evaluate vascular smooth muscle development would help us dissect the responsibility of these two pathways that likely contribute to the abnormalities seen in Sdc2<sup>−/−</sup> mice.

It may be the case, however, that animal phenotypes such as delayed VSMC ingrowth and protection from pulmonary arterial hypertension are due to contributions from multiple pathways dependent on Sdc2. Among these pathways are Notch3-dependent transcriptional effects, Syntenin1-dependent cytoskeletal effects and possibly also pathways that determine arterial guidance and arterial lumen size.

**Conclusion**

Taken together, these findings contribute to the increasing significance of Syndecans in the development of the vasculature and cell signaling. Furthermore, they demonstrate domain dependence of physiologic processes that have clinical
implications. Thus, Syndecans have clinical applicability and present exciting therapeutic opportunities.
Figure 1. The developing retina in Sdc2^SMKO^ mice exhibits abnormalities in vascular smooth muscle coverage. (A and D) Arterioles proximal to the center of the retinal artery in a P5 wild-type and Sdc2^SMKO^ mice stained for smooth muscle actin; the latter shows abnormal gaps in coverage. (B and E) Low-power image of a wild-type and Sdc2^SMKO^ mice at P12 stained for smooth muscle actin; the latter demonstrates reductions in radial extent of smooth muscle coverage and early branching morphology, as noted in (C: 1941 ± 69μm in WT, 1764 ± 42μm in Sdc2^SMKO^, p<0.05) and (F: 1433 ± 79μm in WT, 890 ± 88μm in Sdc2^SMKO^, p<0.0001). (G and H) Proximal retinal arterioles in wild-type and Sdc2^SMKO^ mice demonstrating arterioles that are wider, as noted in (I: 15.5 ± 0.26μm in WT compared with 16.2 ± 0.19μm in Sdc2^SMKO^, p<0.05).
Figure 2. Sdc2SMKO mice at P14 exhibit disorganized vascular smooth muscle coverage in all tissues examined. Wild type mouse (A) internal carotid artery, (B) anterior cerebral artery, (C) intramyocardial coronary artery and (D) tail lateral artery demonstrate regular and lamellar patterning of vascular smooth muscle cells. Sdc2SMKO (E) internal carotid artery, (F) basilar artery, (G) intramyocardial coronary and (H) tail lateral artery demonstrate irregular cells, lack of uniform layering and poor cell contact. Immunohistochemistry for smooth muscle actin.
Figure 3. **Sdc2\textsuperscript{SMKO} mice display abnormal reductions in resting mean arterial pressure.** (A) Resting mean arterial pressures in wild-type (94.36±11.32 mmHg) and Sdc2\textsuperscript{SMKO} mice (75.46±9.93 mmHg, p<0.05). (B) Pressures after phenylephrine infusion (50μg/kg) show differences between groups that are reflective of baseline differences but no difference at any timepoint apart from resting blood pressure.
Figure 4. Sdc2^{SMKO} mice exhibit protection from hypoxically-induced pulmonary arterial hypertension. (A) After three weeks of habitation in an atmosphere of 10% oxygen, wild-type mice had elevated right ventricular systolic pressures (41.26±1.24 mmHg) compared to Sdc2^{SMKO} mice (34.56±0.52 mmHg, p<0.001) (B) The difference between groups was preserved when controlling for gender of animal.
Figure 5. **Sdc2⁻/⁻ VSMC exhibit abnormalities in Notch3 signaling.** VSMCs cultured at low-passage from wild-type and Sdc2⁻/⁻ exhibit reductions in Notch3, greater reductions in the Notch3-intracellular domain, and consequent reductions in PDFGRβ and EphrinB2 without reductions in SM-MHC and Smooth Muscle Actin.
Figure 6. Sdc2−/− VSMC exhibit reductions in known Notch3 transcriptional targets without reductions in canonical markers of VSMC differentiation. (A) In VSMC lacking Sdc2 there are reductions of known Notch3 transcriptional targets (B) PDGFRβ and (C) EphrinB2. There were no changes in (D) Jagged1 mRNA levels. We observed rises in Notch pathway elements (E) Notch1 and (F) HeyL, which are known to be upregulated in Notch3−/− VSMC and pericytes. There were no known reductions in canonical markers of differentiation (G) Serum Response Factor (SRF) and (H) Myocardin (MyoC).
Figure 7. **Knockdown of Notch3 in VSMC produces reductions in protein expression of transcriptional targets.** siRNA-mediated knockdown of Notch3 produces reductions in PDGFRβ and EphrinB2, which are known to be changed on the transcriptional level in the absence of Notch3.
Figure 8. **The intracellular domain of Sdc2 augments Notch3 signaling and target gene expression.** Expression of GFP and HA-Sdc4 with adenoviruses are insufficient to restore reductions of expression in Notch3 and its target proteins PDGFRβ and EphrinB2. Expression of HA-Sdc2^ΔECTM^ and expression of HA-Sdc2 are capable to rescuing expression. Partial rescue may be achieved by expressing HA-Sdc2^ΔICD^. There was no rescue with HA-Sdc2^PDZ^.
Figure 9. *Sdc2*-/- VSMC exhibit no defect in rate of proliferation but have delayed migration. (A) Plating equal numbers of cells in the Roche XCELLigence machine shows no difference in rate of proliferation between wild-type and *Sdc2*-/- VSMCs. (B) When normalized to cell index at 96 hours, measurements of cell index at 24 hours reveal that *Sdc2*-/- cells have a mild migratory defect relative to wild-type VSMCs.
Figure 10. **Sdc2−/− VSMCs display abnormal distributions of filamentous actin and focal adhesions.** Staining for focal adhesions with vinculin and filamentous actin with phalloidin in (A and B) wild-type and (C and D) Sdc2−/− VSMCs demonstrates an abnormal cytoskeleton in Sdc2−/− VSMCs. Filamentous actin and focal adhesions are distributed to the periphery the cell in Sdc−/− compared with a uniform distribution of focal adhesions and stress fibers that traverse the center of the cell exhibited by wild-type cells.
**Figure 11. Sdc2−/− VSMCs exhibit abnormal cytoskeletal behavior linked to focal adhesion formation.** (A) Wild-type VSMCs have lower normalized Rac1 activity (1.00±0.07 normalized units) compared with Sdc2−/− VSMC (1.30±0.08 normalized units, p<0.05). (B) Wild type (54.0±3.7%) and Sdc2−/− (58.0±6.1%, p=.37) cells display no change in adhesion to Fibronectin. There was a trend toward increased adhesion to Collagen I (WT: 9.8±5.0%, Sdc2−/−: 25.1±9.0%, p = 0.062) and to Laminin (WT: 4.5±1.8%, Sdc2−/−: 25.6±14.2%, p = 0.063). (C) There were no changes in the activity of RhoA GTPase. (D) Transcription of Itga4, Itga6 and Itgb4 is elevated in Sdc2−/− VSMC without change in transcription of Itgb1.
Figure 12. Cytoskeletal changes in Sdc2\textsuperscript{-/-} cells can be rescued by re-expression of the Sdc2 intracellular domain and are mediated by the PDZ-binding domain. (A) Re-expression of HA-Sdc2 in Sdc2\textsuperscript{-/-} VSMC rescues the (B) peripheral distribution of focal adhesions and (C) distribution of filamentous actin to stress fibers that traverse the center of the cell. (D-F) These defects may not be rescued by HA-Sdc4, but they are rescued by (HA-Sdc2\textsuperscript{ΔECTM}). (J-L) Expression of HA-Sdc2\textsuperscript{PDZ} does not rescue the defects of Sdc2\textsuperscript{-/-} VSMCs.
Figure 13. **Sdc2 binding of Syntenin1 in VSMCs is dependent on the PDZ-binding domain of Sdc2.** Re-expression of full-length HA-Sdc2 and immunoprecipitation by anti-HA antibodies demonstrates binding of Syntenin1 to Sdc2. Deletion of the terminal alanine residue of Sdc2 in its PDZ-binding domain (A202-) results in deficiency of the interaction between Sdc2 and Syntenin1.
Figure 14. **Syntenin1 may be efficiently deleted with siRNA.** Three distinct siRNA delete Syntenin1 efficiently compared with transfection reagent only and control scramble siRNA. There is no change in Syntenin1 level with deletion of Syntenin2. Deletion of Syntenin1 and Syntenin2 has no bearing on expression of PDGFRβ.
Figure 15. Deletion of Syntenin1 produces comparable cytoskeletal changes to deletion of Sdc2. (A, B) Control siRNA produces no changes in cytoskeletal arrangement with respect to focal adhesions (stained with Paxillin) and filamentous actin (stained with phalloidin). (C, D & E, F & G, H) Three distinct siRNA for Syntenin1 result in peripheral redistribution of focal adhesions. (I, J) Only moderate changes are visualized with deletion of Syntenin2.
Supplemental Figure 0. ** Syndecan sequence and structure.** (A) Peptide sequence of Sdc2 in the mouse, reflecting its signal peptide, extracellular domain (ECD), transmembrane domain (TMD) and intracellular domain (ICD). (B) Schematic of Sdc2 protein structure. (C) Alignment of Syndecan intracellular domains reflecting C1, V and C2 domains, as well as sequence differences between Sdc2 and Sdc4 in red. (D) Multiple sequence alignment of all Syndecans. Bold and underlined segments reflect transmembrane domains.
Supplemental Figure 1. Sdc2 may be efficiently knocked out with Sm22α-Cre. Carotid arterial branches in embryonic day 18.5 (E18.5) mice demonstrate absent expression of Sdc2 protein by immunohistochemistry, as noted in (E).
Supplemental Figure 2. **Retina from ROSA26<sup>mtmG/mtmG</sup>; tg(Sm22α-Cre) mouse.** Retina from Cre reporter mouse demonstrating specificity of recombination to (B) vascular smooth muscle cells of arterioles among (C) all vessels as stained for with IsolectinB4.
Supplemental Figure 3. **Sdc2<sup>SMKO</sup> mice exhibit no abnormalities in cardiac function.** There were no differences between wild-type and Sdc2<sup>SMKO</sup> mice in any parameters of LV function as measured by transthoracic echocardiography.
Supplemental Figure 4. **Sdc2<sup>SMKO</sup> mice exhibit no abnormality in baseline characteristics before treatment with hypoxia.** (A) There were no differences in baseline right ventricular systolic pressures between wild-type (25.50±0.85 mmHg) and Sdc2<sup>SMKO</sup> mice (25.00±1.43 mmHg, p=0.78). (B) There were no differences in baseline weight between wild-type (24.80±0.40 g) and Sdc2<sup>SMKO</sup> mice (23.00±1.15 mmHg, p=0.20).
Supplemental Figure 5. **Vascular smooth muscle cells may be cultured effectively and specifically.** Using culture methods described, cells staining negative for CD31 and positive for (A) Smooth Muscle Actin or (B) Smooth muscle myosin heavy chain may be cultured from mouse aortas.
Supplemental Figure 6. Schematic of mutant Sdc2 constructs generated for rescue experiments. (A) Full-length native Sdc2. (B) Full-length native Sdc4. (C) Chimera HA-Sdc2ΔICD with HA tag generated by overlap extension PCR. (D) Chimera HA-Sdc2ΔECTM with HA tag generated by overlap extension PCR. (E) HA-Sdc2PDZ with HA tag. (F) HA-Sdc2ΔC1. (G) HA-Sdc2ΔV. (H) HA-Sdc2ΔC2. (I) Sequences of intracellular domain of domain-substitution mutants.

ICD Sdc2 ΔC1 (R173K): RMKKDEGSYDLGKRPSS--AAYQKAPTK-EFYA
ICD Sdc2 ΔV : RMRKKDEGSYDLGKPK-----IYQKAPTK-EFYA
ICD Sdc2 ΔC2 (Q193K): RMRKKDEGSYDLGKRPSS--AAYKAPTK-EFYA
ICD Sdc2 ΔC2 (K198N) : RMRKKDEGSYDLGKRPSS--AAYQKAPTN-EFYA
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