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Endothelial caveolin and its scaffolding domain in cancer

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Abstract
Since the initial reports implicating caveolin-1 (CAV1) in neoplasia, the scientific community has made tremendous strides towards understanding how CAV1-dependent signaling and caveolae assembly modulate solid tumor growth. Once a solid neoplastic tumor reaches a certain size, it will increasingly rely on its stroma to meet the metabolic demands of the rapidly proliferating cancer cells, a limitation typically but not exclusively addressed via the formation of new blood vessels. Landmark studies using xenograft tumor models have highlighted the importance of stromal CAV1 during neoplastic blood vessel growth from preexisting vasculature, a process called angiogenesis, and helped identify endothelium-specific signaling events regulated by CAV1, such as vascular endothelial growth factor (VEGF) receptors as well as the endothelial nitric oxide (NO) synthase (eNOS) systems. This chapter provides a glimpse into the signaling events modulated by CAV1 and its scaffolding domain (CSD) during endothelial-specific aspects of neoplastic growth, such as vascular permeability, angiogenesis, and mechanotransduction.

Keywords Caveolin-1 · Neoplasia · Vascular endothelial growth factor · Nitric oxide · Endothelium · Cancer · Nitric oxide synthase

1 CAV1 in caveolae biogenesis and signaling

Caveolae are flask-shaped invaginations of the plasma membrane [1] that play an essential role in endocytosis [2] and many other macroscopic plasma membrane–related events [3, 4]. Caveolar assembly in most cell types requires the presence of caveolin-1 (CAV1) [5], a transmembrane protein initially described as a component of caveolae membrane domains phosphorylated in Rous sarcoma virus-transformed fibroblasts [6]. CAV1 forms high molecular weight oligomers that have the unique but complex ability to regulate the clustering and spatial organization of local signaling complexes in cholesterol- and sphingolipid-rich environments [7, 8]. In addition, numerous reports have documented the inhibition of protein signaling activity following interaction with the CAV1 scaffolding domain (CSD), a hydrophobic 20-amino acid sequence at the very heart of the widespread perception that CAV1 can also serve as a molecular inhibitory clamp that reduces client protein activity [9, 10]. However, despite the fact that CAV1 plays multiple and diverse roles in the regulation of cell homeostasis—including caveolar assembly, signaling complex clustering, and direct protein-protein interactions—knock-out of the CAV1 locus is not sufficient to cause embryonic lethality, as shown by three independent studies [11–13]. Nonetheless, these transgenic animals showed a near-complete loss of caveolae in non-cardiac or skeletal muscle tissues as well as a long range of complex anomalies, particularly in the cardiovascular system [10], confirming the role of CAV1 in whole-body homeostasis [14]. Combined with reports depicting a critical link between CAV1 and oncogenic transformation [15, 16], experiments aimed at studying the role of CAV1 and its CSD in the stroma of tumors using various transgenic models of CAV1 expression, and CSD-mimicking approaches highlight the controversial biological significance of CAV1 in the stroma of tumors, particularly in endothelia-specific signaling [10].

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2 CAV1 and CSD during oncogenic transformation

The first evidence supporting the role of CAV1 as an endogenous tumor suppressor came from the observation that normal cells show drastic reduction in caveolae biogenesis and

amount of CAV1 during oncogenic transformation induced by v-Abl, middle T antigen, or Ras [15], three oncogenes that trigger transformation via heterogeneous signaling events [17] and result in accelerated cell hyper-plasticity. Using GST fusion proteins, synthetic peptides, and scanning approaches, amongst others, a growing list of tumoral CAV1-interacting proteins have been identified that are capable of modifying tumor cell behavior [18–20]. Indeed, growth factor receptors, such as epidermal growth factor receptor [21], as well as key intracellular signaling pathways that include PI3 kinase [22], protein kinases A and C[23, 24], heterotrimeric G proteins [25, 26], H-Ras [27], Src family tyrosine kinases [28, 29], extracellular-regulated kinase ERK [30], and STAT3 [31] (reviewed in [32]) are believed to interact directly with CAV1, generally resulting in a state of hypoactivity. Domain mapping studies have identified the critical role of the amino acids 82-101 of CAV1's CSD in mediating direct interactions with many client proteins [33], a concept initially shown to take place *in vivo*, as expression of truncated CAV1 mutants that included sections of the CSD in tumor cells resulted in downregulated Neu tyrosine kinase receptor-dependent signaling of the MAP kinase/Elk pathway in the nucleus [33]. Despite normal expression of CAV1, loss of CSD activity is often observed in human cancer [34], as others have shown using CSD-less CAV1 expression systems that CSD act as a major check point in the control of G2/M cell cycle modulation [31]. While it was obvious that solid tumors derived from transformed cells showed downregulated CAV1 expression compared with untransformed controls, it is of interest to stress that the stromal vasculature, and endothelial cells in particular, can present with robust CAV1 expression [33]. Of particular relevance to this chapter is the mounting evidence that in addition to its role in oncogenic transformation, stromal CAV1 and its CSD play an intricate role in tumor neo-angiogenesis—and regulation of vascular homeostasis in general—likely through their non-exclusive ability to modulate the VEGF receptors and eNOS systems, which both show a high degree of endothelial specificity.

3 CAV1 and CSD in the stroma of tumors

Use of xenograft tumor models in combination with CAV1 null animals allowed the proper testing of the role of host stromal CAV1/caveolae system in response to "exogenous" tumor growth stimulation—thereby eliminating the contribution of tumor cell–derived CAV1 when using inherited (genetic) models of tumor growth. Based on the lessons learned from the study of CAV1-regulated signaling events depicting it as a negative regulator of signal transduction, one would have assumed that loss of stromal CAV1 would result in more sustained growth by tumor xenografts, particularly in the context of unexpectedly high vascular permeability observed in absence of CAV1 [12]. Indeed, tumors benefit from vascular leakage of plasma proteins as it results in increased fibrin deposition in and around tumors [35, 36]—a critical pro-tumor growth characteristic of the stroma (Fig. 1a)—although the loss of caveolae-dependent endocytosis and transcytosis in CAV1 KO tissues did not materialize in the predicted lower permeability in these animals [37, 38]. However, reports have shown that solid tumors derived from melanoma cells implanted in syngenic CAV1 null mice showed abnormally low growth rates, weight, and volumes compared with control animals [14, 39], an unexpected observation considering the absence of the so-called CAV1 clamp and presence of heightened vascular leakage. Interestingly, these melanoma tumors also showed lower capillary density [39] suggesting a role for stromal CAV1 in formation of fresh blood supply to growing tumors. In stark contrast, others have shown increased carcinoma xenograft growth in CAV1 null animals, along with heightened angiogenesis[40]—the formation of new blood vessels from preexisting ones—suggesting a more predictable role for the host's CAV1 system in that particular setting of tumor growth. These unpredicted differences between tumor growth outcomes helped portray a more complex and sometimes controversial view of the CAV1 systems during vascular remodeling and tumor growth. It is therefore likely that the role of stromal CAV1 may be tumor type–dependent, and more importantly that variability in angiogenic response of a particular tumor type may be the reason behind differences in tumor growth potential reported in CAV1 null hosts [14]. This further lends credence to reports describing a critical role for CAV1 in the vasculature [12, 41], with a particular emphasis on the endothelium [42, 43] and that the CAV1- and CSD-dependent regulation of stromal and endothelial signaling is likely more complex than depicted by current models, particularly as new regulatory pathways, such as mechanotransduction, continue to emerge.

4 CAV1 and CSD in tumor-associated endothelial homeostasis, permeability, and angiogenesis

Caveolae are abundant in the vascular endothelia [44], and studies have demonstrated that they are the predominant endothelial plasmalemmal vesicles in large and small blood vessels, constituting up to 30% of their luminal surface area in the capillaries [45, 46]. With these data in mind, and the fact that the endothelium is the predominant determinant of vascular barrier and vasoreactivity functions of blood vessels, one could have predicted that loss of CAV1 and therefore caveolae formation would result in a high degree of cardiovascular abnormalities in CAV1-deficient mice. This could in turn affect tumor-associated permeability and angiogenesis, two crucial steps in establishing a new blood supply network to

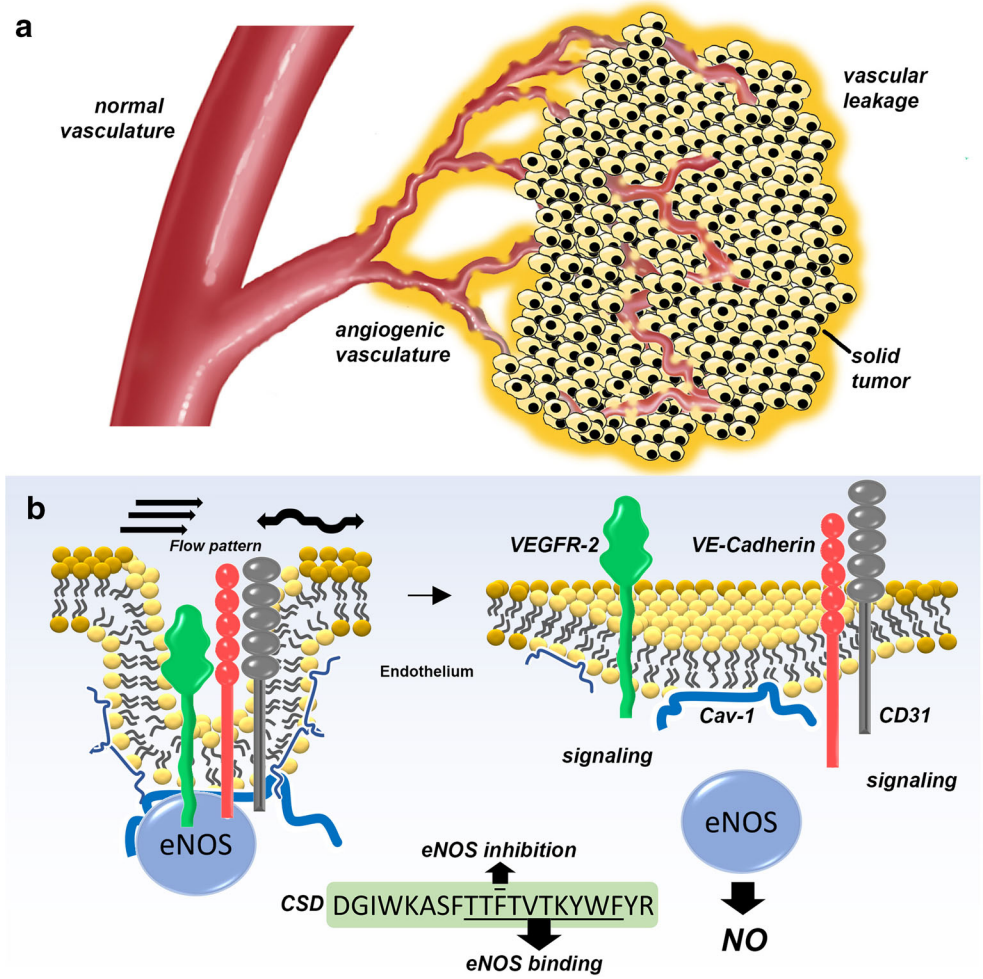

Fig. 1 The complexity of stromal CAV1 signaling as depicted by endothelium-specific signalosome that promotes downstream tumor growth. **a** The typical tumor vasculature is often derived by hijacking a "mother" vessel, resulting in many angiogenic daughter vessels. In contrast to endogenous vessels, an angiogenic vasculature is tortuous, shows high cell turnover, is highly permeable in part due to lack of a basement membrane and pericyte coverage, and results in fibrinogen degradation product deposition around tumor vessels. Much evidence obtained in CAV1-deficient or overexpressing animals suggest that CAV1 and caveolae regulate these critical but complex biological properties of angiogenic vessels. **b** An endothelium-specific signalosome-dependent on CAV1 and caveolae for proper angiogenic signaling. The endothelium is highly enriched in caveolae and CAV1, particularly when endogenous vasculature is exposed to laminar flow. Many endothelial-specific proteins target to caveolae for proper signaling, such as VEGFR-2, eNOS, CD31/PECAM, and VE-cadherin, although direct association with CAV1 and its CSD generally result in attenuated activity. However, the abnormal and sometimes reverse flow patterns of tumor-associated blood flow may affect caveolae formation or signaling. Normal endothelial cells under proper flow-mediated physical stress and stretching can cause caveolae disassembly, resulting in separation from CAV1's inhibitory clamp and angiogenic signaling. eNOS is by far the most frequently described CAV1 client protein, binding directly to CSD amino acids 90–99, whereas the F92 residue is responsible for eNOS inhibition. It remains to be determined whether caveolae-resident proteins continue to signal in cholesterol-rich environments after caveolae disassembly, and how angiogenic endothelium integrates abnormal extracellular forces into intracellular signaling differently than endogenous vasculature

hyperplastic cells. Indeed, angiogenesis is often initiated by the vasodilation and hyperpermeability [47–49] of existing vasculature [50]—a critical step in making a tumor's stroma more hospitable to fast dividing cells—followed by more chronic upregulation of endothelial matrix-remodeling enzymes [51], directional migration/chemotaxis, and proliferation [52] and tri-dimensional assembly of endothelial cells into cylindrical structures [53] capable of carrying the blood towards hypoxic tumors. However, CAV1 null vessels show abnormal vascular reactivity, such as lower phenylephrine contractility and higher vasodilatory response to acetylcholine [12], which are hallmarks of reduced angiogenic stimulation. Interestingly, NOS inhibition with L-NAME resulted in the normalization of CAV1 null vessels compared with controls, establishing a strong *ex vivo* link between the endothelium, loss of CAV1, and hyperactivation of eNOS [12] early during the angiogenic process.

Unfortunately, glimpses of the complexity of CAV1's role in endothelial homeostasis were made more evident by unexpected reports of increased plasma protein leakage in CAV1

null tissues despite loss of CAV1-dependent endocytosis and transcytosis. Indeed, both radio-iodinated albumin [41, 54] and ruthenium red [41], which are respective markers of trans- and para-cellular permeability, were both shown to be cleared from the vasculature at an accelerated rate, a likely consequence of the role of CAV1-dependent regulation of adherens junctions [55], cell-cell, and cell-matrix adhesion molecules [56, 57] such as PECAM/CD31 [58] and integrins but independent of endothelial vesiculo-vacuolar organelles (VVO), a channel-like structure composed of numerous interconnecting vesicles and vacuoles that is positive for CAV1 expression but still present in CAV1 null tissues [14, 35, 59–61]. This is in contrast with other *in vivo* data showing reduced colloidal gold-labeled albumin uptake in CAV1-deficient endothelial cells [62]. While these data are more in line with CAV1 being required for CAV1-dependent caveolae, they also illustrate the multiple pathways that can lead to vascular hyperpermeability and their complexity, in this case blunted caveolae formation vs loss of transcellular filtration properties. This claim is further compounded by microscopy experiments showing 10–20% residual caveolae in dermal microvascular endothelium of CAV1 KO animals [14]. Once again, L-NAME can normalize hyperpermeability in the systemic vasculature of CAV1 KO animals [41], although it must be noted that others have found no difference between the permeability of tumor xenografts in wild-type (WT) vs CAV1 null mice [54]. Interestingly, endothelial-specific overexpression of CAV1 using a preproendothelin promoter–driven CAV1 transgene did not increase caveolae detection, indicating that caveolae biogenesis based purely on CAV1 protein expression level is likely saturated in WT mice[63]. However, as depicted in the following paragraphs, endothelial CAV1 overexpressing animals provided clear evidence of *in vivo* differentiation of CAV1-dependent caveolae formation abilities vs CAV1-dependent signaling as they exhibited lower agonist-induced permeability due to increased endothelial CAV1.

The critical observation that the endothelial-specific rescue of CAV1 expression in global CAV1 KO animals resulted in complete rescue of endothelial caveolae and importantly, the vascular abnormalities observed in KO mice highlighted the importance of endothelial CAV1 and caveolae in vascular and whole-body homeostasis [43]. Taken together with L-NAME rescue experiments mentioned above, the endothelial cell–specific CAV1 rescue data emphasize the significant role of CAV1/eNOS interactions in endothelial barrier function. To err on the side of caution, one must consider that vascular permeability is often considered a heterogeneous phenomenon as basal, unstimulated hyperpermeability tends to occur in the capillaries whereas acute plasma protein leakage—such as that triggered by agonists—generally takes place in post-capillary venules [14, 64, 65]. These differ from chronic, tumorigenesis-associated hyperpermeability, which occurs after the hijacking of a feeding "mother" vessel followed by leakiness of immature "daughter" vessels [66]. When combined, these important differences could result in heterogeneous contributions of CAV1 and eNOS, and help to rationalize the divergence of CAV1's contribution to vascular leakage.

In contrast to vascular permeability, angiogenesis is a more chronic and complex process critical to tumor growth that nonetheless benefits from the vascular extravasation–dependent enrichment of a tumor's micro-environment. Rapidly growing tumors have long been documented to promote the extension of host capillaries after reaching 1–2 mm^3 in size [67] due to the limited diffusion of nutrients from preexisting vasculature [68, 69]. Circumstantial evidence suggest that CAV1 can regulate the changes in endothelial cell plasticity, such as endothelial-to-mesenchymal transition [70], required for their migration and proliferation towards the source of the angiogenic stimulus [71]; however, how it does this is not fully understood, and there remains some degree of controversy. As mentioned above, early data noted lower vessel density in tumor xenografts derived from CAV1 null mice [39], which were corroborated by similar outcomes using similar models [14]. However, subsequent investigations described both pro- and anti-angiogenesis activities by CAV1. Indeed, overexpression of endothelial CAV1 [63] or *in vivo* CAV1 gene delivery [72] both resulted in attenuated angiogenesis in mice, although others have reported increased angiogenesis and associated tumor growth in CAV1 null mice [40]. In contrast, antisense knock-down of CAV1 was shown to inhibit *in vivo* and *in vitro* angiogenesis [73]. Hence, whether the loss of CAV1 up- or downregulates angiogenesis and associated tumor growth remains unclear, casting further doubt about the accuracy of current models of CAV1- and caveolae-dependent modulation of the angiogenic response. Highly mechanistic data nonetheless show that tumor-derived vessels from CAV1 KO mice suffer from deficit in smooth muscle cell (SMC)/pericyte recruitment, resulting in blunted tumor maturation and neovascularization processes [54]. This vascular maturation defect could help explain the lower response to direct bFGF stimulation in CAV1-deficient mice [39], although AdVEGF-mediated angiogenesis was also reduced in CAV1-overexpressing transgenic mice [63]. However, one must stress how novel concepts may help rationalize some of the discrepancies mentioned above, such as that of the "just right amount of CAV1" required for vascular homeostasis [14], a concept also valid for caveolin-3 in skeletal muscle tissue homeostasis [74–77].

If one assumes that vascular and whole-body homeostasis are indeed critically dependent on the "just right" amount of endothelial CAV1, two highly endothelial-specific signaling pathways that are required for tumor growth and regulated by CAV1 and caveolae come to mind, i.e., the VEGF receptors and the eNOS pathways. While VEGF receptors and eNOS are critical for optimal tumor growth [53, 78–81], the dynamic

interplay between them is highly relevant during angiogenesis and even mechanotransduction (see below). Evidence of the endothelial VEGF-eNOS link within the realm of caveolae is supported by the considerable body of evidence describing how VEGF can induce both eNOS-derived nitric oxide (NO) release [82–84] and hyperpermeability [40, 49, 71, 85] *in vivo* and *in vitro* within minutes, whereas VEGF-induced increase in permeability, angiogenesis, and tumor growth are unquestionably downregulated in eNOS null mice [79, 80, 86, 87].

5 Endothelial CAV1 and CSD in VEGF receptor signaling

VEGF and its network of receptors are by most accounts one of the main regulators of whole-body angiogenesis for several key reasons. First, VEGF is an ideal candidate for the early working model depicting how hypoxic tumor cells release soluble "tumor angiogenesis factor" (TAF) that can stimulate the recruitment and growth of remote vasculature [88]. While many angiogenic growth factors and cytokines have been identified [89, 90], VEGF—initially called vascular permeability factor [91, 92]—can be released by most hypoxic tissues [93] including solid tumors and stimulate specific receptors almost exclusively on endothelial cells. Indeed, through tyrosine kinase receptors VEGFR-1/Flt-1 [94] and especially VEGFR-2/Flk-1/KDR [95], the primary VEGF receptor, VEGF, can stimulate every angiogenic step that will result in greater perfusion of downstream tissues. Despite the many controversies that stemmed from anti-VEGF therapies in cancer [96, 97], the significance of the VEGF system to homeostasis and other highly effective FDA-approved therapies [98] more than justifies the intense scrutiny on the role of CAV1 and caveolae play in regulating the endothelial VEGF receptor systems. However, one should stress the complexity of studying how CAV1 and caveolae modulate VEGF receptor signaling since many of the known downstream biological properties of VEGF, such as VEGFR-2-driven permeability and eNOS phosphorylation, are also directly regulated by both CAV1 and caveolae, making the dissociation between CAV1-dependent modulation of VEGF receptor signaling vs downstream biological activity difficult.

With this fundamental challenge in mind, VEGF stimulation of cultured endothelial cells was shown to quickly induce both the appearance of CAV1-positive transcellular structures reminiscent of previously described VVOs and internalization of membrane caveolae during a hyperpermeability response [99]. Proper targeting of VEGFR-2 to cholesterol-enriched membrane microdomains [100, 101] was imperative for optimal VEGF-dependent ERK1/2 phosphorylation [102], although dissociation of a CAV1/VEGFR-2-containing complex was shown to occur in cultured endothelial cells following VEGF stimulation as documented by co-immunoprecipitation experiments [102]. The kinetics of VEGFR-2 dissociation from CAV1 also matched that of VEGFR-2 phosphorylation, supporting the release of signaling molecules from the molecular clamp effect of CAV1 following stimulation, although intracellular delivery of CSD (see below) does not result in inhibition of VEGFR-2 signaling [72, 87]. The dynamic activation of VEGR-2 in caveolae following unclamping from the CSD illustrated the dichotomous effect of CAV1 and associated caveolae formation on signaling. However, *in vivo* data suggest that CAV1-dependent caveolae formation is of greater importance than CAV1-dependent inhibition of VEGFR-2 signaling, as VEGF-mediated pathological angiogenesis is strikingly reduced in CAV1 knock-out mice [14]. Interestingly, culture of mouse endothelial cells (ECs) deficient in CAV1 and caveolae brought a new perspective on the complexity of VEGFR-2 regulation [40, 103]. In mouse lung endothelium, loss of CAV1 resulted in higher VEGFR-2 phosphorylation in response to VEGF and decreased co-association with VE-cadherin, which is a negative endogenous regulator of VEGFR-2 autophosphorylation, and resulted in greater VEGF-induced eNOS phosphorylation and increase in permeability [40]. In contrast, stimulation of CAV1 null endothelial cells of aortic origin resulted in lower VEGF-induced network tube formation and eNOS and ERK phosphorylation due to abnormal caveolar VEGFR-2 targeting [103]. It must be noted that some of these CAV1 null cells showed lower Cav-2 levels than their WT counterparts and require immortalization for growth [40], two significant confounders, although Cav-2 does not have a CSD and therefore is devoid of the traditional molecular clamp effect of CAV1. However, this pathway might be more complex than initially believed, since both loss and overexpression of CAV1 in ovine ECs reduced VEGF-induced ERK1/2 signaling, lending credence to the "just right" amount of CAV1 for proper signaling as depicted earlier [104]. *In vivo*, endothelial overexpression of CAV1 results in inhibition of VEGF-mediated phosphorylation of Akt, higher ERK1/2 phosphorylation but lower AdVEGF-induced angiogenesis overall [63]. Although the exact contribution of VEGFR-2 to these complex endothelial-specific biological activities is unknown, they lend credence to the important observation that angiogenesis inhibitors can interfere with VEGF's ability to downregulate CAV1, resulting in greater inhibitory clamp effect on VEGFR-2 [105] (Fig. 1b).

Another important VEGFR-2-dependent signaling event is the activation of *Src* family kinases. VEGF can stimulate Src and Yes to increase permeability [106], whereas Src can also phosphorylate CAV1 on tyrosine-14, triggering conformational changes to CAV1 that are believed to promote spatial distancing of phosphorylated CAV1 molecules within the CAV1 oligomer. The conformational and/or accessibility changes affects CSD binding to client proteins [107, 108] such

as Src itself, eNOS [109], VEGFR-2, and matrix-remodeling enzymes including membrane-type 1 matrix metalloproteinase [110, 111]. To err on the side of caution, it has been proposed that VEGFR-2/phospho-CAV1/Src interplay may involve larger CSK-containing complexes [111, 112], further illustrating CAV1's ability to orchestrate large signaling complexes. These complexes likely include the lesser investigated VEGFR-1, which is also expressed in vascular endothelial cells and shown to be inhibited by CAV1 [113]. How these large VEGFR-1/VEGFR-2-containing signalosomes fit into newer models of CAV1-dependent caveolae biogenesis and activity at the membrane is poorly understood, particularly as they appear critical for endothelial mechanotransduction of extracellular forces [114, 115].

6 Endothelial CAV1 and CSD in eNOS signaling

The early characterization of the CSD-eNOS interaction by multiple groups [9, 116, 117] combined with the many vascular defects observed in CAV1 null mice [12, 41] substantiate the growing body of evidence highlighting this interaction as a "poster child" of the CAV1 signaling trilogy—caveolae-dependent, CAV1 signalosome–dependent, and direct CSD modulation—that is critical to whole-body homeostasis. eNOS is the primary source of NO, initially called EDRF [118, 119], based on the landmark observations describing how the endothelium and NO can promote vasodilation independently of the agonist used for preconstruction [120]. eNOS expression [121] is highly restricted to the endothelium, as shown by endothelial-only expression of a GFP-tagged eNOS transgene *in vivo* [122]. eNOS can not only promote protective vasodilation and anti-atherogenic properties of the vasculature [123], but also trigger NO-dependent increase in vascular permeability and inflammation in response to agonists such as VEGF [124], amongst others [125]. In addition to the L-NAME- and endothelial cell CAV1 reconstitution–dependent reversal of most of the abnormalities observed in CAV1-deficient mice [41, 43], the angiogenic defect observed in CAV1 gene–deficient mice can be corrected in CAV1/eNOS double KO mice [126], which provided genetic evidence of dysregulated eNOS during angiogenesis in CAV1 KO mice. However, the presence of eNOS is required for maximal tumor growth, as knock-out of the eNOS locus attenuates growth rates of tumor xenografts [87].

eNOS regulation is complex, but modulation of NO release can be achieved through phosphorylation of the enzyme, such as following laminar sheer stress [127] and Akt activation [128, 129]. These are both linked to CAV1 and caveolae biogenesis as well as other less reversible covalent modifications such as palmitoylation and myristoylation [130–133], which are believed to target a special pool of eNOS to caveola and cytoplasmic sections of the Golgi apparatus for activity that differs from lipid raft–based signaling [134]. Substrate [135] and co-factor availability [136] also affect eNOS activity, as well as various protein-protein interactions such as with calmodulin [137–139] and heat-shock protein 90 [139, 140]. However, eNOS docking to CAV1 is one of the very few direct protein-protein interactions with eNOS to result in reduction in NO release [9, 116]. Indeed, immunoprecipitation (IP) of signaling complexes have shown eNOS co-IP with CAV1 and *vice versa* [141], and GST pull-down [109, 116] and yeast two-hybrid assays [142] have directly implicated the CSD in this interaction. Experiments using reconstituted cell systems show lower NO release in cells co-transfected with eNOS and CAV1 than eNOS alone [125], whereas incubation of purified eNOS with a synthetic CSD peptide inhibits eNOS activity. Little is known about the caveolin binding domain of eNOS; early evidence suggested that the reductase portion of the enzyme interacts with CSD [141, 143], although others postulated that the structure and membrane environment of CAV1 may not allow direct interaction between CSD and the CAV1 binding motif of eNOS [144–146].

A significant number of studies have focused on the CSD site in this interaction. Early mutational analyses showed that CAV1's functional inhibitory effect on eNOS *in vivo* is contained within CAV1 residues 82–101 [9, 116], thereby implicating the CSD. The molecular determinants of this direct interaction were further deciphered using cell permeable peptide technology, which helped overcome the complexity associated with the intracellular nature of eNOS binding to CSD. Indeed, addition of the CSD to the penetratin/*antennapedia* (AP) sequence—a fusion peptide termed AP-Cav or cavtratin—allowed its homogeneous intracellular uptake through a non-endocytic, non-degradative pathway [147, 148] and resulted in both attenuation of inflammation-induced microvascular leakage *in vivo* as well as lower eNOS but not iNOS-derived NO release [86]. Profound anti-tumor growth effects were observed with AP-Cav in the xenograft models of HepG2 and Lewis lung carcinoma—two tumor types that do not express eNOS—indicating that a host-dependent vascular effect was responsible for the large areas of tumor necrosis observed following AP-Cav delivery [87]. Confirmation of the role of the host eNOS system in the effect of AP-Cav was obtained using eNOS null mice; these animals showed no effect of AP-Cav on attenuation of the tumor growth rate and Evans blue extravasation, a common marker of inflammation-induced vascular leak. Noteworthy is the observation that VEGF- and bFGF-induced activation of angiogenic signaling was unaffected by AP-Cav, indicating a high degree of eNOS specificity that once again illustrates the significance of the eNOS-CAV1 interaction *in vivo* [87]. As a whole, these data depict the CSD as a critical negative regulator of tumor microvasculature permeability, an essential aspect of tumor survival and growth. In addition to its eNOS

inhibitory activities, AP-Cav was shown to inhibit Erk1/2 [149], MMP-9, and COX-2 expression in various systems [150], although the role of eNOS or NO in this inhibitory effect is unknown. To err on the side of caution, one must consider that synthetic peptides lack a tertiary structure which might cause them to behave differently than a properly folded CSD within full-length CAV1 monomer or oligomer in a normal lipid environment, which might impact its promiscuity with regards to client molecules. This concept is also supported by data showing cross-talk between phosphorylated CAV1 tyrosine 14 residue and the CSD [110]. In addition, the cell permeable sequence may affect intracellular localization of its cargo, as others have shown that CSD and truncated CSD peptides lacking a permeability sequence may be readily taken up by cells and therefore not require a Trojan sequence to mediate some of their activities *in vivo* [151].

7 Identification of multiple signaling domains with the CSD

An unexpected outcome from experiments relying on AP-fused peptides was that it led to the identification of the critical sub-CSD amino acids responsible for eNOS inhibition by CAV1. Mapping studies using truncated CSD-fused cell identified CSD residue F92 as the major eNOS inhibitory amino acid [125] despite acting as a by-stander in CSD binding to eNOS [152], thus providing evidence of CSD subdomain heterogeneity in terms of activity. Instead, CSD amino acids 90–91 and 93–99 appeared to mediate the majority of CSD's binding affinity to eNOS [109] independently of F92-dependent inhibition of eNOS, allowing the F92A mutant CAV1 to act in a dominant negative-like fashion, i.e., binding to eNOS without inhibiting its activity, resulting in greater NO release and endothelial function in general [152]. However, since the F92A CAV1 mutant localizes, oligomerizes, and targets to cholesterol membrane microdomains similar to WT CAV1 in endothelial and reconstituted cell systems [152] without inhibiting eNOS, this represented an important stepping stone towards differentiating between CSD's regulation of signaling events and its participation in other biological processes related to caveolae biogenesis and formation of signaling complexes. In addition, AP-fused CSD peptide with F92A substitution, which was named CavNOxin, was shown to increase NO release *ex vivo* in an eNOS- and CAV1-specific fashion [152]. CavNOxin also upregulated the anti-adhesive properties of the vascular endothelium *in vivo* by decreasing VCAM-1 expression and attenuating oxidative stress, as shown by blunted 4-hydroxynonenal (HNE) and dihydroethidium (DHE) staining [123]. *In vivo*, overexpression of F92A CAV1 specifically in the vascular endothelium increased NO release without affecting caveolae formation, confirming the possibility of modulating the signaling properties of a CAV1 protein without affecting its role in caveolae biogenesis [153] (Fig. 1b). Whether modulation of CAV1-regulated signaling can occur independently of an effect on endothelial caveolae formation and still influence tumor growth is unknown.

8 Endothelial CAV1- and CSD-mediated mechanosensing events: the next frontier?

A novel concept that helps integrate the ever-growing body of literature on CAV1 and its CSD as modulators of endothelial-specific homeostasis and angiogenic signaling may be their capacity to trigger critical signaling activities in response to physical stimulation [154]. Indeed, cells in general and particularly blood flow–exposed endothelial cells are capable of complex mechanosensing and mechanotransduction activities, which are terms that depict how cells sense extracellular forces and transduce them through the plasma membrane to trigger meaningful intracellular signaling events. In the case of the endothelium, the integration of extracellular forces, such as sheer stress, can result in eNOS-derived NO release and flow-mediated vasodilation (FMD) [42, 155], which caveolae are believed to be critical for signaling triggered by the sheer forces on the luminal membrane. This fascinating concept of caveolae-dependent mechanotransduction is based on early evidence demonstrating heightened phosphorylation of endothelial caveolae–resident proteins vs bulk plasma membrane protein fractions following exposure to flow [45]. In combination with recent data depicting rapid caveolae disassembly during mechanical stress [154], these studies indicate that caveolae- and CAV1-dependent signaling can be generated in response to flow. Indeed, cultured endothelial cells exposed to laminar flow show increased levels of caveolae at the membrane [156], and these are likely part of a mechanotransducing complex at the luminal surface of the plasma membrane [157] that results in rapid eNOS dissociation from CAV1 and its association with calmodulin that accounts for heightened NO release [158]. How this occurs is poorly understood, but caveolae are believed to act as a membrane buffer, resulting in their spontaneous disassembly following stretching [154]. The critical importance of endothelial CAV1 in *ex vivo* signaling events was confirmed in transgenic mice with an endothelial-specific rescue of CAV1 that corrected the aberrant flow-induced mechanotransduction and downstream vasodilation found in CAV1 null vessels [42]. How sheer stress and other mechanical forces induce caveolae disassembly and alter signaling of caveolae-resident proteins and CAV1 client protein interactions is unknown, but it is likely that these endothelial-specific signalosomes triggered by flow result in a heightened state of activation due to loss of the CAV1 inhibitory clamp. These newly formed endothelial signalosomes can contain numerous signaling proteins known to mediate

mechanosensing, such as VEGFR-2 [159], eNOS as well as other endothelial-specific membrane proteins such as VE-cadherin and CD31 [58], which have all been implicated in endothelial mechanotransduction events [159]. Consequently, the critical importance of endothelial CAV1 expression in whole-body homeostasis and tumor-derived angiogenesis could be integrated and summarized as a loss of function of the mechanotransduction machinery. Although tumor angiogenesis requires the above-mentioned signaling proteins, how this occurs in the context of a mechanosensitive endothelial plasmalemma remains to be investigated. As previously mentioned, tumor-derived vasculature is known to be critically different than its endogenous counterpart; for instance, newly formed vessels typically show a high degree of tortuosity known to affect flow patterns and directionality [160, 161]—an important factor in caveolae homeostasis (Fig. 1a) [156]. These new vessels also lack a basement membrane [162], present higher endothelial cell turnover [160] along with heightened permeability and propensity to spontaneous collapse due to lack of pericyte coverage and proper basement membrane [163]. These are all key targets of vascular disrupting agents [164], which could cause or contribute to abnormal mechanosensing. Hence, how the endothelium-selective CAV1-CSD-VEGFR2-eNOS signalosome behaves (Fig. 1b) under abnormal mechanosensing conditions in the tumor stroma remains to be explored as a whole, and novel tools to dissociate CAV1 and CSD in caveolae biogenesis and signaling [152, 153] will likely facilitate these investigations. With these concepts in mind, one could therefore create more integrative models of caveolae- and CAV1-dependent signaling in endothelial health and angiogenesis, which could better rationalize the complex and sometimes divergent data obtained in CAV1-deficient mice.

9 Concluding remarks

Despite their humble beginnings as "small caves" at the plasmalemma of endothelial cells, it is likely that the versatility by which endothelial CAV1 and its CSD can influence tumor vascularization, growth, as well as whole-body homeostasis will continue to puzzle the scientific community for decades to come. Of crucial importance will likely be the capacity of scientists to integrate individual, small-scale findings into larger working models that depict how CAV1 and caveolae biogenesis assimilate the large signaling events required for endothelial cell recruitment and growth into blood vessels that vascularize tumors. Whether the interpretation of caveolae-dependent mechanosensing events in angiogenic endothelial cells will be the final chapter in our understanding of caveolae, CAV1, and the CSD in stromal endothelial, or if a yet undiscovered layer of complexity will feed our enthusiasm for more CAV1-related discoveries is unknown, it is nonetheless likely that the key challenges will need to be overcome. These include the relatively small size of tumor-derived angiogenic capillaries, making them difficult to isolate and study, the flow-dependent and dynamic nature of caveolae formation and stability, as well as the rapid capacity of signaling proteins to evade the inhibitory effect of Cav-1 during experimental handling. Better tools will likely improve the pace of future CAV1-related discoveries as well as their significance, which could ultimately translate into measurable societal impacts, such as improved cancer patient care.

Compliance with ethical standards

Conflict of interest The author declares that he has no conflict of interest.

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