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## Caveolins and caveolae in ocular physiology and pathophysiology

Xiaowu Gu<sup>a,b,1,‡</sup>, Alaina M. Reagan<sup>a,b,1</sup>, Mark E. McClellan<sup>a,1</sup>, and Michael H. Elliott<sup>a,b,c,1,\*</sup>

<sup>a</sup>Department of Ophthalmology/Dean McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK, 73104

<sup>b</sup>Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

<sup>c</sup>Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

### Abstract

Caveolae are specialized, invaginated plasma membrane domains that are defined morphologically and by the expression of signature proteins called, caveolins. Caveolae and caveolins are abundant in a variety of cell types including vascular endothelium, glia, and fibroblasts where they play critical roles in transcellular transport, endocytosis, mechanotransduction, cell proliferation, membrane lipid homeostasis, and signal transduction. Given these critical cellular functions, it is surprising that ablation of the caveolae organelle does not result in lethality suggesting instead that caveolae and caveolins play modulatory roles in cellular homeostasis. Caveolar components are also expressed in ocular cell types including retinal vascular cells, Müller glia, retinal pigment epithelium (RPE), conventional aqueous humor outflow cells, the corneal epithelium and endothelium, and the lens epithelium. In the eye, studies of caveolae and other membrane microdomains (i.e., “lipid rafts”) have lagged behind what is a substantial body of literature outside vision science. However, interest in caveolae and their molecular components has increased with accumulating evidence of important roles in vision-related functions such as blood-retinal barrier homeostasis, ocular inflammatory signalling, pathogen entry at the ocular surface, and aqueous humor drainage. The recent association of *CAVI2* gene loci with primary open angle glaucoma and intraocular pressure has further enhanced the need to better understand caveolar functions in the context of ocular physiology and disease. Herein, we provide the first comprehensive review of literature on caveolae, caveolins, and other membrane domains in the context of visual system function. This review highlights the importance of caveolae domains and

\*Corresponding author: Tel: +1 405 271 8001, ext. 30024. michael-elliott@ouhsc.edu.

‡Current address: Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205

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their components in ocular physiology and pathophysiology and emphasizes the need to better understand these important modulators of cellular function.

### Keywords

*Keywords:* caveolin; caveolae; lipid rafts; blood-retinal barrier; vascular permeability; neuroinflammation; glaucoma; ocular hypertension

## 1. INTRODUCTION: Caveolae as specialized lipid rafts, a historical perspective

The “nautical” description of cell membranes as a sea of lipids with randomly-distributed protein “buoys” defined by Singer and Nicolson’s fluid mosaic model (Singer and Nicolson, 1972) was a major advance in the understanding of cellular membrane organization. This maritime definition of cell membranes was later expanded (disputed?) to incorporate observations of thermodynamically-stable clusters of lipids (Karnovsky et al., 1982; Lee et al., 1974) that were eventually described formally as “lipid rafts” (Simons and Ikonen, 1997). The lateral heterogeneity of model membranes is essentially without question but the challenge of defining such domains in a cellular context has consumed cell biologists, biochemists, and biophysicists since the raft hypothesis was “floated” (pun intended). The purpose of this review is not to argue the evidence supporting the existence of lipid rafts in cell membranes as this has been reviewed extensively (Carquin et al., 2015; Munro, 2003; Pike, 2006, 2009; Shogomori and Brown, 2003). Instead, we will focus on caveolae, specialized lipid rafts, that by virtue of their ability to be visualized ultrastructurally and to be defined molecularly, have been the best studied and accepted membrane platforms traveling on the lipid seas. We will further focus our discussion on caveolae and their molecular components in the context of vertebrate visual system function and dysfunction.

With the advent of electron microscopy to elucidate cellular ultrastructure, George Palade, in 1953, first described plasma membrane invaginations in capillary endothelial cells, which he referred to as plasmalemmal vesicles (Fig. 1) (Palade, 1953). Shortly thereafter, a similar vesicular structure was observed in epithelial cells and named “*caveola intracellularis*” or “intracellular cave” (YAMADA, 1955). The appearance of caveolae led to the early postulation that they could be pinched off from the plasma membrane to mediate the transport of fluid or other molecules across the cell, thus representing a mechanism for transcellular capillary permeability (Palade, 1953, 1961). Based on the electron microscopic description, caveolae possess the following morphological characteristics: 1) flask-shaped membrane invaginations that are typically associated with the plasma membrane and occasionally seen with a narrow neck or a diaphragm; 2) typical sizes are of 50–100 nm without an apparent electron-dense coating compared clathrin-coated pits (Palade, 1953; Palade and Bruns, 1968; YAMADA, 1955). Further electron microscopic experiments demonstrated that morphologically identifiable caveolae could be found in almost every cell type, but were particularly abundant in adipocytes, fibroblasts, smooth muscle cells, and endothelial cells (Gabella, 1976; NAPOLITANO, 1963; Palade, 1953). However, they were

reportedly absent in certain cell types (lymphocyte cell lines (Fra et al., 1994) and most neurons (Gorodinsky and Harris, 1995)).

The initial discovery of caveolae in the 1950's relied on ultrastructural analyses which did not provide details on their molecular composition. Nearly 40 years passed from their initial identification until the first protein component of caveolae, now known as "caveolin-1" (Cav-1) was identified. Cav-1 was discovered as a phosphorylated substrate with a molecular weight of 22 kDa in an antibody screen of phosphotyrosine-modified proteins in Rous sarcoma virus-transformed chicken fibroblasts (Glenney and Zokas, 1989). Importantly, the authors of this breakthrough noted the concentrated punctate immunolocalization of the protein at cell margins (Glenney and Zokas, 1989). The eventual localization of this 22-kDa protein to morphological caveolae by immuno-electron microscopy resulted in the naming of this protein, "caveolin" (Rothberg et al., 1992) which was subsequently changed to "caveolin-1" after the Lisanti laboratory identified a second caveolin family member (Scherer et al., 1996). Despite the later discovery of cytosolic and secreted forms (Liu et al., 1999; Moon et al., 2014; Tahir et al., 2001), Cav-1 normally behaves as an integral protein embedded as a hairpin loop into the inner leaflet of the lipid bilayer with both the N- and C-termini facing the cytoplasm (Fig. 1) (reviewed in (Cohen et al., 2004; Parton and Simons, 2007)). It contains a cholesterol-binding domain (Murata et al., 1995), a highly conserved caveolin scaffolding domain (CSD) (Li et al., 1996a) and the previously identified tyrosine phosphorylation site which are collectively thought to play important roles in modulating cell signaling. The CSD was originally discovered in an *in vitro* experiment as a short peptide of 20 amino acids (82–101 residues of Cav-1) (Li et al., 1996a). In a large body of literature (reviewed in (Cohen et al., 2004; Patel et al., 2008)), Cav-1 has been shown to interact with G proteins, receptor tyrosine kinases (e.g., epidermal growth factor receptor, vascular endothelial growth factor receptor), non-receptor protein kinases (e.g., Src family, protein kinase C), other enzymes (e.g., endothelial nitric oxide synthase) and cytoskeletal proteins that localize to caveolae. Importantly, CSD interactions tend to negatively regulate the activities of many associated proteins (Couet et al., 1997a; Couet et al., 1997b; García-Cardena et al., 1997; Labrecque et al., 2003; Li et al., 1996a; Li et al., 1995; Lisanti et al., 1994a; Lisanti et al., 1994b). These interacting partners share caveolin binding motifs present in their protein sequence:  $\Phi X\Phi XXXX\Phi$  and  $\Phi XXXX\Phi XX\Phi$  (or combined  $\Phi X\Phi XXXX\Phi XX\Phi$ ), where  $\Phi$  is an aromatic residue (Phe, Tyr, or Trp) and X is any amino acid (Couet et al., 1997a). Caveolae and caveolin-rich domains were proposed to act as cell signaling regulatory platforms (Lisanti et al., 1994a). Although the overarching hypothesis that the CSD is a general regulator of signal transduction has recently been challenged (Collins et al., 2012), there is clear evidence that the CSD is involved in endogenous inhibition of endothelial nitric oxide synthase (eNOS) in vascular endothelium and that mechanical uncoupling of eNOS from Cav-1 results in NO production (García-Cardena et al., 1997; Yu et al., 2006). Recent evidence using an eNOS-activating CSD mutant (Bernatchez et al., 2011) has allowed for the separation of this CSD signal regulation from the formation of caveolae domains (Kraehling et al., 2015). Besides the CSD, the originally-identified phosphotyrosine modification at Tyr14 provides an additional means for Cav-1 to modulate signaling (Glenney and Zokas, 1989; Li et al., 1996b). The Tyr14 phosphorylation

of Cav-1 is tightly regulated in normal cells and may serve as a docking site for SH2 domain signaling proteins (Cao et al., 2002; Lee et al., 2000; Li et al., 1996b).

In addition to Cav-1, two additional caveolin family members, caveolin-2 (Cav-2) and caveolin-3 (Cav-3) were subsequently discovered (Scherer et al., 1996; Tang et al., 1996; Way and Parton, 1996). Cav-2 generally coexists with Cav-1 to form hetero-oligomeric complexes in caveolae (Scherer et al., 1997; Scherer et al., 1996) and Cav-2 requires Cav-1 for proper membrane localization/stabilization such that ablation of Cav-1 results in downregulation of Cav-2 (Razani et al., 2001). The third family member, Cav-3, is exclusively expressed in muscle cells where it is essential for caveolae formation (Tang et al., 1996; Way and Parton, 1996). The discovery that caveolins also bind cholesterol (Murata et al., 1995) and that caveolae are enriched in cholesterol and glycosphingolipids (Ortegren et al., 2004) led to the definition of caveolae as specialized lipid rafts (Fig. 1).

As mentioned above, several lymphocyte cell lines do not express caveolins nor do they possess morphologically identifiable caveolae (Fra et al., 1994). Astonishingly, they were capable of making caveolae when Cav-1 was introduced, suggesting that Cav-1 is a key element for caveolar biogenesis (Fra et al., 1995). The generation of each of the caveolin knockout (Cav-1<sup>-/-</sup>) mouse lines has unequivocally confirmed that Cav-1 but not Cav-2 is essential for the formation of caveolae in non-muscle cells (Drab et al., 2001; Razani et al., 2001; Razani et al., 2002) and that Cav-3 is essential for making caveolae in muscle cells (Galbiati et al., 2001). However, although Cav-1 is necessary for caveolae biogenesis, it is not sufficient to generate morphologically identifiable caveolae and requires a more recently discovered family of proteins called “cavins” which have not yet received attention in the eye. Cavin-1, originally named “polymerase I and transcript release factor” (PTRF) because of its role in transcription, was subsequently found to be essential to stabilize mature caveolin and form morphologically identifiable caveolae (Hill et al., 2008; Liu et al., 2008). Lack of cavin-1 destabilizes membrane-bound Cav-1 and promotes its lysosomal degradation (Hill et al., 2008; Liu et al., 2008). Therefore, the necessity of caveolins and cavins in caveolae biogenesis is likely critical to understanding why certain types of cells (e.g., Müller glia) do not possess caveolae but still abundantly express Cav-1, likely in planar rafts (Fig. 1) (Nelson et al., 2011; Roesch et al., 2008). In fact, the elucidation of the functions of extra-caveolar caveolins is only recently being realized in the context of cell migration and cancer (Hill et al., 2012; Moon et al., 2014; Nassar et al., 2015; Tahir et al., 2013). Given that Müller glial cells in the retina only appear to express extra-caveolar Cav-1, future studies of Cav-1 function in this cell type could provide fundamental insight into novel Cav-1 functions.

Since their discovery in the 1950s, the functions of caveolae and caveolins have been extensively studied outside of the visual system. In this introduction, we have focused on a historical perspective and several groundbreaking research contributions. For more information on general caveolae functions, the interested reader is pointed to several outstanding reviews that highlight the myriad functions attributed to caveolae/caveolins including lipid trafficking, transcytosis, extracellular matrix (ECM) remodeling, mechanotransduction/mechanoprotection and cell signaling (Cheng and Nichols, 2016; Chidlow and Sessa, 2010; Cohen et al., 2004; Echarri and Del Pozo, 2015; Nassoy and

Lamaze, 2012; Parton and del Pozo, 2013; Parton and Simons, 2007; Sotgia et al., 2012). The remainder of this review will focus on putative caveolae/caveolin functions with specific relevance to visual physiology and pathophysiology. We will concentrate broadly on caveolins/caveolae in: 1) the retina and blood-retinal barrier; 2) primary open angle glaucoma and intraocular pressure/aqueous humor outflow; and 3) the ocular surface and lens.

## 2. Caveolins/caveolae in the neuroretina and RPE

### 2.1. Localization of caveolins in the adult retina

Caveolins and/or caveolae have been observed widely in retinal vascular cells (Caldwell and Slapnick, 1992; Chen et al., 2007; Feng et al., 1999a; Feng et al., 1999b; Gardiner and Archer, 1986a; Gu et al., 2014a; Opreanu et al., 2011; Raviola and Butler, 1983; Sagaties et al., 1987; Stitt et al., 2000a; Stitt et al., 2000b), Müller glia (Gu et al., 2014b; Hauck et al., 2010; Li et al., 2012; Nelson et al., 2011; Reagan et al., 2016; Roesch et al., 2008), RPE (Bridges et al., 2001; Chen et al., 2003; Chowers et al., 2004; Forbes et al., 2007; Kook et al., 2008; Mora et al., 2006; Omri et al., 2011; Sethna et al., 2016), and photoreceptors (Berta et al., 2011; Boesze-Battaglia et al., 2002; Corley and Albert, 2011; Elliott et al., 2003; Elliott et al., 2008; Kachi et al., 2001; Nair et al., 2002; Senin et al., 2004). In the adult mouse retina, Cav-1 is the major detectable caveolin protein and is abundant in retinal and choroidal vascular beds and in Müller glia with detectable but lower expression in the retinal pigmented epithelium (RPE) (Li et al., 2012)(Fig. 2a). When visualized *en face* in neuroretinal wholemounts (Fig. 2b), Cav-1 immunoreactivity is apparent in all branches of the vasculature with the most intense immunoreactivity detected in retinal venules (Gu et al., 2014a). The vascular localization is consistent with ultrastructural studies showing caveolae in retinal vascular endothelium and mural cells (Caldwell and Slapnick, 1992; Gardiner and Archer, 1986a; Gu et al., 2014a; Raviola and Butler, 1983; Sagaties et al., 1987) (Fig. 2c). The strong Cav-1 immunoreactivity in Müller glia in the neuroretinal compartment agrees well with transcriptional data indicating that Cav-1 is a Müller cell-enriched transcript compared to retinal neurons (Roesch et al., 2008). Although enriched in Müller glia relative to photoreceptors, Cav-1 protein has been found in biochemical isolates of photoreceptor membranes (Boesze-Battaglia et al., 2002; Elliott et al., 2008; Martin et al., 2005; Nair et al., 2002; Senin et al., 2004) and in isolated photoreceptor preparations where it is preferentially observed in inner segment blebs (Elliott et al., 2003). Consistent with this, Cav-1 has also been found associated with rod photoreceptor ribbon synapses by immunogold EM (Kachi et al., 2001). As discussed later, the function of photoreceptor-intrinsic Cav-1 is unclear as photoresponse deficits resulting from Cav-1 ablation do not seem to result from loss of photoreceptor-intrinsic Cav-1 (Li et al., 2012). Instead, it is more likely that the loss of Cav-1 in photoreceptor support cells (e.g., RPE and Müller cells) contribute to secondary photoreceptor functional deficits (Sethna et al., 2016). Cav-1 expression in native and cultured RPE can support the formation of rare ultrastructurally-identifiable caveolae with both apical and basolateral localizations (Mora et al., 2006) but immunogold localization (Bridges et al., 2001) and later functional studies (Sethna et al., 2016) suggest that Cav-1 may function outside of caveolae in these cells.

In human retina (Fig. 3), the localization of Cav-1 is consistent with that observed in murine (Li et al., 2012; Reagan et al., 2016) and other species (Berta et al., 2011; Berta et al., 2007b). Immunoreactivity is enhanced in the nerve fiber layer, presumably in Müller glial endfeet (Berta et al., 2007a)(Fig. 3). Of note, Müller glial immunoreactivity is increased in diabetic retina around areas with cystic lesions (Fig. 3). Whether this represents increased expression in the diabetic retina or more efficient immunolabeling is not clear at present.

## 2.2. Developmental expression of caveolins in the retina

The developmental expression of caveolins has been addressed in several important studies. In zebrafish, Cav-1 plays a critical role in development as its depletion by morpholinos resulted in significant reductions of caveolae and profound systemic developmental abnormalities (Fang et al., 2006; Nixon et al., 2007). In the zebrafish eye, Cav-1 silencing resulted in defects in RPE differentiation, eye pigmentation, and ocular cell organization (Fang et al., 2006). This essential role of Cav-1 in eye development in fish is not recapitulated in mammals as Cav-1<sup>-/-</sup> mice, which also lack caveolae, develop structurally normal RPE and retinae (Li et al., 2012). However, subtle defects in ocular development have not been rigorously tested in Cav-1<sup>-/-</sup> mice.

The expression of caveolins during postnatal retinal development has been studied in mice (Gu et al., 2014b; Nelson et al., 2011) and hamster (Berta et al., 2011), respectively, with largely consistent findings. As represented in Fig. 4 (reproduced from (Gu et al., 2014b)), Cav-1 expression at postnatal (P) days 0 and 2 is most pronounced in the newly developing retinal vasculature and in the choroidal vasculature with detectable expression also localized to the RPE at these early timepoints. Cav-1 expression in the mouse RPE declines from P0–P21 consistent with western blotting data from rat RPE (Mora et al., 2006). Expression in the neuroretinal compartment is weak until P7–P10 at which point the localization appears in cells with Müller glial morphology. This coincides with increased expression of glutamine synthetase in these same cells (Gu et al., 2014b). The predominant Müller glial expression of Cav-1 is maintained into adulthood which agrees with the relative enrichment of Cav-1 mRNA in these cells compared to retinal neurons (Roesch et al., 2008). These findings are also consistent with increased expression of Cav-1 and Cav-2 mRNAs at the time when Müller cells terminally differentiate (Nelson et al., 2011). Further evidence that Cav-1 expression is associated with Müller glia differentiation state comes from analysis of expression in gliotic/dedifferentiated cells responding to retinal insult. Following up on their elegant transcriptomic analysis of isolated Müller cells (Roesch et al., 2008), the Cepko laboratory performed gene expression studies of Müller glia at the peaks of rod and cone degeneration in rhodopsin knockout mice (Roesch et al., 2012). Using their raw data, we plotted the expression of Cav-1 and glial fibrillary acidic protein (GFAP), a marker of Müller gliosis (Fig. 5). Intriguingly, Cav-1 mRNAs were dramatically reduced in Müller cells at the peak of rod degeneration and returned to control levels by the peak of cone degeneration. These expression changes negatively correlated with the expression of GFAP. Collectively, these findings imply that Cav-1 is predominantly expressed in differentiated Müller glia and not in cells that have either dedifferentiated or have not yet differentiated. The role that Cav-1 plays in Müller cell differentiation is as yet unexplored and merits rigorous attention.



### 2.3. Functional consequences of Cav-1 deficiency in the retina

The expression and localization of caveolins and morphological caveolae in the retina suggest that they make important contributions to retinal function. At this time, our working hypothesis was that photoreceptor-intrinsic Cav-1 played an important role in modulating phototransduction either through direct interactions with phototransduction machinery or via control of photoreceptor lipid composition. This hypothesis was based on biochemical and biophysical data showing that phototransduction components either associated with Cav-1 or were present in cholesterol-rich lipid microdomains and that the heterotrimeric G-protein, transducin, was sequestered in such domains in an activity-dependent manner (Boesze-Battaglia et al., 2002; Ding et al., 2008; Elliott et al., 2003; Elliott et al., 2008; Martin et al., 2005; Nair et al., 2002; Senin et al., 2004; Seno et al., 2001; Wang et al., 2008). Earlier work had indicated that the cholesterol content of rod photoreceptor disks is spatially heterogeneous with newly formed, basal disks having a higher cholesterol content than older, apical disks (Boesze-Battaglia et al., 1990; Boesze-Battaglia et al., 1989) and that a higher cholesterol content impairs rhodopsin activation (Niu et al., 2002). Given Cav-1's established role in cholesterol transport, we predicted that Cav-1 deficiency would perturb photoreceptor lipid content leading to functional deficits.

To test this prediction, electrophysiological studies were carried out on Cav-1-deficient mice (Li et al., 2012). As shown in Fig. 6A–C, genetic ablation of Cav-1 results in significant deficits in both a- and b-wave responses as measured by scotopic electroretinography (ERG). To assess rod photoreceptor function in more detail, a computational model of phototransduction (Hood and Birch, 1994) was fit to the leading edges of a-wave responses revealing a significant impairment in phototransduction amplification. Using a second *in vivo* functional test, manganese-enhanced magnetic resonance imaging, Li et al. (2012) found significantly suppressed ion uptake under dark-adapted conditions in Cav-1<sup>-/-</sup> mice implying a reduction in photoreceptor dark current. These results supported the initial hypothesis that photoreceptor-intrinsic Cav-1 is important to support photoreceptor function. However, rigorous analyses of photoreceptor structure and biochemistry, including the finding that cholesterol content and fatty acid compositions were indistinguishable between Cav-1<sup>-/-</sup> and control mice, failed to yield a photoreceptor-intrinsic explanation for reduced retinal function. The penultimate experiment that eliminated a significant contribution of photoreceptor-intrinsic Cav-1 was performed in collaboration with Gordon Fain's laboratory at UCLA where isolated photoresponses were measured from Cav-1<sup>-/-</sup> and control rods by the suction electrode method revealing that dark current and flash intensity/response relationships were indistinguishable between genotypes (Fig. 6D). A subtle defect in the dim flash (single photon) response was noted but did not explain the more significant functional deficits revealed by ERG analysis. The observation of functional deficits, *in vivo*, without significant deficits in isolated rod responses led to the revised hypothesis that photoreceptor-extrinsic Cav-1 is important in the maintenance of a retinal environment conducive to neuronal function.

One possibility is that Cav-1 deficiency alters the ionic milieu surrounding photoreceptors, *in situ*. Ionic and fluid homeostasis in the interphotoreceptor space is largely controlled by transport activities in the apical RPE including the activity of the  $\alpha 1$ -sodium/potassium

ATPase (Na/K-ATPase) (reviewed in (Strauss, 2005)). The Na/K-ATPase contains consensus Cav-1 binding motifs, interacts with Cav-1 in non-ocular cells, and its activity is sensitive to cholesterol (Wang et al., 2004; Yeagle, 1983). In Cav-1-deficient RPE, the Na/K-ATPase has reduced affinity for potassium in the concentration range found in the subretinal space (Li et al., 2012). Thus, the observed retinal functional deficits in Cav-1<sup>-/-</sup> mice could result from defects in the ability of the RPE to support photoreceptor function. Additional evidence of RPE dysfunction due to specific ablation of Cav-1 in the RPE will be discussed in the next section. The lack of evidence that photoreceptor-intrinsic Cav-1 modulates phototransduction does not eliminate the potential impact of ribbon synapse-localized Cav-1 (Kachi et al., 2001). This could be addressed through rod-specific conditional deletion of Cav-1 but remains an open question.

#### 2.4. The role of RPE-specific Cav-1 in phagolysosomal digestion

The RPE fulfills several critical functions to maintain photoreceptor health and function including epithelial transport, formation of the outer blood-retinal barrier (BRB), maintenance of the retinoid visual cycle, polarized secretion of anti- and pro-angiogenic factors, and removal and clearance of debris from the diurnal shedding of photoreceptor outer segments (POSs) (reviewed in (Strauss, 2005)). RPE cells are post-mitotic professional phagocytes that efficiently engulf and digest large quantities of photoreceptor material in the process of photoreceptor renewal (Young and Bok, 1969). Unique among most epithelia is the bipolar localization of caveolae and caveolins with a significant proportion of caveolae found on the apical, photoreceptor-facing surface (Mora et al., 2006). When cultured RPE cells are challenged with POS material, Cav-1 displays time-dependent increases in expression suggesting a role in the phagocytic process (Chowers et al., 2004). Cav-1 deficiency/impairment in another professional phagocyte, macrophages, reduces their phagocytic capacity (Fu et al., 2012; Li et al., 2005; Rodriguez et al., 2006; Tsai et al., 2011).

To begin to assess cell-specific Cav-1 functions, *in vivo*, we recently generated the first conditional, tissue-specific Cav-1 knockout mice including a model with Cav-1 specifically deleted within the RPE (Fig. 7A–H modified from (Sethna et al., 2016)). Ablation of Cav-1 specifically in the RPE resulted in significant reductions in both rod photoreceptor-driven a- and b-wave responses as assessed by ERG (see (Sethna et al., 2016)). The reduction in ERG amplitudes in RPE-specific Cav-1 mutants at least partially explains the photoreceptor-extrinsic functional deficits observed in global Cav-1<sup>-/-</sup> mice (Li et al., 2012). To better understand the RPE dysfunction induced by loss of Cav-1 in the RPE, we collaborated with Silvia Finnemann's laboratory to assess the potential role of Cav-1 in RPE phagocytosis. RPE-specific Cav-1 ablation, *in vivo*, did not affect the RPE's ability to bind or engulf POS but instead resulted in a delay in phagosome clearance from POS laden RPE (Fig. 7I,J). This delayed digestion resulted from defects in the upregulation and activity of the lysosomal enzymes, cathepsin D (Fig. 7K–M) and  $\beta$ -N-acetyl-glucosaminidase (see (Sethna et al., 2016)) which are essential for POS-opsin digestion and deglycosylation, respectively. Intriguingly, the upregulation of cathepsin D immediately after diurnal POS shedding observed in control mouse RPE, was significantly delayed in RPE with specific Cav-1 ablation. Similar delays in phagosome clearance and upregulation of cathepsin D levels and



activity were found in POS-challenged cultured RPE-J cells in which Cav-1 expression was silenced. Overexpression of both wildtype and a CSD mutant of Cav-1 that fails to target to the plasma membrane accelerated POS clearance indicating that Cav-1's impact on digestion was independent of its scaffolding domain. As chronic accumulation of indigestible debris is pathological to the RPE in conditions such as age-related macular degeneration (AMD), these findings suggest that increasing Cav-1 expression in the RPE could be a therapeutic strategy to facilitate debris removal. These findings may also be relevant to other professional phagocytes in the eye (e.g., trabecular meshwork cells, retinal microglia) but this remains to be studied.

## 2.5. Caveolins/caveolae in the blood-retinal barrier (BRB)

The BRB consists of two distinct but analogous interfaces between the neuroretina and the circulatory system. The “inner” BRB is analogous to the blood-brain barrier originally described by Reese and Karnovsky (Reese and Karnovsky, 1967) and is formed by the retinal vascular endothelial cells which have well-developed tight junctions and reduced transcellular transport. The second “outer” BRB is formed by the well-developed tight junctions of the RPE which is adjacent to the fenestrated and inherently “leaky” choroidal vasculature. We will focus our discussion on the inner BRB as unpublished studies from our laboratory using RPE-specific Cav-1<sup>-/-</sup> mice suggest that caveolae do not contribute significantly to outer, RPE barrier integrity. The roles that caveolae play in the control of retinal vascular permeability has been reviewed extensively in a previous volume of this journal (Klaassen et al., 2013). In this section, we will expand upon this previous review to include additional studies that implicate caveolae in BRB maintenance under basal and pathological conditions. We begin with a general review of the evidence of caveolar participation in endocytosis and transcytosis in non-barrier endothelium before focusing on studies of the BRB.

Caveolae have long been thought to be key players in clathrin-independent endocytosis (reviewed recently in (Cheng and Nichols, 2016)). However, the surprising stability of caveolae at the plasma membrane (Thomsen et al., 2002) and the small proportion of caveolae that actually undergo endocytosis under steady state conditions (Shvets et al., 2015) suggests that their direct contribution to large-scale endocytosis is relatively low. Regardless of their direct contribution, caveolins and caveolae clearly influence clathrin-independent endocytosis as ablation of Cav-1 or PTRF/cavin-1 suppresses this endocytic pathway (Chaudhary et al., 2014). Due to this crosstalk and the relatively small direct contribution of caveolae to endocytosis, a clear understanding of the importance of caveolar endocytosis is as yet incomplete.

Caveolae have also been implicated in transcytosis, a specialized form of endocytosis, to deliver cargoes across vascular endothelia via either caveolae vesicles or transendothelial channels formed by the fusion of caveolae (Ghitescu et al., 1986; Milici et al., 1987; Predescu et al., 1994). There is also evidence that the caveolar pathway can be engaged for rapid transendothelial transport, *in vivo* (Oh et al., 2007). Furthermore, recent evidence suggests that caveolae transcytosis participates in the early stages of blood-brain barrier breakdown following ischemic injury (Knowland et al., 2014). The contribution of caveolae

to transcytosis remains controversial (Cheng and Nichols, 2016; Rippe et al., 2002). If caveolae are key players in endothelial transcytosis, then why are paradoxical increases in vascular permeability widely reported in mice in which Cav-1 is deleted or silenced (Gu et al., 2014a; Miyawaki-Shimizu et al., 2006; Schubert et al., 2002)? One mechanism proposed to explain the paradoxically increased permeability caused by caveolae deficiency is a compensatory increase in paracellular permeability via loss of junctional integrity due to crosstalk between the caveolae and junctional pathways (Komarova and Malik, 2010). Alternatively, the increased permeability could be explained by increased capillary hydraulic pressure due to upstream vasodilation induced by eNOS hyperactivity secondary to caveolae loss (Rosengren et al., 2006).

In the retina, the question of whether caveolae contribute to transendothelial permeability has also been debated. Classical EM studies have revealed that caveolae in the retinal vascular endothelium (like that in the brain) are largely polarized to the abluminal surface facing the basement membrane rendering them less accessible to blood components in circulation (for a qualitative example of this localization, see the left panel of Fig. 2C) (Caldwell and Slapnick, 1992; Raviola and Butler, 1983; Sagaties et al., 1987). In addition, the numbers of caveolae in the retinal vascular endothelium is reduced compared to non-barrier endothelia (Sagaties et al., 1987). Collectively, these results imply that under normal conditions, caveolae in the retinal vascular endothelium are unlikely to support blood-to-tissue transendothelial transport. This is in keeping with the concept that a relatively impermeable BRB is formed by: (1) well-developed endothelial cell junctions and (2) reduced numbers of caveolae that do not participate in transendothelial transport (Raviola, 1977). In fact, when horseradish peroxidase (HRP) was injected into the circulation of several mammalian species, HRP reaction product failed to cross to the endothelial basal lamina (Gu et al., 2014a; Raviola and Butler, 1983) nor was it taken up by morphologically-identifiable caveolae within the endothelium (Gardiner and Archer, 1986b). When HRP was injected intravitreally, HRP reaction product filled the vascular basal lamina and abluminal caveolae (Gardiner and Archer, 1986a; Raviola and Butler, 1983). In one study, HRP appeared within endothelial vesicles as well as rare caveolae open to the lumen suggesting the presence of a caveolae-dependent unidirectional transport from the neuropil to the blood (Raviola and Butler, 1983) but this interpretation was later questioned (Gardiner and Archer, 1986a). Collectively, these results question the contribution of caveolae to transendothelial transport under normal conditions.

What contribution does caveolar transcytosis make to increased permeability during pathological BRB breakdown? As indicated above, ischemic brain injury induces an initial increase in transcellular, caveolae-associated permeability that precedes any paracellular, junctional leakage (Knowland et al., 2014). In the retina, several lines of evidence suggest that caveolae transcytosis is upregulated in pathological conditions such as diabetes. In the early 1980's, Ishibashi and colleagues examined retinal vascular permeability to HRP in streptozotocin (STZ)-induced diabetic rats and found a dramatic increase in the number of HRP-laden vesicles in the diabetic compared to control group (Ishibashi et al., 1980). An elegant freeze fracture EM study of retinal vasculature from STZ-induced diabetic rats revealed that while the number of luminal plasmalemmal vesicles (caveolae) did not increase, the size of these vesicles was abnormally large and the number of "double

vesicles” that appear to represent fused caveolae was increased compared to controls (Caldwell and Slapnick, 1992). Of note, the number of caveolae on the abluminal surface of *pericytes* did increase dramatically in diabetic animals. In agreement with these earlier studies, the expression of molecular components of caveolae, including Cav-1 (Klaassen et al., 2009) and plasmalemmal vesicle-associated protein (PLVAP, also known as PV-1 or PAL-E antigen), are upregulated in hyper-permeable, diabetic retinal vasculature (Schlingemann et al., 1997; Schlingemann et al., 1999; Wisniewska-Kruk et al., 2014). In cultured retinal microvascular endothelial cells, advanced glycation end-product-modified proteins, which are implicated in diabetic complications, bind to receptors within, and are internalized by, caveolae (Stitt et al., 2000b). *In vivo*, the delivery of AGE-modified proteins to normoglycemic rats induced BRB breakdown that coincided with increases in endothelial caveolae (Stitt et al., 2000a). Treatment of retinal vascular endothelial cells with vascular endothelial growth factor (VEGF), a potent inducer of vascular permeability (Senger et al., 1983), induces permeability via increased caveolae-associated transcytosis (Feng et al., 1999a). Intriguingly, VEGF receptor-2 (VEGFR-2) is localized to caveolae (Feng et al., 1999b; Labrecque et al., 2003). A recent study shows that retinal vascular endothelium displays polarized responsiveness to VEGF with more pronounced VEGFR-2-mediated responses localized to the abluminal endothelium (Hudson et al., 2014) where caveolae are most abundant. Intraocular injection of VEGF in monkeys resulted in increased vascular permeability that did not result from tight junction opening but instead was associated with a dramatic increase in the number of caveolae on the luminal surface of the retinal endothelium (Hofman et al., 2000). Similar VEGF-induced increases in caveolae numbers have recently been reported in human retinal explants and this correlates with increased PLVAP expression which is not normally expressed in barrier-forming endothelia (Wisniewska-Kruk et al., 2016). Importantly, silencing PLVAP expression blocked this VEGF-induced caveolae formation without reducing the basal numbers of caveolae. Silencing PLVAP, *in vivo*, also reduced BRB permeability in a model of oxygen-induced retinopathy. Thus, pathological upregulation of PLVAP in retinal vasculature likely increases BRB permeability through upregulation of caveolae transcytosis. Collectively, these results suggest that transendothelial permeability mediated by caveolae is an important contributor to pathological BRB breakdown.

To determine the role of Cav-1/caveolae in the BRB directly, Gu et al. (2014) examined the integrity of the retinal vascular barrier in mice globally deficient in Cav-1. Ablation of Cav-1 resulted in a complete loss of morphologically-identifiable caveolae in the retinal vasculature (see Fig. 2C, *right panel*). Using several methodologies including measurements of endogenous albumin extravasation and permeability to intravenously delivered contrast agents, a consistent and significant increase in BRB permeability was observed (Fig. 8). These results agree with studies showing increased vascular permeability in non-barrier endothelium from Cav-1<sup>-/-</sup> mice (Schubert et al., 2002). In retinal wholemounts, where the entire retinal vasculature can be examined, *en face*, the location of leakage was most pronounced in large retinal veins (Fig. 8C). At the ultrastructural level, we observed extravasation of intravenously administered HRP only in Cav-1<sup>-/-</sup> vessels that stained the vascular basement membrane and the cytoplasm of Cav-1-deficient endothelium (Fig. 8D,E). In wildtype endothelium, numerous caveolae, completely devoid of HRP reaction

product (*arrowheads* in Fig. 8D), were apparent on the abluminal side of both the endothelium (“EC”) and pericyte (“P”) in wildtype mice and basement membranes were free of HRP reaction product. Although in some cases we observed HRP extravasation at junctional sites in Cav-1<sup>-/-</sup> vessels, no obvious changes in junction protein localization was observed. These findings suggest that under normal conditions caveolae do not participate in transcellular transport in the retinal vascular endothelium and, in fact, indicated that the absence of caveolae renders retinal vessels hyper-permeable.

Careful analysis of retinal veins revealed a significant increase in venous caliber (compare Fig. 8F to 8J for example) as well as phenotypic changes in venous smooth muscle cells. These include increased expression of alpha smooth muscle actin ( $\alpha$ SMA) (Fig. 8F–N) and a concomitant decreased expression of the mural cell marker, chondroitin sulfate proteoglycan-4 (also known as NG2) (Fig. 8F–N). These results imply that Cav-1 deficiency leads to phenotypic alterations in the contractile properties of venous smooth muscle cells. Currently, we favor the hypothesis that increased BRB permeability in Cav-1-deficient retinal vasculature results from reduced retinal blood flow resulting in venous stasis and subsequent passive leakage from pooled venous blood. The enlarged venous caliber may result from blood engorgement due to venous stasis and suggests that the increase in contractile phenotype of venous smooth muscle cells may be a compensatory response to venous engorgement. It is becoming increasingly apparent from work outside the eye that caveolae regulate resistance vessel autoregulation in part through regulation of eNOS activity (Murata et al., 2007; Yu et al., 2006). Loss of Cav-1 results in eNOS hyperactivity and consequent dilation of resistance vessels. Surprisingly, although eNOS hyperactivity is expected to reduce myogenic tone and, consequently, systemic blood pressure, the mean arterial pressure in Cav-1<sup>-/-</sup> mice is not different from controls (Rosengren et al., 2006). This apparent paradox is reconciled by the finding that small arterioles in Cav-1<sup>-/-</sup> mice are more responsive to adrenergic stimulation than controls which compensates for the reduced myogenic tone resulting from eNOS hyperactivity (Albinsson et al., 2007). In the retina, this presents a dilemma as retinal arterioles lack autonomic innervation and thus rely on intrinsic mechanisms such as myogenic autoregulation to regulate retinal blood flow (reviewed in (Kur et al., 2012)). Thus, the retinal vasculature is likely to be intrinsically more reliant on caveolae-regulated myogenic control and thus more sensitive to caveolae deficiency than systemic vasculature. This hypothesis is consistent with our current data and merits more attention given the association of *CAVI* polymorphisms with primary open angle glaucoma (POAG) and the potential of vascular tone dysregulation to play a role in POAG (Kang et al., 2014; Pasquale, 2016).

## 2.6. Caveolins/caveolae in choriocapillaris transport

The role of caveolae in transcellular transport across the choroidal vasculature has not been as well studied as that in the inner retinal vasculature. Although caveolae are observed in fenestrated vascular beds, including the choroid (Bernstein and Hollenberg, 1965; Gardiner and Archer, 1986b), caveolae are not necessary for fenestrae formation (Sorensson et al., 2002). An early HRP tracer study suggested that caveolae do not participate in transendothelial transport across the choroid and that such transport would be unnecessary due to the rapid exchange pathway provided by choroidal fenestrae (Gardiner and Archer,

1986b). However, while HRP, by virtue of its relatively small size (40 kDa, Einstein-Stokes radius of 30 Å), can readily pass through fenestrae of the choriocapillaris, larger molecules, such as albumin, hemoglobin, and lactoperoxidase are largely restricted from passage (Pino, 1985; Pino and Essner, 1981). Thus, a recent study from the Luty laboratory directly assessed the role of caveolae in regulated transendothelial transport of albumin across the choriocapillaris (Nakanishi et al., 2016). They found that the absence of caveolae in Cav-1-deficient mice dramatically reduced the transport of albumin into the outer retina. These results have implications for AMD as a previous quantitative mass spectrometric analysis revealed a significant reduction in both Cav-1 and PLVAP protein abundances in Bruch's membrane/choroid complexes from AMD donor eyes compared to age-matched controls (Yuan et al., 2010). Given the crucial role caveolae play in lipoprotein transport (Frank et al., 2009) and that lipoprotein deposits in the Bruch's membrane/choroid complex are a feature of AMD pathogenesis (Curcio et al., 2010), the role that caveolae transendothelial transport plays in AMD merits further study.

### 2.7. Caveolar transcytosis: A route for retinal drug delivery?

Delivery of systemically-administered drugs to central nervous system locations that reside behind a tight barrier remains a major pharmacological challenge. Given their role in endothelial transcytosis, caveolae represent attractive routes to bypass blood-CNS barriers. Groundbreaking work from Jan Schnitzer's laboratory demonstrated the power using caveolae for targeted delivery to the lung. They originally screened and identified candidate molecules (e.g., aminopeptidase-P) that were specific components of lung endothelial caveolae (Oh et al., 2004) and subsequently demonstrated rapid and lung-specific uptake of intravenously delivered antibody against aminopeptidase-P (Oh et al., 2007). Although a similar targeting strategy has not been used in the CNS, phosphodiesterase 5 (PDE5) inhibitors have been found to improve drug delivery to metastatic brain tumors, in part, via caveolae-mediated transport (Hu et al., 2010). Other mechanism to bypass the BRB have been tested, including transient disruption of endothelial tight junctions (Campbell et al., 2011; Campbell et al., 2009), but, to our knowledge, hijacking caveolae transcytosis has not yet been attempted. A potential hurdle to utilizing caveolae targeting in the retinal vasculature is the relatively low number of caveolae that face the luminal endothelium surface (Gardiner and Archer, 1986a; Raviola, 1977; Raviola and Butler, 1983; Sagaties et al., 1987). However, as the number of luminal endothelial caveolae in retinal vasculature may increase under pathological conditions (Hofman et al., 2000), targeting caveolae for ocular drug delivery may still be worth pursuing.

### 2.8. Caveolins/caveolae and retinal inflammation

As the first line of host defense, the innate immune system uses pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) present on the surfaces of foreign invaders, to elicit rapid host immune responses to resolve the invasion (Janeway and Medzhitov, 2002). One class of PRRs are Toll-like receptors (TLRs), which are transmembrane proteins with an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (Medzhitov, 2001). The TIR domain is also found in MyD88 and TIR domain-containing adaptor protein (TIRAP), which both function as adaptor proteins in TLR signaling pathways (Akira et al., 2001; Kawai and Akira, 2010;

Kumar et al., 2009; Medzhitov, 2001). Gram-negative bacterial lipopolysaccharide (LPS) is a classical PAMP that is recognized and bound by TLR4 to initiate inflammatory signaling (Beutler and Rietschel, 2003). The binding of TLR4 and LPS recruits MyD88 through the TIR domain to initiate signaling via the MyD88-dependent pathway, culminating in the early-phase activation of NF- $\kappa$ B (Akira et al., 2001; Medzhitov, 2001). Alternatively, the complex is endocytosed into endosomes where it triggers the TRIF-dependent pathway that signals to induce late-phase NF- $\kappa$ B activation (Akira et al., 2001; Kawai and Akira, 2010). NF- $\kappa$ B activation is required for the production of a battery of inflammatory cytokines (Akira et al., 2001; Beutler and Rietschel, 2003; Kawai and Akira, 2010; Kumar et al., 2009; Medzhitov, 2001). Notably, the TRIF-dependent pathway additionally activates IRF3 to induce type I interferon expression (Kawai and Akira, 2010).

There is a growing body of evidence that caveolins and caveolae can regulate innate immune responses (Chidlow and Sessa, 2010; Jin et al., 2011). Importantly, TLR4 (and other TLRs) contain a consensus Cav-1 binding motif in the TIR domain suggesting that Cav-1 could directly modulate TLR function (Wang et al., 2009). In the immune system, caveolins are widely expressed in myeloid cells and have been best studied in macrophages where they are important for monocyte/macrophage differentiation (Fu et al., 2012), inflammatory signaling (Tsai et al., 2011; Wang et al., 2009), and phagocytosis (Li et al., 2005). However, no simple picture has yet evolved to indicate whether caveolin expression generally promotes or suppresses inflammation.

Caveolins seem to exert cell-specific inflammatory regulatory properties best exemplified in several elegant studies of acute lung injury (reviewed in (Jin et al., 2011). In a model of pulmonary sepsis, Garrean and colleagues (Garrean et al., 2006) found that Cav-1-deficiency blunted the NF- $\kappa$ B response to systemic lipopolysaccharide (LPS) challenge. Cav-1<sup>-/-</sup> mice challenged with LPS showed attenuation of immune cell infiltration, microvascular leakage, and edema and had reduced LPS-induced mortality compared to controls. In lung alveolar cells, overexpression of Cav-1 aggravated LPS injury and increased the production of the TLR4-induced, pro-inflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF $\alpha$ ) providing positive evidence that Cav-1 promotes TLR4 signaling (Lv et al., 2010). Two non-exclusive mechanisms have been proposed to describe how Cav-1 promotes TLR4 signaling in pulmonary inflammation: (1) an indirect, nitric oxide-dependent mechanism whereby eNOS hyper-activity secondary to loss Cav-1 induces nitration of TLR4 signaling components and suppression of downstream signaling (Mirza et al., 2010); (2) a direct effect whereby tyrosine phosphorylated Cav-1 interacts with TLR4 and promotes the assembly of the TLR4-MyD88 signaling complex (Jiao et al., 2013). Surprisingly, in RAW264.7 macrophages, Cav-1 seems to have an opposite effect on TLR4 signaling where it acts as a pro-inflammatory effector in immune cells (Wang et al., 2009). This effect occurs through the direct interaction of Cav-1 with the TIR domain which blocks the formation of the TLR4-MyD88 complex. Thus, the combined effect of anti-inflammatory action in tissue and pro-inflammatory effects in immune cells clearly illustrates the importance of understanding caveolin/caveolae inflammatory regulation in cell-specific contexts to determine if Cav-1 might represent a therapeutic target for inflammatory disease.



In 2010, Hauck and colleagues published results of an elegant quantitative proteomic screen of differentially-expressed retinal membrane proteins in equine autoimmune uveitis (Hauck et al., 2010). Cav-1 was found to be upregulated by a remarkable 16-fold in uveitic versus healthy retinas. The uveitis-induced upregulation was found predominantly in Müller glial cells which may, in part, reflect their relatively high levels of Cav-1 expression and their abundance in the retina (Reagan et al., 2016). The expression of PTRF/cavin-1, which is necessary along with Cav-1 to build caveolae (Hill et al., 2008), is dramatically increased in uveitis. This suggests that Müller cells may acquire the ability to build caveolae under pathological conditions but this remains to be determined. The dramatic upregulation of caveolae components in uveitis implies that retina-intrinsic Cav-1 may play a role in chronic inflammation in the retina.

Given the results in the lung inflammation model and the intriguing suggestion that Cav-1 may be induced during retinal inflammation, we set out to interrogate the Cav-1-TLR4 axis in the endotoxin-induced uveitis model (Li et al., 2014). Similar to the pulmonary sepsis model, we found that global Cav-1 deficiency significantly blunted the production of pro-inflammatory cytokines in the retina after intraocular delivery of LPS (Fig. 9A). In naïve, uninjected eyes, BRB permeability was increased in Cav-1<sup>-/-</sup> eyes in agreement with previous results (Gu et al., 2014a). However, while delivery of LPS to wildtype eyes induced dramatically increased permeability, this response was largely blunted in Cav-1<sup>-/-</sup> mice (Fig. 9B). Thus, the presence of Cav-1 promotes inflammatory signaling and pathological BRB breakdown, *in vivo*. It is unclear if the reduction in BRB permeability results from inflammatory suppression or from the lack of ability to upregulate caveolae transcytosis under inflammatory conditions (Knowland et al., 2014). To our surprise, we observed a paradoxical increase in recruitment of innate immune cells to the retina even in the context of significant suppression of pro-inflammatory cytokine production (Fig. 9C,D). The explanation behind this paradox is not yet understood but we speculate that the pro-inflammatory effects of Cav-1 deficiency in immune cells (Wang et al., 2009) and systemic immune dysfunction may contribute. This is supported by increased numbers of circulating immune cells in Cav-1<sup>-/-</sup> mice even without LPS challenge (Fu et al., 2012; Li et al., 2014). Alternatively, venous stasis or vascular endothelial dysfunction from Cav-1 ablation may allow more efficient immune cell extravasation even in the context of the blunted cytokine response. Understanding this paradox is key to the development of rational, Cav-1-targeting therapeutics to modulate local immune responses.

The suppression of local cytokine production that results from Cav-1 deficiency could also impact retinal neuroprotection. Interleukin-6 family cytokines (e.g., ciliary neurotrophic and leukemia inhibitory factors) are potent neuroprotective cytokines in the retina that mediate protection through the gp130/STAT3 signaling pathway (Ueki et al., 2009; Wen et al., 2012). Retina-specific Cav-1 deficiency results in suppression of STAT3 phosphorylation following retinal injury (Reagan et al., 2016). The blunted STAT3 response likely results from suppressed IL-6 family cytokine production rather than an effect of Cav-1 on the gp130/STAT3 pathway as STAT3 activation in response to exogenous IL-6 family cytokine stimulation is not affected by Cav-1 ablation. This highlights the possibility that endogenous PAMPs produced during retinal injury may activate TLR4 in the absence of pathogen and that Cav-1 may modulate this response.

The retinal cell type(s) in which Cav-1 might modulate TLR4 signaling are not yet identified. Likely cellular candidates include microglia, astrocytes, Müller glia, and RPE, all of which are reported to express TLR4 (Jiang et al., 2009; Karlstetter et al., 2015; Kindzelskii et al., 2004; Kumar and Shamsuddin, 2012). Although TLR4 is expressed in retinal astrocytes and microglia, we have not observed detectable Cav-1 immunoreactivity in either cell type in retinal flatmounts even following LPS activation (unpublished observation). Thus, at present we favor Müller glia and/or RPE, both of which express both Cav-1 and TLR4, as the cell types in which Cav-1 may modulate TLR4 signaling. Our newly developed tissue-specific conditional Cav-1<sup>-/-</sup> mice should help us to answer this question in future studies.

As demonstrated in the previous sections, caveolins and caveolae make several important contributions to retinal inflammatory signaling and BRB maintenance under both normal and inflammatory conditions. As shown in the model in Fig. 10, under normal conditions caveolae are predominantly localized to the abluminal side in both vascular endothelium and pericytes/smooth muscle cells and Müller glia are decorated with extra-caveolar caveolin-1. Under these conditions, caveolae do not contribute significantly to transendothelial transport and the BRB is essentially impermeable. Upon inflammation (e.g., diabetic insult, uveitis, ischemic injury), caveolin-1 expression is increased in all of the cell types of the neurovascular unit, caveolae numbers increase and their localization in vascular cells becomes more bipolar. The permeability of the BRB increases via both transcellular and paracellular pathways and the Müller glia with increased caveolin-1 expression can potentiate TLR signaling and pro-inflammatory cytokine release.

### **3. Caveolins/caveolae in primary open angle glaucoma (POAG) and intraocular pressure (IOP)**

#### **3.1. Introduction to POAG, IOP regulation, and aqueous outflow**

There are several excellent recent reviews on glaucoma risk factors, genes, and IOP control and the interested reader is pointed to these (Janssen et al., 2013; Nickells et al., 2012; Stamer et al., 2015; Wang and Wiggs, 2014). In this review will briefly introduce a few important concepts before focusing on recent evidence of the genetic and functional links of caveolins/caveolae to POAG. Glaucoma is a complex group of diseases, with POAG being the most prevalent, that is a major cause of blindness (Quigley and Broman, 2006) resulting from progressive damage to the optic nerve and loss of retinal ganglion cells (reviewed in (Nickells et al., 2012)). Although the vision-compromising insult impacts retinal neurons, the primary risk factor for POAG is IOP (Janssen et al., 2013) and lowering IOP is currently the primary therapeutic strategy even in the case of normal tension glaucoma (Anderson and Study, 2003).

IOP is maintained by the balanced production of aqueous humor from the ciliary body and its drainage from the eye by two pathways, the conventional and uveoscleral outflow tracts (Tamm et al., 2015). The conventional outflow pathway consists of the trabecular meshwork (TM) and a unique drainage vessel called the Schlemm's canal (SC) (Kizhatil et al., 2014; Tamm, 2009). The conventional outflow pathway is pressure-dependent and thus maintains

IOP in a normal range by varying its resistance to aqueous outflow in response to IOP fluctuations. Conventional outflow is the major pathway for normal drainage and is the pathological site of ocular hypertension in POAG making it a key target for the development of IOP-lowering therapeutics (Stamer and Acott, 2012). The TM is anatomically organized into three regions: the innermost uveal meshwork (close to the anterior chamber), the middle corneoscleral meshwork and the deep juxtacanalicular connective tissue (JCT; adjacent to the endothelial lining of Schlemm's canal). Unlike the uveal and corneoscleral regions that are formed of regular trabecular lamellae, the JCT is a loose connective tissue with scattered cells surrounded by irregular fibrillar extracellular matrix (Tamm, 2009; Tian et al., 2000). Given that the uveal and corneoscleral tissues are highly porous (thus providing little resistance to aqueous humor outflow), it is thought that IOP originates from resistance at the JCT and/or the endothelial lining of SC (Overby et al., 2009; Tamm, 2009). A unifying hypothesis for how this region of tissue generates resistance has been proposed that takes into account fluid dynamics, cellular organization and biomechanics (Overby et al., 2009). The inner wall of the SC (the wall closest to the anterior chamber) and the JCT are subject to large and fluctuating mechanical loads and must have mechanisms to withstand these loads and alter outflow resistance to maintain IOP. Likely as a result of the large gradient associated with aqueous outflow, the inner endothelial lining of the SC can generate large (0.1 to 2  $\mu\text{m}$ ) giant vacuoles in which the SC endothelium is stretched thin and frequently forms pores that allow aqueous humor egress (Overby et al., 2014). Pore density is lower in glaucoma patients (Johnson et al., 2002) and the dynamic process of pore formation is impaired in glaucomatous SC cells (Overby et al., 2014). Pathological outflow resistance in POAG is also likely the result of extracellular matrix remodeling in the TM (recently reviewed in (Vranka et al., 2015)).

### 3.2. Genetic studies associating *CAV1/2* loci with POAG and IOP

Among the multifactorial risk factors for POAG there are clear genetic risk variants. Genome-wide association studies (GWAS) by genotyping single nucleotide polymorphisms (SNPs) have emerged as powerful, efficient, unbiased tools to identify disease-associated genes in complex diseases, such as POAG (Fan et al., 2006; Hirschhorn and Daly, 2005). Using this method, Thorleifsson et al. (2010), located a common risk variant (rs4236601) for POAG to chromosome 7q31, near a region that encodes *CAV1* and *CAV2* (Thorleifsson et al., 2010). This association was originally made in a large cohort from Iceland and confirmed in additional cohorts of POAG patients and controls from Sweden, United Kingdom, Australia, and China (Thorleifsson et al., 2010). The association of rs4236601 with POAG has been replicated in several subsequent studies and new SNPs in the same genetic region have also been associated (Huang et al., 2014; Loomis et al., 2014; Rong et al., 2016; Wiggs et al., 2011). In addition, several of these *CAV1/2* POAG-associated SNPs have been recently associated with IOP (Chen et al., 2015; Hysi et al., 2014; Kim et al., 2015; Ozel et al., 2014; van Koolwijk et al., 2012). Although replicated in multiple populations, associations of the *CAV1/2* loci with POAG have not been found in all populations (Abu-Amero et al., 2012; Burdon et al., 2015; Kuehn et al., 2011; Liu et al., 2013; Williams et al., 2015).

Vascular tone is an additional contributor to POAG risk and impaired autoregulation and blood flow has been reported (reviewed in (Flammer et al., 2002; Hayreh, 2001; Pasquale, 2016). Genes (including *CAVI*) involved in local control of vascular tone are more strongly associated with POAG with early paracentral visual field loss (Kang et al., 2014; Loomis et al., 2014). Patients with this POAG subtype are more likely to have systemic vascular dysfunction (Park et al., 2012). The association of this subtype of glaucoma with *CAVI* polymorphisms is interesting given the retinal vascular phenotypes observed in *Cav-1<sup>-/-</sup>* mice (Gu et al., 2014a) which are suggestive of defects in local autoregulation. Furthermore, *Cav-1<sup>-/-</sup>* mice display systemic vascular dysfunction resulting in pulmonary hypertension and heart failure (Cohen et al., 2003; Zhao et al., 2002).

### 3.3. The role of caveolae in IOP maintenance and aqueous humor outflow

Caveolae are abundant features of the SC endothelium and TM (Tamm, 2009). Caveolins are also expressed in the human and murine outflow pathway, *in situ*, and in outflow pathway cell in culture (Aga et al., 2014; Elliott et al., 2016; Gonzalez et al., 2000; Kuehn et al., 2011; Surgucheva and Surguchov, 2011) In fact, treatments of TM cells with dexamethasone or soluble CD44 induce expression (Clark et al., 2013) or phosphorylation (Giovingo et al., 2013) of Cav-1, respectively. Recently, Aga et al. (2014) transiently silenced Cav-1 and Cav-2 in *ex vivo* anterior segment organ cultures and in TM cells and found that silencing *CAVI* increased outflow facility (aqueous humor drainage) while silencing *CAV2* reduced facility. These authors also found overall increased matrix turnover in Cav-1/Cav-2 silenced TM cells (Aga et al., 2014). In contrast, in Cav-1-deficient mice which chronically lack caveolae and have reduced expression of Cav-2, we observed significantly *increased* IOP and significantly *reduced* outflow facility (Elliott et al., 2016). The John laboratory has found similarly increased IOP and reduced aqueous humor drainage in Cav-1<sup>-/-</sup> mice (Dr. Simon John, The Jackson Laboratory, *personal communication*) and a recent study reported similar findings (Lei et al., 2016). The apparent conflict between results from Cav-1<sup>-/-</sup> mice and those of Aga et al. are likely due to differences between chronic caveolae deficiency and transient silencing. Transient loss of caveolins/caveolae may uncover immediate cellular responses such as rapid hyperactivation of eNOS that would be expected to transiently increase aqueous drainage as eNOS activity lowers IOP and increases outflow facility (Chang et al., 2015; Stamer et al., 2011). Chronic Cav-1/caveolae loss may lead to the development of outflow pathway dysfunction and our recent results imply that caveolae deficiency renders the conventional outflow tract more susceptible to IOP-induced injury (Elliott et al., 2016). Furthermore, Lei et al. (Lei et al., 2016) propose that eNOS hyperactivity resulting from Cav-1 ablation may lead to nitration of outflow tract proteins. Collectively, these results indicate that caveolins/caveolae play important roles in IOP maintenance and, specifically, in aqueous humor outflow.

Mechanistically, what Cav-1-dependent actions might be at play to explain the ocular hypertension and reduced outflow resulting from Cav-1 deficiency? In cells in which they are abundant, caveolae act as mechanosensors and membrane reservoirs that can donate membrane to buffer mechanical perturbation and provide mechanoprotection (Albinsson et al., 2008; Cheng et al., 2015; Dulhunty and Franzini-Armstrong, 1975; Joshi et al., 2012; Lo et al., 2015; Prescott and Brightman, 1976; Sinha et al., 2011; Yu et al., 2006). Reduced

IOP-dependent outflow observed in Cav-1<sup>-/-</sup> eyes indicates that caveolae influence the ability of the outflow tissue to respond to IOP fluctuations. It is also possible that the observed functional defects in the SC and TM result as a consequence of the inability of caveolae-deficient tissue to withstand chronic fluctuations in mechanical load. This hypothesis remains to be tested rigorously in future experiments but recent results suggest that caveolae participate in buffering mechanical perturbations of outflow pathway cells.

## 4. Caveolins/caveolae in other ocular cells

### 4.1. Caveolae functions in the cornea

To date, caveolins and caveolae have received less attention in the cornea. The limited literature on corneal caveolins has focused in two main areas: epithelial wound healing and pathogen uptake/inflammatory modulation. Caveolin-1 is normally expressed weakly in the basal corneal epithelium and more strongly in the endothelium (Amino et al., 1997). Although not normally abundant in corneal keratocytes in the stroma, caveolae seem to be induced in corneal fibroblasts and myofibroblasts suggesting that they may be upregulated in stromal injury (Guo et al., 2007; Karamichos et al., 2011). In the corneal epithelium, Cav-1 may play a crucial role in re-epithelialization following injury. An early study demonstrated that the caveolar localization of a plasmalemmal calcium pump is reduced in regenerating epithelium indicating that caveolae composition is modified during epithelial wound healing (Amino et al., 1997). In human corneal epithelial cells, Cav-1 expression increases with age and this increased expression is negatively-associated with wound healing capacity (Rhim et al., 2010). While this association does not indicate causation, work from our laboratory (Griffith GL et al., IOVS 2014; 55: ARVO E-Abstract 4698) indicates that Cav-1 deficiency does, in fact, accelerate re-epithelialization in agreement with the original correlational study (Rhim et al., 2010). In fact, the faster re-epithelialization results, at least in part, from increased proliferation of Cav-1-deficient corneal epithelium. We also found that healing could be accelerated in wildtype mice by transiently silencing Cav-1 via RNA interference (RNAi). These results highlight the exciting possibility that Cav-1 could be targeted therapeutically to accelerate wound healing.

A small but growing body of evidence indicates that caveolae are also involved in pathogen uptake and inflammatory responses at the ocular surface. Recent work indicates that adenoviruses utilize a caveolae-mediated pathway for entry into corneal cells (Mukherjee et al., 2015; Yousuf et al., 2013). Importantly, disruption of caveolae and cholesterol-rich membrane domains by cholesterol depletion dramatically reduced viral infection (Yousuf et al., 2013). Studies designed to examine viral-mediated gene transfer to the corneal epithelium revealed improved transduction efficiency of recombinant adenovirus to the corneal epithelium by the PDE5 inhibitor, sildenafil, which acted by redistributing caveolin-1 expression from the normal basal epithelial location to suprabasal epithelium thus facilitating viral uptake (Saghizadeh et al., 2010). Sequestration of cholesterol by filipin abrogated the effect of sildenafil on viral transduction. Collectively, these results demonstrate that both pathological adenovirus infection and adenoviral gene transfer utilize caveolae to infect the corneal epithelium. In addition to viral pathogens, infection of the corneal epithelium with *Pseudomonas aeruginosa* drives inflammatory signaling via

macrophage migration inhibitory factor (MIF) by assembling caveolin-1-rich signaling platforms (Reidy et al., 2013). The *Pseudomonas aeruginosa* and MIF-dependent activation of the pro-inflammatory cytokine, interleukin-8, was suppressed by silencing of Cav-1. This finding agrees with studies in the retina (Li et al., 2014) and lung (Garrean et al., 2006) suggesting that Cav-1 promotes pro-inflammatory cytokine production and highlights the potential of targeting Cav-1 to reduce inflammation in the eye.

Caveolin-1 plays an important role in modulating angiogenic signaling. As indicated previously, VEGFR-2 is localized to caveolae (Feng et al., 1999a; Labrecque et al., 2003). Outside of the eye, studies on the effect of caveolae on angiogenesis have been confounding with some studies suggesting that Cav-1 promotes angiogenesis (Chang et al., 2009; Sonveaux et al., 2004; Woodman et al., 2003) while others indicate anti-angiogenic activity (Bauer et al., 2005; Lin et al., 2007). A study using the corneal VEGF pocket assay in Cav-1<sup>-/-</sup> mice showed that Cav-1 expression promotes VEGF-induced corneal angiogenesis (Morais et al., 2012). In these studies, Cav-1<sup>-/-</sup> mice had a significantly blunted angiogenic response to VEGF. In agreement with this work in cornea, another study silenced Cav-1 expression with inhibitory RNAs delivered into the vitreous and showed that both vascular permeability and pathological angiogenesis were suppressed in retinas in the oxygen-induced retinopathy model (Tian et al., 2012). Our work also suggests that inflammation-induced retinal vascular permeability is blunted by global Cav-1 deletion (Li et al., 2014). However, as noted previously, Cav-1 deficiency promotes a modest increase in basal blood-retinal barrier permeability (Gu et al., 2014a). Although the mechanism by which Cav-1 inhibition suppresses pathological angiogenic responses is unknown, additional studies are warranted to assess the anti-angiogenic potential of Cav-1 inhibition in the cornea and retina.

#### 4.2. Caveolae/caveolins in the lens

Like the corneal epithelium and RPE both morphological caveolae and Cav-1 have been found in the lens epithelium and lens fiber cells (Cenedella et al., 2006; Lo et al., 2004; Rujoi et al., 2003; Sexton et al., 2004). In the lens epithelium, caveolae have been implicated in the transcytosis of albumin (Sabah et al., 2007) and in the structure and function of lens epithelial gap junctions (Lin et al., 2004; Lin et al., 2003). In lens fiber cells, Cav-1 may regulate aquaporin-0 membrane particles to form square array junctions (Biswas et al., 2014). In Cav-1<sup>-/-</sup> lenses, fiber cells form abnormally large square array junctions possibly due to defective cholesterol trafficking as a result of Cav-1 deficiency. However, these dramatic fiber cell alterations do not result in induction of lens opacity and Cav-1<sup>-/-</sup> lenses retain their transparency. Thus, although Cav-1 is expressed in lens cells its function has yet to be completely elucidated.

### 5. Conclusions and future directions

Since their discovery in the 1950s, caveolae and their associated molecular components have received extensive attention outside of the eye (reviewed in (Cheng and Nichols, 2016; Chidlow and Sessa, 2010; Cohen et al., 2004; Echarri and Del Pozo, 2015; Nassoy and Lamaze, 2012; Parton and del Pozo, 2013; Parton and Simons, 2007; Sotgia et al., 2012). Given the expression of caveolins in important ocular cell types and the myriad functional



tests to assess visual system function, the eye provides a unique model system to advance our basic understanding of these membrane domains. Furthermore, the association of polymorphisms in the *CAVI2* gene loci with POAG highlights the clinical importance of understanding caveolin/caveolae function in the aqueous outflow pathway, retina, and optic nerve. With the advent of new mouse models to conditionally manipulate the *Cav1* gene using Cre/lox technology (Reagan et al., 2016; Sethna et al., 2016), there are now unique opportunities to study cell-specific caveolin/caveolae functions, *in vivo*. As caveolae domains provide a nexus for a variety of signaling pathways relevant to ocular physiology and pathophysiology (e.g., mechanotransduction, VEGF signaling, G-protein signaling), the understanding of caveolin function in ocular contexts provides an opportunity to develop novel therapeutic strategies to modulate IOP, retinal vascular permeability, neuroinflammation, and corneal infection and injury.

The cell biology community has focused recently on the function of caveolae in mechanotransduction and mechanoprotection as growing evidence indicates that these domains can directly induce cellular responses to mechanical perturbations (reviewed recently in (Cheng and Nichols, 2016; Echarri and Del Pozo, 2015; Nassoy and Lamaze, 2012; Parton and del Pozo, 2013)). Cellular responses to biomechanical fluctuations are likely key to our understanding of glaucoma-relevant processes such as IOP regulation by the conventional aqueous outflow pathway and the protection of the optic nerve head at the lamina cribrosa. Caveolae-dependent, flow-mediated activation of eNOS, a well-studied mechanism to modulate vascular autoregulation and vascular remodeling (Yu et al., 2006), has potentially profound relevance to ocular blood flow and BRB permeability. Much research has focused on the maintenance of BRB integrity via regulation of paracellular, junctional permeability (reviewed in (Antonetti et al., 2012; Kaur et al., 2008; Runkle and Antonetti, 2011)) but much less is known about the role of caveolar, transcellular permeability in the context of retinal vascular disease (Klaassen et al., 2013). Historical evidence that there are fewer caveolae in barrier-forming endothelium of the retina and brain compared to more permeable non-barrier endothelia (Sagaties et al., 1987; Stewart and Tuor, 1994) and that caveolae numbers and caveolin expression positively correlate with increased pathological BRB permeability (Feng et al., 1999a; Hofman et al., 2000; Klaassen et al., 2009; Stitt et al., 2000a) suggest that upregulation of transcellular permeability via caveolar vesicles contribute to pathological BRB breakdown. The concept that caveolae contribute to basal transcellular permeability in the barrier-forming retinal endothelium is controversial (Gardiner and Archer, 1986a, b). However, it remains an open question if caveolae transcytosis plays a role in pathological BRB breakdown that deserves careful study. Finally, caveolae seem to play important roles at the ocular surface both in pathogen uptake (Mukherjee et al., 2015; Yousuf et al., 2013), corneal inflammation (Reidy et al., 2013), and wound healing (Amino et al., 1997; Rhim et al., 2010) but these areas are yet to receive further attention.

### 5.1. Therapeutic potential of targeting caveolins/caveolae

As highlighted throughout this review, caveolins, by virtue of their ability to modulate myriad cellular responses in cell-specific contexts, represent challenging but intriguing therapeutic targets for a variety of ocular disorders. On one hand, because of their ability to

modulate the activity of multiple signaling pathways, targeting caveolae to suppress inflammation and vascular dysfunction could provide broader therapeutic efficacy than targeting individual signaling pathways. On the other hand, this comprehensive targeting approach has the potential to cause unwanted side effects if critical caveolae functions are affected. The fact that global ablation of caveolae either by deletion of caveolins or PTRF/cavin-1 does not induce lethality in mice (although it is acknowledged that the health of such animals is compromised) highlights the potential of caveolae targeting as a rational therapeutic intervention. Most current efficacious therapies (e.g., steroids, anti-VEGF, anti-ocular hypertensives) are not without potential side effects but the ability to target delivery and dosages for the ocular environment (topical or intraocular administration) make the eye an ideal organ to assess therapeutic benefit while reducing risk of systemic side effects. Furthermore, several experimental results have suggested the possibility of targeting pathological but not basal caveolae functions. Thus, we conclude this review by highlighting potential means to manipulate caveolae functions for therapeutic benefit.

There are several potential strategies available to inhibit caveolins/caveolae functions. There has been significant work on *in vivo* delivery of Cav-1 silencing RNAs to target lung vasculature (Sparks et al., 2012). Silencing of Cav-1 expression in lungs has been shown to paradoxically suppress inflammation-induced permeability (Hu et al., 2008) while increasing basal permeability (Miyawaki-Shimizu et al., 2006) as we have previously observed in retinas from global Cav-1 knockout mice (Gu et al., 2014a; Li et al., 2014). Silencing of Cav-1 expression by intravitreal delivery of inhibitory RNAs can suppress angiogenesis and vascular permeability induced by the oxygen-induced retinopathy model (Tian et al., 2012). Besides targeting caveolin expression by RNAi, a recent study showed that suppressing expression of PLVAP significantly reduced VEGF-induced upregulation of caveolae and reduced BRB leakage (Wisniewska-Kruk et al., 2016). Thus, silencing expression of caveolae components by inhibitory RNAs could target inflammation and pathological BRB breakdown.

In addition to RNAi-based approaches, several plant-derived polyphenolic compounds (e.g., daidzein, quercetin, and green tea polyphenols) can suppress Cav-1 expression in non-ocular cells (Arya et al., 2011; Choi et al., 2010; Dasari et al., 2006; Kamada et al., 2016; Li et al., 2009; Sharma et al., 2012; Sobey et al., 2004; Woodman et al., 2004). Quercetin also suppresses oxidant-induced expression of Cav-1 in RPE cells and protects them from oxidative stress (Kook et al., 2008). Intriguingly, quercetin has also recently been shown to reduce fibrotic changes in human corneal fibroblasts, *in vitro* (McKay et al., 2015). Whether the anti-inflammatory, anti-oxidant, and anti-fibrotic properties of polyphenolic compounds are mediated through suppression of caveolin expression remains to be determined. Furthermore, the mechanism by which these polyphenolic compounds suppress caveolin expression has not been fully established. It is clear that the *CAVI* gene has two oxidative-stress response elements that bind the transcription factor, Sp1 and that quercetin blocks oxidant-induced Cav-1 expression (Dasari et al., 2006). These results suggest that possibility that polyphenolic compounds could specifically target Cav-1 upregulation in response to pathologic stimulation without affecting basal Cav-1 expression levels. Thus, a clearer understanding of the mechanism(s) by which such compounds modulate Cav-1 expression could lead to the development of more specific therapeutics to manipulate caveolins.

Downregulating the expression of caveolae components may be beneficial under some circumstances (e.g., inflammation and inflammatory vascular barrier breakdown, corneal re-epithelialization). However, under some conditions it may be important to therapeutically augment caveolin expression. For example, Cav-1 expression in the brain declines with age and neuron-targeted re-expression promotes neuronal structural and functional improvement (Head et al., 2011; Head et al., 2010; Mandyam et al., 2015). Furthermore, there is a significant body of evidence that caveolins are necessary for ischemic- and anesthetic-induced preconditioning in the heart and brain (reviewed in (Schilling et al., 2015; Sary et al., 2012)). As preconditioning by light and ischemia can induce endogenous neuroprotection in the retina (Caprara and Grimm, 2012; Kast et al., 2016; Liu et al., 1998; Ueki et al., 2009), determining whether the mechanisms of retinal preconditioning require caveolins is warranted. Strategies to induce expression of caveolins are less well-understood and most experimental approaches have relied on transgenic overexpression (e.g., (Head et al., 2011; Murata et al., 2007)). One notable exception is evidence that Cav-1 expression is induced by peroxisome proliferator-activated receptor-gamma (PPAR $\gamma$ ) (Chen et al., 2010). Cav-1 expression is induced by thiazolidinediones such as rosiglitazone, a PPAR $\gamma$  agonist used to treat type 2 diabetes (Llaverias et al., 2004; Tencer et al., 2008). In the retina, these compounds suppress retinal and choroidal neovascularization (Higuchi et al., 2010; Murata et al., 2001; Murata et al., 2000), reduce diabetes-induced retinal vascular inflammation and BRB breakdown (Muranaka et al., 2006), and promote neuroprotection (Doonan et al., 2009; Li et al., 2011; Normando et al., 2016). It remains to be determined whether these effects are mediated through Cav-1 expression in the retina.

The approaches described above focus on methods to manipulate the expression of caveolae components but another potential therapeutic strategy is to manipulate caveolae signaling via peptides based on caveolin protein sequences. The Sessa laboratory at Yale University has developed a compelling rationale for the use of peptide sequences derived from the caveolin scaffolding domain (CSD) to suppress inflammation, inhibit or activate eNOS, and block fibrosis. In their early studies, they developed a chimeric peptide containing the CSD sequence fused to a cell-permeable, “Trojan Horse” peptide (called “cavtratin”), that could be delivered, *in vivo*, to suppress inflammation and vascular permeability predominantly through its ability to inhibit eNOS (Bucci et al., 2000; Gratton et al., 2003). Through mutational analysis of the CSD they were able to define the essential amino acid, phenylalanine-92 (F92), that mediates eNOS inhibition (Bernatchez et al., 2005) and subsequently to generate a F92A mutant peptide (called “cavnoxin”) that activates eNOS (Bernatchez et al., 2011). It remains to be determined whether CSD-derived peptides could be beneficial in suppressing inflammation and vascular hyperpermeability in the eye. Furthermore, given the potential of nitric oxide to facilitate aqueous humor outflow and reduce IOP (Chang et al., 2015; Stamer et al., 2011), there may be therapeutic potential to activating endogenous eNOS activity via delivery of cavnoxin to the outflow tract.

There is a large body of literature describing caveolin functions and the potential of caveolae-targeted therapies outside of the eye but much less is known in eye-specific contexts. In this review, we have provided comprehensive coverage of this topic to facilitate further research in the hopes of taking advantage of diverse caveolae functions to identify novel therapeutics for ocular disorders.

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## Abbreviations

<b>AMD</b>	age-related macular degeneration
<b><math>\alpha</math>SMA</b>	alpha smooth muscle actin
<b>BRB</b>	blood-retinal barrier
<b>Cav-1/<i>CAVI</i></b>	caveolin-1
<b>CSD</b>	caveolin scaffolding domain
<b>eNOS</b>	endothelial nitric oxide synthase
<b>ERG</b>	electroretinogram/electroretinography
<b>GFAP</b>	glial fibrillary acidic protein
<b>GWAS</b>	genome-wide association studies
<b>HRP</b>	horseradish peroxidase
<b>IL</b>	interleukin
<b>LPS</b>	lipopolysaccharide
<b>Na/K-ATPase</b>	alpha-1 sodium/potassium ATPase
<b>NG2</b>	chondroitin sulfate proteoglycan-4
<b>PAMPs</b>	pathogen-associated molecular patterns
<b>PDE5</b>	phosphodiesterase 5
<b>PLVAP</b>	plasmalemmal vesicle-associated protein
<b>POAG</b>	primary open angle glaucoma
<b>PRRs</b>	pattern recognition receptors
<b>POS</b>	photoreceptor outer segment
<b>PTRF</b>	polymerase I and transcript release factor
<b>RNAi</b>	RNA interference

<b>RPE</b>	retinal pigment epithelium
<b>SC</b>	Schlemm's canal
<b>SNPs</b>	single nucleotide polymorphisms
<b>STZ</b>	streptozotocin
<b>TLRs</b>	Toll-like receptors
<b>TM</b>	trabecular meshwork
<b>VEGF</b>	vascular endothelial growth factor
<b>VEGFR-2</b>	vascular endothelial growth factor receptor-2

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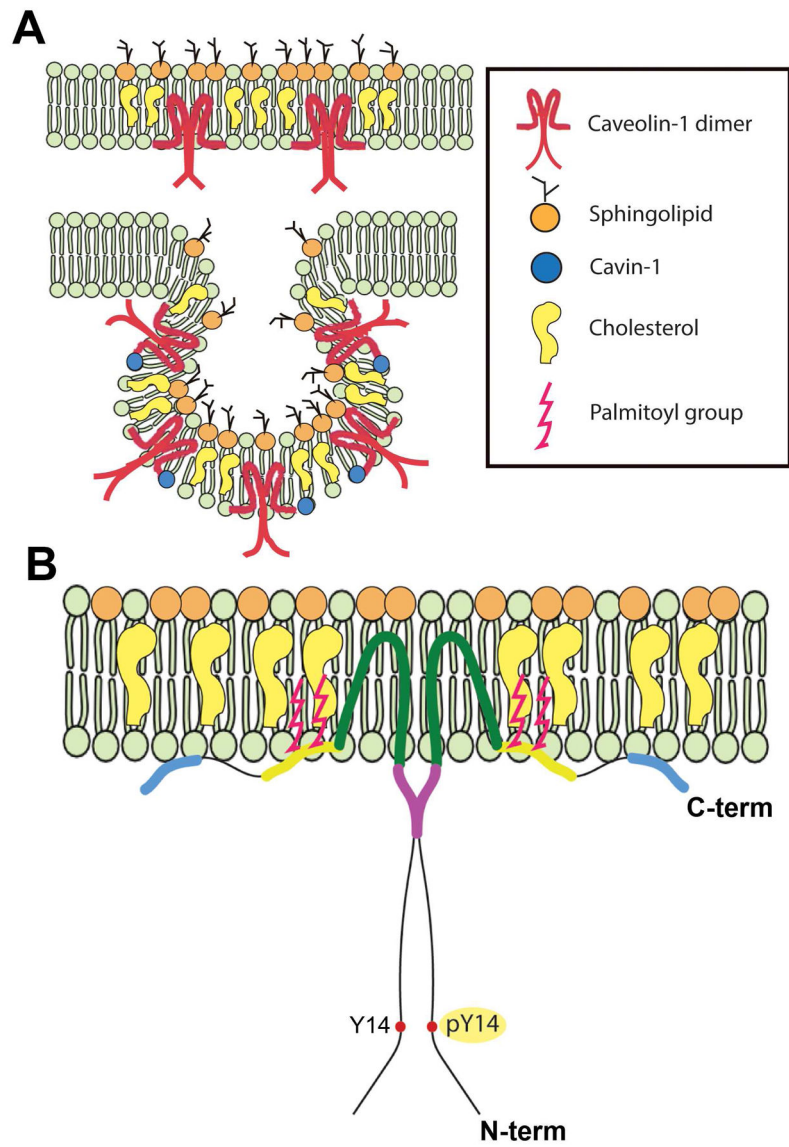
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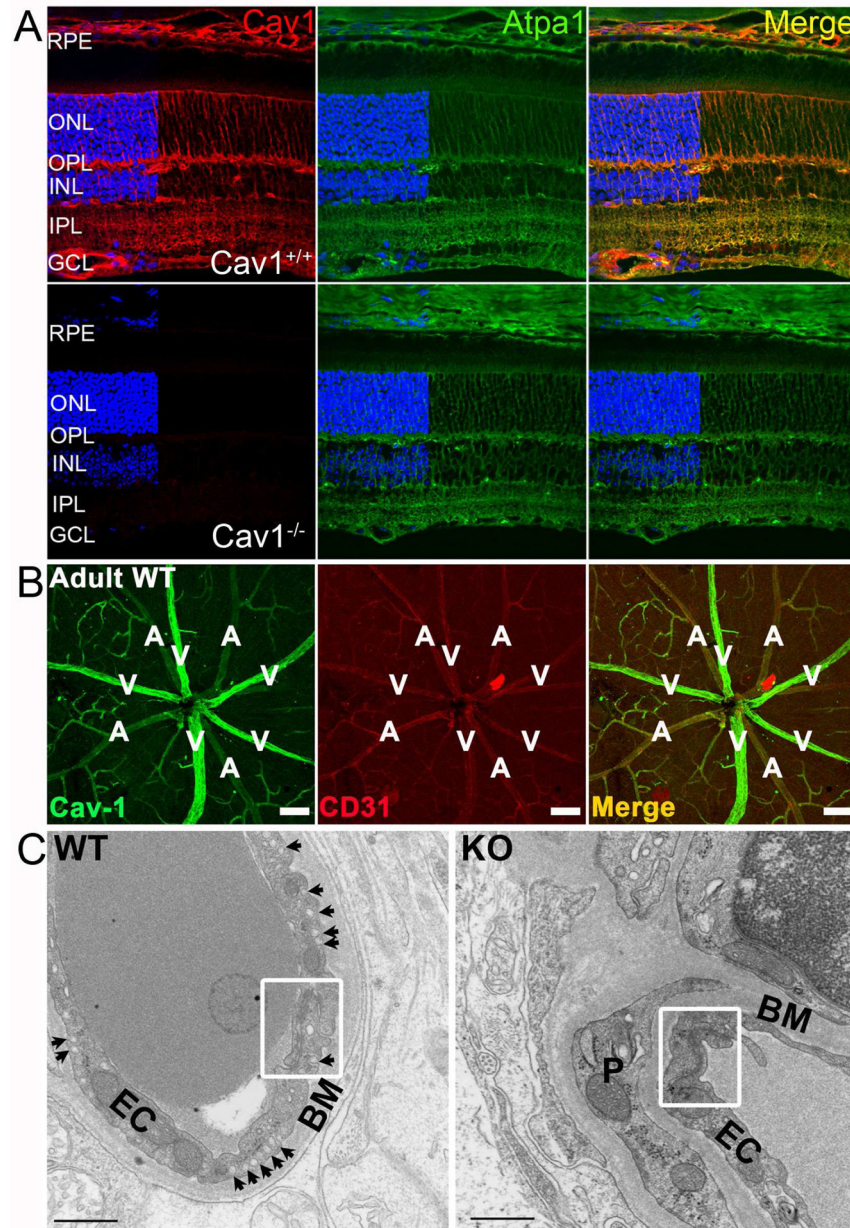
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**Fig. 1.** Caveolae membrane domains and the signature Cav-1 protein. (A) Illustrations of a planar Cav-1-enriched membrane microdomains with “extracaveolar” caveolin-1 and a typical caveola. (B) Model of the insertion of a caveolin-1 homodimer with the CSD (purple) and the Tyr-14 which can be phosphorylated by several tyrosine kinases.





**Fig. 2.** Localization of caveolin-1 and caveolae in the murine retina. (A) Retinal cross-sections from *Cav1*<sup>+/+</sup> (upper panel) and *Cav1*<sup>-/-</sup> mice (lower panel) stained for Cav-1 (red) and the Na/K-ATPase. Cav-1 immunoreactivity is predominantly localized to Müller glia, retinal and choroidal vasculature, and to the RPE (modified from (Li et al., 2012)). (B) Vascular localization of Cav-1 (green) and CD31 (red) revealed in retinal whole mount. Cav-1 is detected throughout the retinal vasculature with enhanced immunoreactivity in large retinal veins. Scale bar = 100 μm; “A”, artery; “V”, vein. (C) Ultrastructure of *Cav-1*<sup>+/+</sup> and *Cav-1*<sup>-/-</sup> retinal vessels. Note the numerous abluminal caveolae (arrows in the left panel) and the absence of caveolae in *Cav-1*<sup>-/-</sup> vessel. Well-developed tight junctions (white boxes)

are visible in both genotypes. BM, basement membrane; EC, endothelial cell; P, pericyte. Scale bar = 500 nm. Panels B and C from (Gu et al., 2014a).

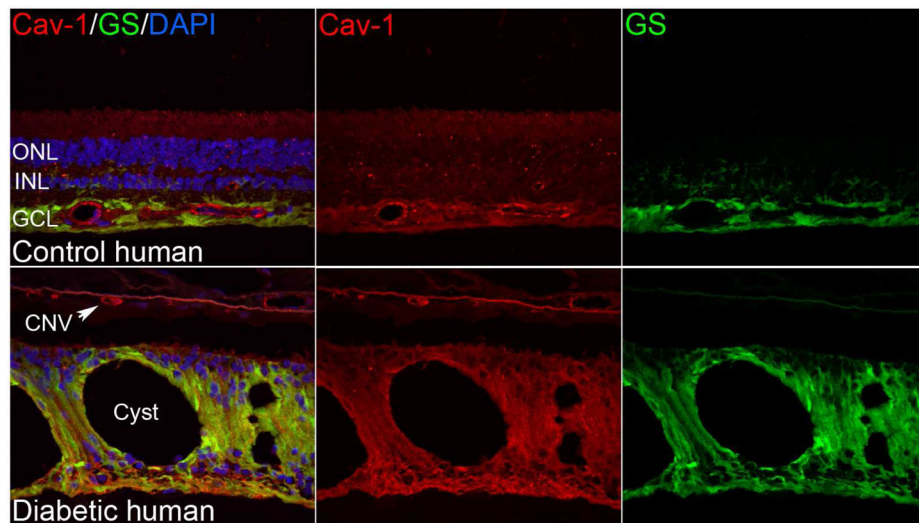
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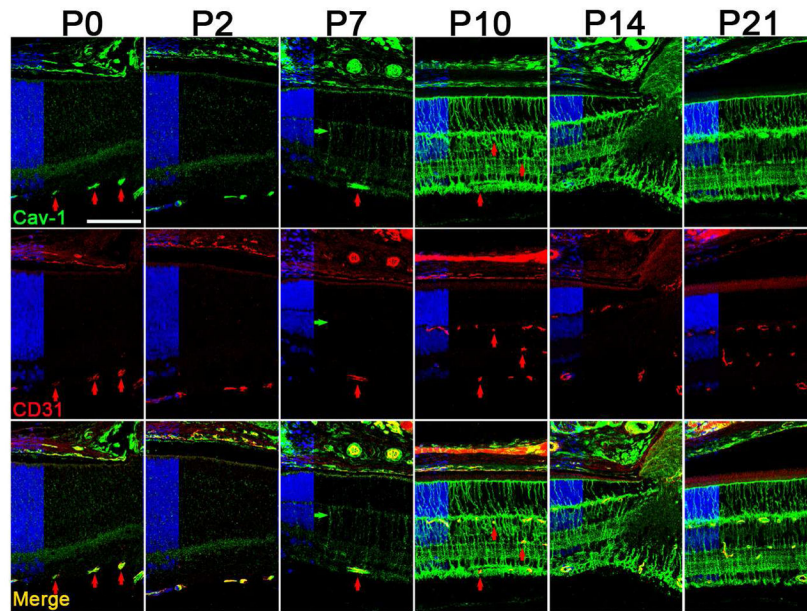
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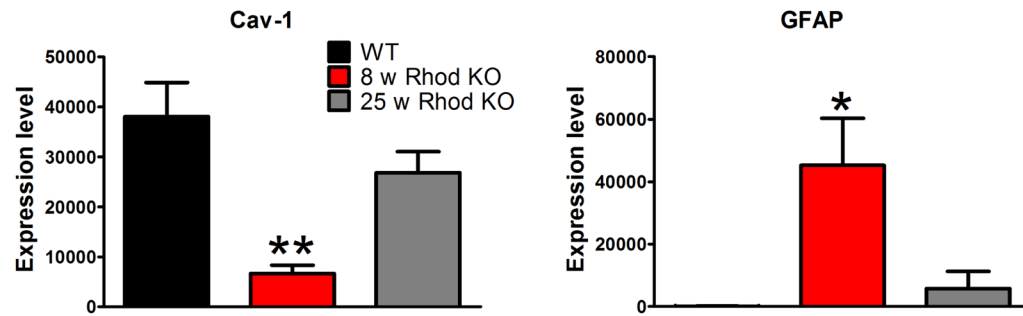




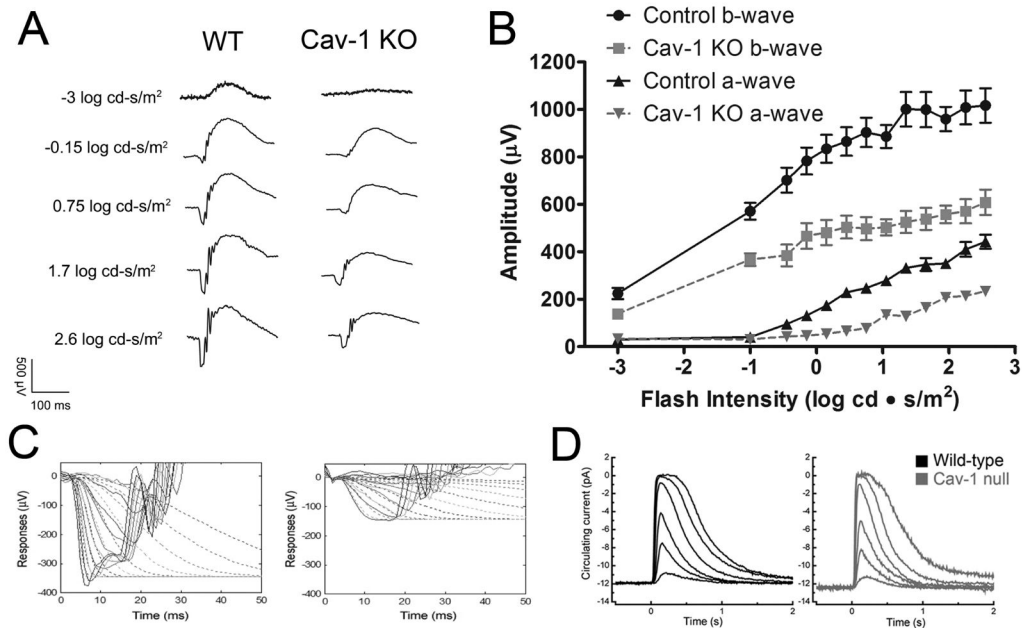
**Fig. 3.** Localization of caveolin-1 and caveolae in control and diabetic human retinae. Like the murine retina, Cav-1 immunoreactivity is detectable in retinal vasculature and Müller glia (labeled with glutamine synthetase, “GS”, in green). In the cystic, diabetic retina in the lower panels Cav-1 immunoreactivity is enhanced in Müller glia and is also present in the choroidal neovascular (CNV) lesion. (Elliott laboratory, *unpublished images*).



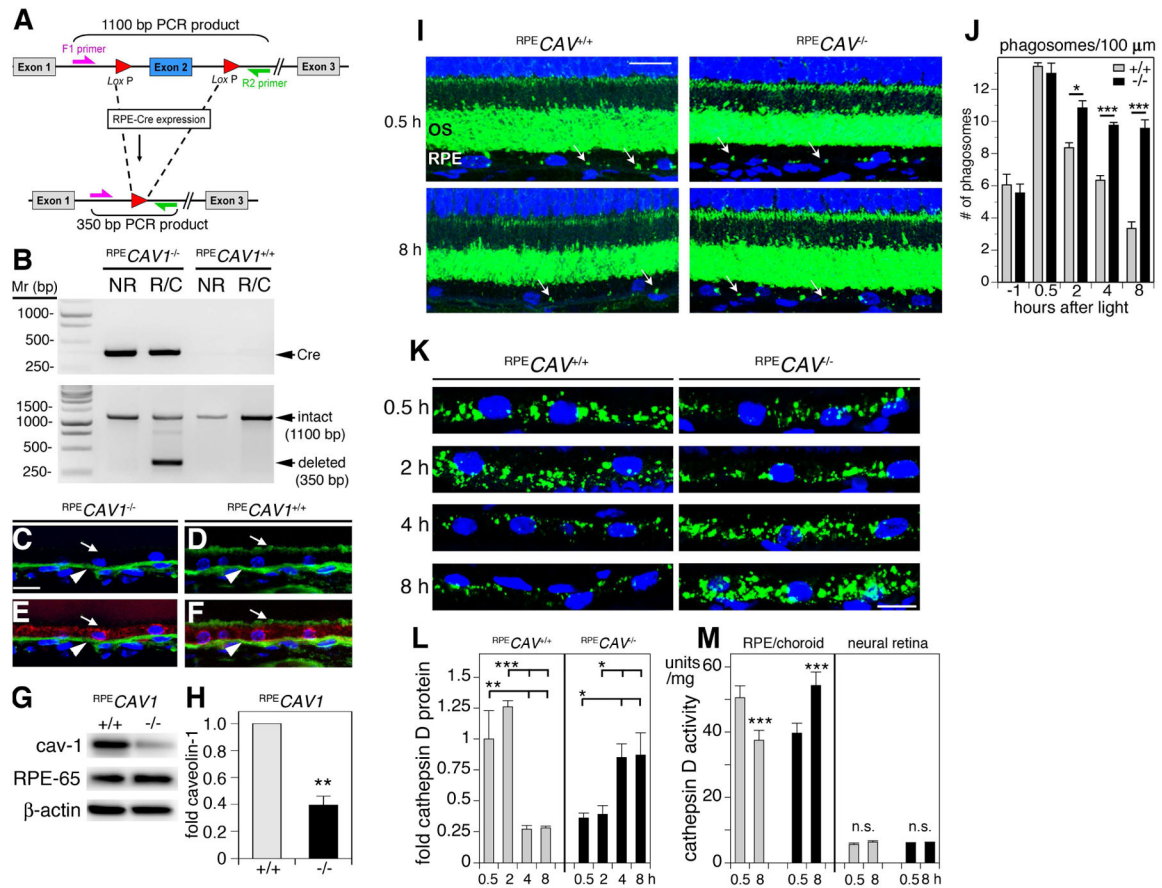
**Fig. 4.** Developmental expression of Cav-1 in the murine retina. Cav-1 (green) is expressed in developing vasculature (colabeled with CD31 in red) as early as P0. In the neuroretinal compartment, Cav-1 expression increases dramatically from P7–P10 in cells with Müller glial morphology. Reproduced from (Gu et al., 2014b).



**Fig. 5.** Cav-1 mRNA expression is significantly reduced in isolated Müller glia at the peak of rod degeneration in rhodopsin knockout mice. The Müller gliotic gene, GFAP, shows the opposite pattern of expression. These results suggest that Cav-1 expression is associated with differentiated Müller glia. The gray bar shows expression in Müller glia at the peak of cone degeneration in the rhodopsin knockout model. These data were plotted from the raw data presented in (Roesch et al., 2012).



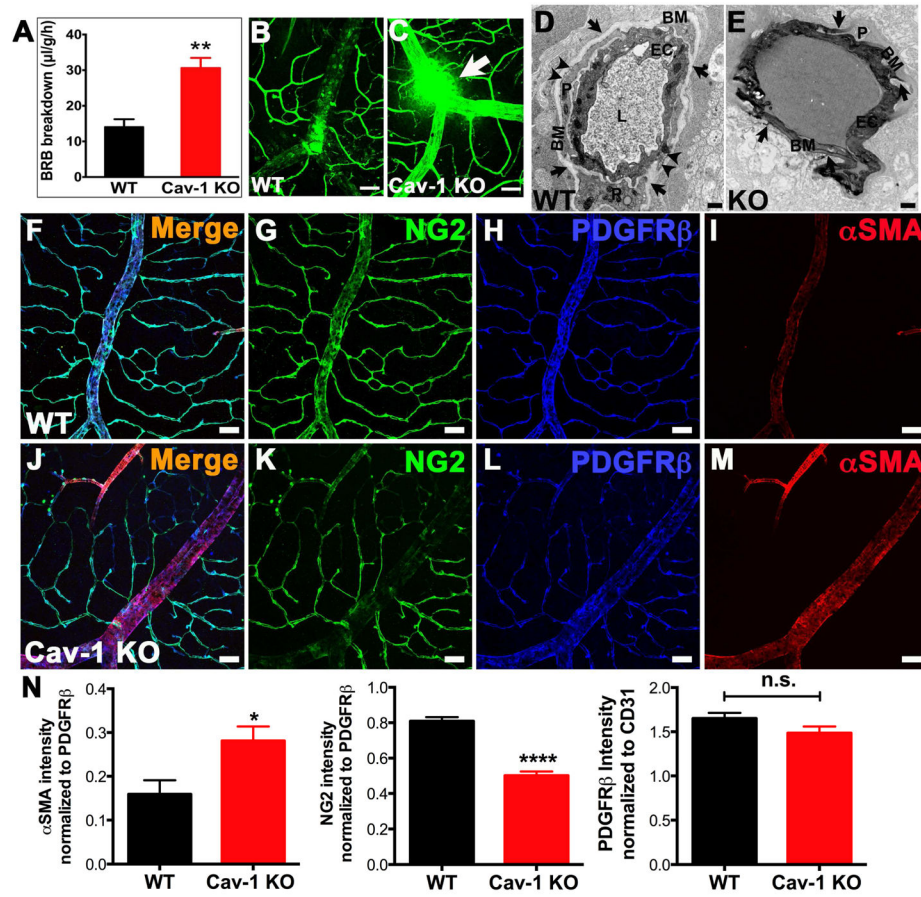
**Fig. 6.** Electrophysiological studies from Cav-1<sup>-/-</sup> mice. ERG analysis (A–C) revealed significantly reduced photoreceptor a-wave and second order neuronal (b-wave) responses, *in vivo*. (D) Suction electrode recordings of rod light responses to graded series of flash intensities reveal normal photoresponses from isolated rods. (*left panel*) Mean current traces in wildtype rods (black traces) to flashes at intensities of 4, 17, 43, 160, 450, and 1122 photons  $\mu\text{m}^{-2}$ . The average dark current in wildtype rods was  $14.1 \pm 0.6$  pA ( $n = 45$ ). (*right panel*) mean current traces from Cav-1<sup>-/-</sup> to the same flash intensities. The average dark current in Cav-1 null rods was  $12.5 \pm 1.1$  pA ( $n = 9$ ), not significantly different from wildtype ( $p = 0.24$ , Student's t-test). The flash sensitivity of the Cav-1<sup>-/-</sup> rods was  $0.26 \pm 0.03$  pA/photon/ $\mu\text{m}^2$ , not significantly different ( $p = 0.20$ ) from wildtype rods ( $0.31 \pm 0.02$  pA/photon/ $\mu\text{m}^2$ ). These results indicate that retinal function *in situ* is defective but that the functional deficit is not intrinsic to rods. Reproduced from (Li et al., 2012).



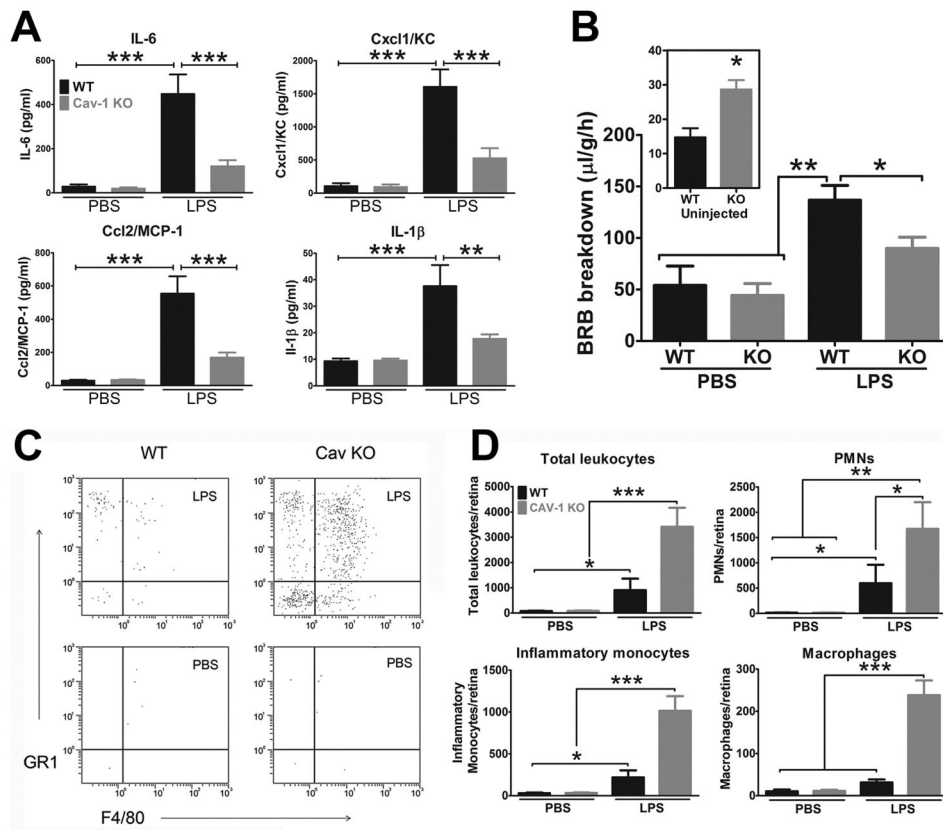
**Fig. 7.** RPE-specific deletion of Cav-1 impairs phagosome clearance by RPE cells, *in vivo*. (A) Schematic representation of Cre/lox mediated deletion of Cav-1 via RPE-specific Cre recombinase expression. (B) Representative gel of PCR products from genomic DNA from neural retina (NR) and RPE/choroid (R/C) from littermate RPE-Cre-expressing and RPE-Cre-negative mice showing Cre (top panel) and caveolin-1 floxed products. The 350 bp CAV1 deletion product (bottom panel) is detected only in RPE/choroid from Cre-carrying mice following doxycycline induction. (C–F) Representative images showing cross sections of RPE/choroid from  $RPE^{CAV1-/-}$  (C, E) and  $RPE^{CAV1+/+}$  (D, F) labeled with caveolin-1 (green; C–F) and RPE-65 (red; E, F). Arrows indicate apical RPE surface showing absence or presence of caveolin-1 in  $RPE^{CAV1-/-}$  (C, E) and  $RPE^{CAV1+/+}$  (D, F), respectively. Arrowhead indicates caveolin-1 signal in the choroid. (G) Representative western blot showing RPE ablation of caveolin-1 in RPE/choroid lysates from  $RPE^{CAV1+/+}$  and  $RPE^{CAV1-/-}$  mice. RPE-65 and  $\beta$ -actin are loading controls. (H) Quantification of experimental conditions as indicated in (G). (I–J) Representative images showing cross sections of retina from  $RPE^{CAV1+/+}$  and  $RPE^{CAV1-/-}$  mice as indicated sacrificed at 0.5 h (top panels) or 8 h (bottom panels) after light onset labeled with opsin N-terminus antibody B6-30 (green) and nuclei counterstain (blue). Arrows indicate POS phagosomes residing in the RPE. ONL, outer nuclear layer; OS, outer segment layer. Bar = 10  $\mu$ m. (I) Quantification of phagosome content of 100  $\mu$ m-stretches of RPE counted from images and samples as

shown in A. Bars show mean  $\pm$  s.e.m.; n = 4 mice per group with phagosomes counted in at least 6 images per mouse. Gray bars:  $RPE^{CAV1^{+/+}}$  mice, black bars:  $RPE^{CAV1^{-/-}}$  mice. (K–M) RPE-specific deletion of Cav-1 reverses the diurnal rhythm in activity of phagolysosomal enzymes in the RPE *in situ*. (K) Representative images showing close-up views of RPE in retina cross sections from  $RPE^{CAV1^{+/+}}$  and  $RPE^{CAV1^{-/-}}$  mice as indicated sacrificed between 0.5 and 8 h after light onset as indicated labeled with cathepsin D antibody (green) and nuclei counterstain (blue). Bar = 10  $\mu$ m. (L) Quantification of total cathepsin D protein levels in RPE *in situ* from images and samples as shown in A. Bars show mean  $\pm$  s.e.m.; n = 4 mice per group with cathepsin D signal quantified in 4 images per mouse. Gray bars:  $RPE^{CAV1^{+/+}}$  mice, black bars:  $RPE^{CAV1^{-/-}}$  mice. M, Comparison of cathepsin D enzyme activity at 0.5 and 8 h after light onset in posterior eyecups enriched in the RPE and neural retina as indicated from  $RPE^{CAV1^{+/+}}$  (gray bars) and  $RPE^{CAV1^{-/-}}$  mice (black bars). Bars show mean  $\pm$  s.e.m., n = 4 mice per condition. Reproduced from (Sethna et al., 2016).



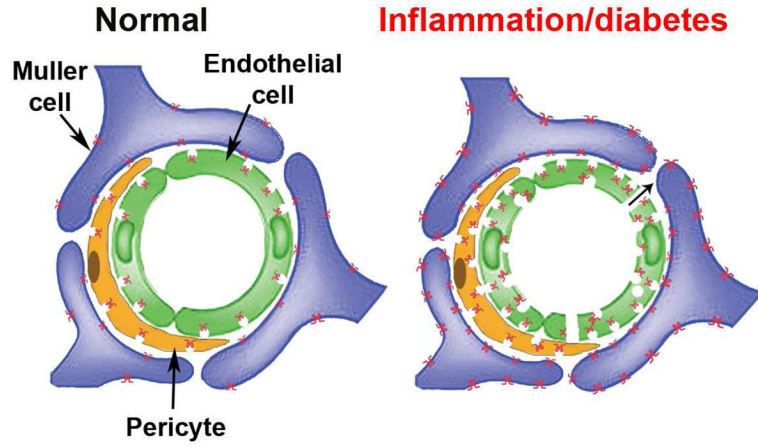


**Fig. 8.** Loss of Cav-1 results in BRB hyperpermeability (A–E), venous enlargement and a transition of contractile phenotype (increased alpha-SMA and decreased NG2) in mural cells (F–N). Reproduced from (Gu et al., 2014a).



**Fig. 9.** (A) Cav-1 deficiency suppresses LPS-induced pro-inflammatory cytokine production. (B) Although basal BRB permeability is higher in Cav-1<sup>-/-</sup> retinas (inset), inflammatory BRB breakdown induced by LPS is significantly reduced with Cav-1 deficiency. (C–D) Flow cytometry shows that immune cell infiltration in the retina in response to LPS is paradoxically increased in Cav-1<sup>-/-</sup> retinas. Reproduced from (Li et al., 2014)

# Caveolae/Caveolin in the Retinal Neurovascular Unit



**Fig. 10.** Illustration of Cav-1/caveolae in the neurovascular unit of normal and inflamed retinal vasculature. Under normal conditions, caveolae show a predominant abluminal localization in vascular endothelium (green) and mural cells (orange). Cav-1 protein, but no caveolae are detectable in Müller glia (purple). During inflammatory conditions (e.g., diabetic retinopathy), caveolae increase in number and show bipolar localization in both endothelial and mural cells possibly promoting transcellular permeability. Cav-1 expression in Müller glia is dramatically increased.