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Caveolins as regulators of stress adaptation

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Running Title: Caveolins and stress

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List of non-standard abbreviations: Cav, caveolins; I/R, ischemia-reperfusion; RISK, reperfusion injury salvage kinase; IPC, ischemic preconditioning; GLUT, glucose transporter; MBCD, methyl-beta-cyclodextrin; KO, knockout; AC, adenylyl cyclase; SR, sarcoplasmic reticulum; PAH, pulmonary arterial hypertension.

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Abstract

Caveolins have emerged over the past few decades as key regulators of cell physiology. They are ubiquitously expressed and regulate a number of processes that ultimately impact efficiency of cellular processes. Though not critical to life, they are central to stress adaptation in a number of organs. The following review will focus specifically on the role of caveolin in stress adaptation in the heart, brain, and eye, three organs that are susceptible to acute and chronic stress as well as show declining function with age. In addition, we consider some novel molecular mechanisms that may account for this stress adaptation and also offer potential to drive the future of caveolin research.

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Introduction

Caveolins (Cav) are ubiquitously expressed proteins found in many cell types and have been abundantly studied as major regulators of cell function and physiology. As form follows function, the initial discovery and description was limited to structural characterization of caveolae, Latin for "little caves", first identified in the early 1950's by Palade and Yamada (Palade, 1953; Yamada, 1955) using electron microscopy. Though initially considered artifacts of tissue processing for electron microscopy, in nearly four decades following the initial discovery of the caveolar structure to eventual molecular characterization, multiple studies described the physical presence of caveolae in numerous cell types and organ systems (Abrahams et al., 1980; Costello and Shafiq, 1979; Frank et al., 1980; Gabella, 1978; Mugnaini et al., 1977; Oguchi and Tsukagoshi, 1980; Sakata et al., 1983; Severs, 1981) and manipulation of the structure through a number of interventions (i.e., swelling, osmotic stress, stretch) (Gabella and Blundell, 1978; Kordylewski et al., 1993; Parton et al., 1994; Sage and Jennings, 1988). What emerged from these largely structural electron microscopy studies was the sense that caveolae were dynamic and versatile structures that could be manipulated by extracellular stressors. In the early 1990s, the protein caveolin, the structural and scaffolding component of caveolae, was discovered (Rothberg et al., 1992) allowing over the next two decades the molecular characterization of structural caveolae. Caveolins served as a means to explain compartmentation of signaling molecules that could be assembled into these "little caves" via enrichment of caveolins and various lipids creating a microenvironment for efficient signaling.

Besides the discovery of caveolin proteins, the one tool that has most dramatically shaped and guided caveolin research is the generation of caveolin knockout mice (Galbiati et al., 2001; Park et al., 2002b; Razani et al., 2001; Razani et al., 2002). Three isoforms of caveolin have been identified with caveolins -1 and -2 being ubiquitously expressed and caveolin-3 being restricted predominantly to muscle (Williams and Lisanti, 2004). Though the various caveolin knockout mice showed a number of phenotypes (i.e., altered life span, muscular dystrophy, cardiomyopathy, altered adiposity, pulmonary hypertension,

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neoplasia, etc. (Capozza et al., 2005; Galbiati et al., 2001; Park et al., 2003; Park et al., 2002a; Park et al., 2002b; Razani et al., 2001; Razani et al., 2002; Williams and Lisanti, 2004; Woodman et al., 2002b)), knockout of one or effectively all caveolins did not result in lethality. Such an observation questions the relative importance and central focus that has been attributed to caveolin as a key regulator of such critical and important cellular functions; compatibility with life of the various single and double knockout mice would suggest some sort of potential compensation (Insel and Patel, 2007). Interestingly, restoring Cav-1 specifically in the endothelium of Cav-1 global knockout mice restores many of the specific pathophysiologies observed in the global Cav-1 knockout mouse (Murata et al., 2007). This suggests that cell specific expression of caveolin as well as expression of caveolin in specific cells may be the most critical feature to organism health. Since the discovery of the caveolin proteins, over 6500 publications appear on PubMed with the term caveolin and nearly of sixth of these make some use of caveolin knockout phenotypes (i.e., knockout, deficient, null). A broad perspective on this literature suggests an alternative view of the importance of caveolin to biology, that loss of caveolin may not result in embryonic lethality, but rather that its expression and ultimate manifestation in caveolae allows the cell, organ, and organism to sense and respond in an efficient manner to external stimuli and the larger environment for stress adaptation. What has emerged from this larger view is that in general increased expression of caveolin is adaptive (i.e., survival positive) whereas loss of caveolin is maladaptive (i.e., survival negative) to stress (Figure 1). Though caveolin is ubiquitously expressed and has important implications for physiology, pathophysiology, and stress adaptation in multiple organs and various cell types (i.e., see the following thorough reviews for lung (Jin et al., 2011; Maniatis et al., 2012; Royce and Le Saux, 2014; Thompson et al., 2014) and liver (Fernandez-Rojo and Ramm, 2016)), we will focus on three particular organs. If one were to age long enough with no other issues, three things would likely fail, the brain, heart, and eyes due to declining function of terminally differentiated cells that largely make up these organs. As such, these become important organs for consideration of caveolin expression and stress adaptation as a function of age. This review will focus on the role of caveolin in the heart, brain,

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and eye in terms of stress adaptation and offer perspective on potential novel caveolin dependent molecular determinants of this adaptation.

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Heart

Terminal differentiation of cells offers a means to develop specialization of cells based on structure, shape, and function with potential maintenance of the cell for a lifetime. In the heart, the cardiac myocyte serves this purpose. It is largely non-dividing and therefore its survival is critical to stress adaptation. Stress in the heart can be described as acute, chronic, and building across a lifetime.

In terms of acute stress, in 1986**,** Murry and colleagues (Murry et al., 1986) discovered that multiple, brief episodes of ischemia, applied before a sustained ischemic insult, did not contribute to ischemic injury, but rather induced an increased tolerance against ischemic damage. Termed ischemic preconditioning (IPC), this intervention has proven to be the most robust and potent application to confer protection against myocardial ischemia/reperfusion (I/R) injury. Preconditioning is mediated via a molecular signaling cascade that has become known as the reperfusion injury salvage kinase (RISK) pathway (Hausenloy et al., 2005). The role of caveolin in regulating IPC and the signaling network of survival kinases is well described. Ischemia/reperfusion injury activates p42/44 and p38 MAPK, redistributes Cav-3 and down-regulates expression of Cav-1 (Ballard-Croft et al., 2006). Ischemic preconditioning may modulate the microenvironment of caveolae and caveolin-associated protein interactions so as to enrich for proteins that promote cardiac protection. This idea is consistent with findings indicating that eNOS and the glucose transporter GLUT4 translocate to caveolae after preconditioning (Koneru et al., 2007). Myocytes treated with methyl-β-cyclodextrin (MBCD) to deplete membrane cholesterol and disrupt caveolae fail to display IPC and opioid-mediated cardiac protection (Patel et al., 2006), whereas, transgenic mice with cardiac myocyte-specific overexpression of Cav-3 have increased tolerance to myocardial I/R injury. Hearts from Cav-3 overexpressing mice have improved functional recovery after myocardial ischemia and reperfusion, and increased basal Akt and GSK3β phosphorylation suggesting augmentation of the RISK signaling pathway in Cav-3 overexpressing mice (Tsutsumi et al., 2008). Such findings suggest that

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caveolae/caveolins may be major regulatory control points for cardiac homeostasis and pathophysiology in the acute setting predominantly by providing a scaffold for existing and trafficking proteins.

The protective role of caveolin in the setting of acute stress may be potentially ubiquitous. Caveolin is critical to protection in the heart and kidney and may regulate common signaling pathways. Pharmacological agents such as volatile and intravenous anesthetics and opioids that have the potential to activate G protein coupled receptors (GPCRs) to induce acute protection in multiple organs may utilize caveolin as a key signaling protein in mediating this response (Horikawa et al., 2008; Patel et al., 2007; See Hoe et al., 2014; Song et al., 2010; Tsutsumi et al., 2010; Zhu et al., 2017). These agents appear to utilize caveolin and its binding partners to enhance signaling efficiency in these various organs suggesting a common network of cellular protection may be activated. Importantly, these agents also show the ability to modulate caveolin and caveolae suggesting that there may be pharmacological means to manipulate these proteins and structures that may advance therapeutic potential in the future.

As stress becomes more chronic, the network of proteins and structures have added pressures that lead to modulation of expression of various proteins and signaling networks. Heart failure is a major cause of morbidity and mortality with very large human, social, and economic costs (Lloyd-Jones et al., 2009). The quality of life and life expectancy of patients with heart failure are poor despite optimal therapy.

Both Cav-1 and -3 have been shown to be involved in cardiac hypertrophy. Knockout of Cav-1 (Cav-1 KO) results in cardiac hypertrophy and induces contractile dysfunction (Augustus et al., 2008b). Hearts from Cav-1 KO mice show a progressive cardiac hypertrophy characterized by increased cardiomyocyte size and interstitial fibrosis. Impairment of heart function in Cav-1 KO mice is characterized by a dilated cardiomyopathy with an enlarged left ventricular diameter, wall thinning, decreased systolic function, and decreased contractility (Cohen et al., 2003). Although gross histological and functional changes within the heart are a hallmark of Cav-1 depletion, Cav-1 KO mice exhibit hyper-activation of the p42/44 mitogen-

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activated protein kinase cascade in isolated cardiac fibroblasts (Cohen et al., 2003) and nitric oxide synthase in endothelial cells. It is known that caveolae contain numerous signaling molecules involved in cardiac hypertrophy including but not limited to alpha adrenergic receptors, G_q proteins, phospholipase C, epidermal growth factor receptors, Ras, MAPKs, Src kinases, natriuretic peptide receptors and Cav-3 (Krajewska and Maslowska, 2004). Woodman et al (Woodman et al., 2002a) have shown that knocking out the gene for Cav-3 results in hyper-activation of the Ras/extracellular signal-regulated kinases (ERK 1/2) signaling pathway, cardiac hypertrophy, and reduced cardiac function. Cav-1/Cav-3 double KO mice completely lack morphologically identifiable caveolae and develop a severe cardiomyopathic phenotype with left ventricular hypertrophy and dilation (Park et al., 2002b). Cav-3 KO mice develop cardiomyopathy characterized by hypertrophy, ventricular dilation and reduced contractility (Woodman et al., 2002a). Koga et al (Koga et al., 2003) have demonstrated that *in vitro* overexpression of Cav-3 in neonatal cardiac myocytes attenuated phenylephrine and endothelin induced ERK1/2 activation and blocked myocyte hypertrophy. Cardiac-specific overexpresison of caveolin results in blunted hypertrophy and cardiac dysfunction in response to transverse aortic constriction (Horikawa et al., 2011).

When considering the organizations of signaling networks critical to heart failure, the adrenergic nervous system is a key part of the neurohumoral response to heart failure and a major focus of heart failure research and therapy (Triposkiadis et al., 2009). Decreased cardiac function in the early stages of heart failure leads to increased sympathetic neuronal activity, increased circulating norepinephrine, and activation of cardiac β-adrenergic receptors (βARs) as a means to increase cardiac output by increasing heart rate and myocardial contractility. However, heart failure progression is associated with a decrease in β_1AR number and in coupling of β_1 and β_2ARs to downstream effectors (Dorn and Liggett, 2009). Persistent βAR stimulation worsens heart failure and therapy with βAR agonists can be detrimental. βAR antagonists are now used to treat patients with heart failure. Deleterious effects of persistent βAR activation appear to result from activation of the β_1AR pathway while beneficial effects from β_2AR

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activation may ameliorate such deleterious changes (Xiao et al., 2004). β_1ARs and β_2ARs are the principal βARs in the heart but β_3ARs may also contribute to cardiac βAR activity and may be a therapeutic target for heart failure. Work in recent years has shown that subcellular localization/compartmentation of βAR subtypes within cardiac myocytes influences functional responses that result from receptor activation. Altered distribution of βARs in cardiac myocytes may be a critical derangement in heart failure progression (Dorn, 2010; Macdougall et al., 2012; Nikolaev et al., 2010).

Recent data show that ideas about GPCR signaling must take into account subcellular localization of the receptors and post-receptor signaling components (Calebiro et al., 2009; Kamal et al., 2012; Maurice et al., 2012; Timofeyev et al., 2013). The localization of β_1ARs and β_2ARs differs in cardiac myocytes (Steinberg, 2004). β_1AR are broadly expressed in the sarcolemma while β_2AR primarily localize to caveolar microdomains. βAR subtypes in the different locales have different signaling properties: β_1AR activation produces inotropic and chronotropic responses via global G stimulatory (G_s)-coupling and activation of adenylyl cyclase (AC; AC 5 and 6 are the two major AC isoforms in the heart), protein kinase A (PKA), Epac, L-type calcium channels and other downstream targets (Chen-Izu et al., 2000; Han et al., 2013; Pereira et al., 2013; Timme et al., 2000; Xiang and Kobilka, 2003). In contrast, β_2AR couple to both G_s and G inhibitory (G_i) pathways. β_2AR localized in caveolae produce transient PKA-dependent inotropic responses adjacent to L-type calcium channels, followed by decreased contraction due to sequential coupling to G_s and then G_i proteins (Xiang and Kobilka, 2003). G_i proteins are enriched in caveolae (Chen-Izu et al., 2000). The different localization of βAR sub-types has implications for cell death responses that occur in heart failure: β_1AR stimulation can promote myocyte apoptosis while β_2AR activation can be anti-apoptotic via a G_i-, phosphoinositide 3-kinase (PI3K)-, Akt-dependent pathway (Communal et al., 1999; Xiao et al., 2004).

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β_2AR within caveolae localize to transverse (T)-tubules and produce localized cAMP signals in cardiac myocytes (Head et al., 2005; Nikolaev et al., 2010). T-tubules are enriched in cholesterol and caveolin-3 (Cav-3), molecules that characterize caveolar microdomains (Carozzi et al., 2000). The loss of T-tubule structure likely contributes to progression of heart failure and changes in subcellular distribution of βARs and their signaling components (Wei et al., 2010). With the development of heart failure, the restricted localization of β_2ARs in T-tubules can be disrupted, such that β_2ARs are found on the myocyte plasma membrane where β_1ARs are normally the dominant βAR subtype. As a result, β_2ARs then can produce a diffuse cAMP signal that is similar to what occurs with β_1AR stimulation and thus, may enhance myocyte dysfunction (Nikolaev et al., 2010). Importantly, the abnormal distribution of βARs in heart failure can be mimicked by chemical disruption of the T-tubule/caveolae microenvironment (Nikolaev et al., 2010).

Cav-3 preferentially interacts with β_2ARs vs β_1ARs (Steinberg, 2004). Caveolae and caveolins play a key role in organizing and regulating cell signaling pathways, including βAR signaling (Patel et al., 2008). Caveolae contain major components of β-adrenergic signaling, including βARs, G_s and G_i, ACs, G protein-coupled receptor kinases (GRKs), PKA subunits, and L-type calcium channels (Balijepalli et al., 2006; Krajewska and Maslowska, 2004). Localization of L-type calcium channels to caveolae is essential for their regulation by β_2ARs (Balijepalli et al., 2006) and localization of β_2ARs to caveolae in association with Cav-3 is critical for localized β_2AR signaling in cardiac myocytes: Disruption of caveolae converts β_2AR responses to a β_1AR-like response (Calaghan et al., 2008). Cav-3 knockout mice have a 40% increase in myocardial cAMP content, suggesting that absence of Cav-3 enhances cAMP synthesis (Augustus et al., 2008a). Other data imply that cAMP synthesis in caveolae is influenced by G_i signaling with a key role for PI3K and cyclic nucleotide phosphodiesterase (PDE) (Kerfant et al., 2006). PI3K signaling in this response depends on macromolecular complexes within caveolae while close apposition of the T-tubular membrane (containing caveolae) with the sarcoplasmic reticulum (SR) membrane gives PDE access to the SR compartment. Caveolae and T-tubules share structural and

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functional similarities; both are enriched in cholesterol and Cav-3, increase the functional surface area of the sarcolemma, and share analogous mechanisms of biogenesis (Carozzi et al., 2000). Cav-3 plays a major role in organizing and maintaining T-tubule structure and function in cardiac myocytes (Ziman et al., 2010). Chronic adrenergic stimulation and heart failure can decrease Cav-3 expression (Ruiz-Hurtado et al., 2007; Yamamoto et al., 1999) which may ultimately impact the caveolar structure to alter networks for stress adaptation.

Finally, when considering stress adaptation over a lifetime one must consider the aging heart. Age is an important predictor of mortality linked to cardiovascular disease (Boersma et al., 2000). Protective networks are lost in aged myocytes (Mio et al., 2008). Preclinical studies reveal increased sensitivity and decreased tolerance to I/R injury in the aged heart (Headrick et al., 2003; Willems et al., 2005). Recent studies indicate that the heart may be a central player in dysfunction of peripheral organs such as brain, liver and kidney, underscoring a key idea: a better functioning heart and the resultant enhancement in tissue perfusion will positively influence multiple organ systems with age-related loss in function, contribute to healthier aging and perhaps enhance lifespan.

Importantly, a decrease in the expression of cardiac Cav-3 (Kawabe et al., 2001) is observed with age and aging results in dissociation of Cav-1 and -3 from membrane caveolae (Ratajczak et al., 2003). Though Cav-1 KO mice show a decreased lifespan (Park et al., 2003), it is not clear if this a consequence of the cardiovascular aspects regulated by Cav-1; however, these mice are resistant to cardiac protective stimuli (Patel et al., 2007). Cav-3 KO mice develop a progressive cardiomyopathy (Woodman et al., 2002a) and are also resistant to cardiac protective stimuli (Horikawa et al., 2008) but lifespan does not appear to be impacted. However, this is growing evidence to suggest that Cav-3 may have impact on the function of the aging heart. Cav-3 and caveolae are reduced with age and may be associated with dysfunctional survival signaling (Peart et al., 2007; Peart et al., 2014). Protein kinase C (PKC) is critical to cardiac protection and this is largely dependent on trafficking to mitochondria. Recently, Cav-3 was shown to be

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critical for this transport and age resulted in reduced mitochondrial PKC (Kang et al., 2017). Such data point to the potential survival positive benefits of caveolin in the heart and a potential molecular target to impact acute, chronic, and lifetime stress.

Brain

Neurons, glia, and the BBB. Neurons are absent of morphological caveolae but do possess planar microdomains enriched in cholesterol, glycosphingolipids, and sphingomyelin and express all three caveolin isoforms (Head and Insel, 2007; Shyng et al., 1994). Cav-1 is also expressed in CNS endothelia (Sowa, 2012), pericytes (Virgintino et al., 2002), and astrocytes (Cameron et al., 1997). Within cellular components of the blood brain barrier (BBB), Cav-1 regulates extracellular matrix (ECM) proteins which include metalloproteinases (MMPs) (Virgintino et al., 2002) and tight junction (TJ) proteins, that in turn modulate BBB physiology (Abbott et al., 2006; Gu et al., 2011; Gurnik et al., 2016; Lakhan et al., 2013; Liu et al., 2012; Zhao et al., 2014). Cav-1 has also been shown to be critical in hypoxia-induced astrocyte injury (Xu et al., 2016) which can lead to BBB damage and leakage. Knockdown of Cav-1 using Cav-1 small siRNA exacerbated astrocyte cell damage and impaired cellular glutamate uptake after oxygen-glucose deprivation (OGD). In contrast, overexpression of the Cav-1 CSD (caveolin scaffolding domain) peptide attenuated OGD-induced astrocyte apoptosis via ERK signalling (Xu et al., 2016), thus further demonstrated the importance of Cav-1 in BBB physiology (Fu et al., 2014; Gu et al., 2012).

Cav-1 is expressed in both microglia and astrocytes (Salgado et al., 2012). Cav-1 expression was detected in activated microglia (using kainic acid) in several brain regions with the highest expression measured after 3 days, implicating a potential role of Cav-1 in microglial activation (Takeuchi et al., 2013). Moreover, work from our group demonstrated that Cav-1 protein decreased and redistributed from the plasmalemma to cytoplasmic vesicles in inactive microglial, while the active (amoeboid-shaped) microglia exhibited increased Cav-1 expression (Niesman et al., 2013). Additional published findings demonstrated that Cav-1 KO mice exhibited significant astro- and microgliosis as well as an early aging

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phenotype in the brain (decreased hippocampal synapses, altered cerebrovascular, and reduced pro-growth signalling components) (Head et al., 2010), suggesting that loss of Cav-1 may in part contribute to neuropathological conditions in the brain.

Neuronal Cav-1, neuroprotective signalling and plasticity. Specifically, within neurons, Cav-1 scaffolds and organizes neurotransmitter and neurotrophin receptors (NMDARs and TrkB) in raft microdomains *in vitro* (Head et al., 2011; Head et al., 2008) and *in vivo* (Mandyam et al., 2017). Knockdown of Cav-1 using small interfering RNA (siRNA) knockdown blunted NMDAR and TrkB-mediated signalling (Head et al., 2011; Head et al., 2008). Studies employing either a sublethal ischemia (SLI) or NMDA preconditioning model increased expression of phosphorylated (P) Cav-1, P-Src, and P-ERK1/2 in primary cortical neurons from rats or mice (Head et al., 2008). Primary neurons treated with Cav-1 small interfering RNA or isolated from Cav-1 KO mice lacked NMDA-mediated increase in P-Src and P-ERK, as well as SLI- and NMDA-induced preconditioning. Cav-1 re-expression (using a viral vector) in Cav-1 KO primary neurons restored NMDA-mediated increases in P-Src and P-ERK1/2 and redistributed NMDAR2B to membrane rafts.

In addition to a neuroprotective role, additional studies have shown that Cav-1 promotes neuronal and synaptic plasticity and improves neurobehavior (Egawa et al., 2017a; Egawa et al., 2017b; Head et al., 2011; Mandyam et al., 2017). Using a neuron-specific synapsin promoter to express Cav-1 (termed *SynCav1*) specifically in neurons, Cav-1 overexpression enhanced cellular cholesterol accumulation and raft formation, augmented receptor-mediated cAMP production, functional NMDAR (P-Src, P-CaMKII, P-ERK1/2) and TrkB signalling (P-TrkB, P-Akt), dendritic growth and arborisation *in vitro* (Head et al., 2011) and *in vivo* (Mandyam et al., 2017) and prevented hippocampal-dependent learning and memory loss and improved motor function in a murine model of traumatic brain injury (TBI) (Egawa et al., 2017a). Further work from our group showed that when delivered to the hippocampus *in vivo*, *AAV9-SynCav1* increased hippocampal neuroplasticity, improved fear learning and memory in adult and aged

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mice (Mandyam et al., 2017), and promoted ultrastructural and functional indicators of synaptic plasticity (Egawa et al., 2017b), suggesting that Cav-1 and raft microdomains alter aspects of synapse biology necessary for functional neuronal and synaptic plasticity.

Eye

Along with the heart and brain, the eye is an organ sensitive to external stressors and one that shows a clear aging phenotype. The aging eye shows retinal deterioration, macular degeneration, changes in the lens, drainage issues, and many other changes that ultimately lead to declining and failing vision (Salvi et al., 2006). Though studies involving caveolin in the eye have been lagging compared to other organ systems, there is a recent growing interest in studying the relationship of caveolin to the eye (Gu et al., 2017). This interest has been fueled by a number of recent studies suggesting Cav-1/Cav-2 as potential glaucoma susceptible genes in multiple populations (Chen et al., 2014; Loomis et al., 2014; Wiggs et al., 2011; Yoshikawa et al., 2017). Cav-1 has been shown to protect retinal ganglion cells to a similar survival kinase activation mechanisms involving Akt as in the heart (Zhang et al., 2017). This was observed in a stress model involving ocular hypertension induced injury and suggests Cav-1 as a potential therapeutic target. Studies with pressure overload and hypertension in the heart (Horikawa et al., 2011; Markandeya et al., 2015) suggest the same benefit on protection of terminally differentiated cardiac myocytes with the unique observation that cardiac myocytes and hearts with overexpression do not hypertrophy whereas wild-type hearts nearly double in size. Such preserved responses in myocytes and neurons suggest a generalized response to hypertensive stress (i.e., physical or receptor initiated stress) with common, as of yet unidentified, downstream signaling that is caveolin dependent and may be a novel therapeutic target. Further studies using Cav-1 KO mice suggest that Cav-1/caveolae may be mechanoprotective in the eye in response to increases pressure that may be linked to alteration in aqueous humor drainage and this is likely dependent on nitric oxide (Elliott et al., 2016; Song et al., 2017). At the cellular level, this mutation likely impacts a variety of cells in the eye including the trabecular meshwork cells (Aga et al., 2014). Further exploration is needed to confirm a molecular function for caveolin in glaucoma. In addition, a

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number of studies have linked caveolin to retinal-blood barrier changes (Gu et al., 2014; Klaassen et al., 2009; Tian et al., 2012) that have important implication of primary and secondary diseases related to the eye. As the field grows, it is expected that the eye will present a fruitful target for caveolin modulations as potential therapy for stress adaptation.

Novel Molecular Mechanisms

Many different domains have been described for caveolin (i.e., scaffolding domain, oligomerization domain, a number of post-translational modification sites) that are implicated in a number of cellular and disease processes (Patel and Insel, 2009; Patel et al., 2008). Studies related to these domains have led to significant insights into caveolin regulation of cell biology. However, detailed mechanistic functions of the C-terminus of Cav-1 are not well described and this area of caveolin research is fairly new but may hold promise for defining novel functions of caveolin. During the last decade, many patients with juvenile pulmonary arterial hypertension (PAH) and various lipodystrophy phenotypes were identified with C-terminal Cav-1 mutations (Austin et al., 2012; Garg et al., 2015; Schrauwen et al., 2015). Importantly, a heterozygous frameshift mutation that leads to a premature stop codon and truncation at amino acid 160 results in a neonatal progeria like phenotype (Schrauwen et al., 2015). The fibroblasts from this patient show a near complete loss of Cav-1 expression suggesting an importance for caveolin in regulating "aging" phenotypes in the cell.

The two main functions of the C-terminus were originally described as 1) membrane attachment (proximal third) and 2) protein/protein interaction (distal third) (Razani et al., 1999; Schlegel and Lisanti, 2000). Mutated caveolin (Cav-1 deltaC) proteins show that the C-terminus is required for homo-oligomers to interact (Li et al., 1998). Additionally, functional Cav-1 is required to recruit Cav-2 to the membrane (Li et al., 1998). Furthermore it has been described that the Cav-1 C-terminal region interacts with both the N- and C-terminal region specifically and is required for homo-oligomerization (Song et al., 1997), and both, the N- and C-terminus interact with endothelial nitric-oxide synthase (eNOS) oxygenase

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domain (Ju et al., 1997). The C-terminal region is important in conjunction with the scaffolding domain to mediate endothelin B signaling (Yamaguchi et al., 2003). A splice variant of caveolin-2 mRNA was identified which lacked the C-terminal region. This variant localized to the endoplasmic reticulum, while the full version localized with Cav-1 to the Golgi apparatus and plasma membrane suggesting an important role of the C-terminus for caveolin localization (Kogo et al., 2002). There appears to be a gap in the literature when it comes to assessing the structure of the C-terminal region and just recently, the C-terminal secondary structure of Cav-1 has been described as helical using NMR spectroscopy (Plucinsky and Glover, 2015). C-terminal tagging of ectopically expressed Cav-1 with fluorescence proteins (EGFP, mCherry, and myc) showed enhanced aggregation and/or degradation of a wild-type and mutant form, while the endogenous forms remained mainly intact (Han et al., 2015). Antibodies against the C-terminal domain are currently under investigation to inhibit Cav-1 mediated signaling in cancer cells (Kuo et al., 2012). Taken together, these studies imply a crucial role for the C-terminal region of Cav-1 in signaling, localization, and assembly of caveolin oligomers. Though detailed mechanisms have not been worked out, a description of these C-terminal mutations identify a new region of caveolin, one largely ignored, as a potential hot spot for regulation of cell physiology critical to stress adaptation.

Caveolins can be post-translationally modified, though limited insights exist regarding the physiological consequence of such modification. Caveolin was originally discovered as a target of Src (Rothberg et al., 1992) which in turn is a major stress regulated kinase. In the heart, studies in Cav-1 knockout mice suggest that Cav-1/Src interactions are critical to adaptation to ischemic stress (Patel et al., 2007) and cell-cell communication and arrhythmogenecity (Yang et al., 2014). Cav-1 tyrosine 14 is the primary Src phosphorylation target which has been implicated in focal adhesion and cancer biology (Meng et al., 2017; Ortiz et al., 2016), endothelial cell signaling in sepsis-induced lung injury (Jiao et al., 2013), regulation of mechanotransduction (Joshi et al., 2012; Zhang et al., 2007), and insulin signaling (Chen et al., 2008). Additionally, caveolins can undergo S-nitrosylation, which is important in the setting of oxidant stress. This may have implications for acute cardiac ischemia (Sun et al., 2015), as well as, the

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structure of caveolin oligomers (Bakhshi et al., 2013) and caveolae in pathophysiology. Limited information exists regarding modifications specific to Cav-3; however, it has been suggested that in addition to S-nitrosylation (Sun et al., 2015), Cav-3 may undergo SUMOylation leading to regulation of GPCR and eNOS signaling (Fuhs and Insel, 2011).

In addition to potential membrane specific changes related to caveolin, caveolae and caveolin have become important considerations in the regulation of mitochondrial structure and function. Mitochondria, being major regulators of energy homeostasis and regulators of stress signaling in the cell, are a major control point for stress adaptation in the cell. In the heart, caveolae are found in close proximity to mitochondria and stress induces a transfer for caveolin to the mitochondrial leading to preserved mitochondrial structure and function in response injury (Fridolfsson et al., 2012). Such an observation was also seen in cancer cells and *C elegans* suggesting a potential generalized mechanism of caveolin protection of mitochondria (Fridolfsson et al., 2012). Cav-1 has also been described to modulate mitochondrial function (Asterholm et al., 2012; Bosch et al., 2011) where decreased Cav-1 expression led to mitochondrial cholesterol accumulation (Bosch et al., 2011) and down regulation in mitochondrial genes (Asterholm et al., 2012).

Recently it has been shown that in Cav-1$^{-/-}$ mouse embryonic fibroblasts cellular senescence and mitochondrial dysfunction was observed. This was attributed to the p53-p21 pathway and the downregulation of cardiolipin. In regards to mitochondrial function, respiration was decreased with a reduced activity of complex I (CI), inactivated SIRT1, and decreased NAD^+/NADH ratios (Yu et al., 2017). From a cancer perspective, the influence of Cav-1 on cell metabolism, mitochondrial function, glutaminolysis, fatty acid metabolism, and autophagy has been reviewed (Nwosu et al., 2016). In regards to mitochondrial function, increased Cav-1 levels in tumor cells increased mitochondrial number and respiration, mitochondrial ROS, mitochondrial complex I-V, Ca^{2+}-signaling, altered intracellular cholesterol flux, and decreased apoptosis (Nwosu et al., 2016). Furthermore, Cav-1 has been attributed

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roles in maintaining mitochondrial integrity and function in the setting of increased free radicals (Volonte et al., 2016). Knockdown of Cav-1 results in metabolic switching with decreased glycolytic intermediates, increased fatty acids, and autophagy activation (Shiroto et al., 2014). Furthermore, Cav-1 is down regulated in stromal fibroblasts which leads to accelerated epithelial breast cancer growth and this has been termed reverse Warburg effect or "two-compartment tumor metabolism" where the stromal fibroblasts supply the tumor cells with metabolites (Salem et al., 2012). Taken together these multiple roles of caveolin in regulating mitochondrial dynamics seem to play a crucial role in cell fate and offer multiple sites for possible therapeutic intervention. Nevertheless, role of caveolin in end-differentiated cells like cardiac myocytes and neurons in regards to mitochondrial function is less well described and warrants further investigation.

Conclusions

Caveolins/caveolae have come a long way since the early structural description of caveolae over six decades ago. Over this time our understanding has grown dramatically from structure to function with a particular focus on the caveolin proteins. What has emerged is an appreciation that these proteins are indeed critical features of cells and act as regulatory control points for cell adaptation to stress. This is readily apparent in terminally differentiated cells such as cardiac myocytes and neurons but have important implications for proliferative cells as well such as smooth muscle, fibroblasts, glia, cancer, and many others. We can learn a lot from mutations in these proteins and their impact on cell biology as well as the regulation of intracellular structures by caveolin. What remains to be identified and what will likely fuel the next six decades of research on caveolin is to utilize this cell specific knowledge that has and will be gathered and then apply targeted therapeutics to modulate caveolin and caveolin specific pathways in precise ways to modulate disease manifestation and treatment.

Authorship Contributions

Wrote and contributed to the writing of the manuscript: Schilling, Head, and Patel.

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Figure Legend

Figure 1. Caveolin in cell death and survival. Cartoon depicting the critical role for caveolin in maintaining cell survival via regulation of multiple cellular sites including the membrane and mitochondria, as well as, the negative aspects of loss of caveolin expression on cell death.

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Figure 1