UNIVERSITY OF CALIFORNIA, SAN DIEGO

Posttranslational Regulation of Caveolin by Small-Ubiquitin-like Modifier (SUMO) Proteins.

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in

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by

Stephen Rush Fuhs

Committee in Charge:

Professor Paul A. Insel, Chair
Professor Tracy Handel
Professor Tony Hunter
Professor Susan Taylor
Professor JoAnn Trejo

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Chapter 2, in part, is currently being prepared for publication. The dissertation author is the primary author and Paul Insel is the principal investigator.

Chapter 3, in part, will be prepared for future publication pending further experiments to be performed in part by the dissertation author and by Anna Busija. The dissertation author will be the primary author, Anna Busija will be a secondary author and Paul Insel will be the principal investigator.

VITA

1994-1998	BS, Environmental, Population & Organismic Biology, University of Colorado at Boulder
1999-2000	Postbaccalaureate Studies, University of Puget Sound
2005-2010	PhD, Biomedical Sciences, University of California at San Diego

PUBLICATIONS

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ABSTRACT OF THE DISSERTATION:

Posttranslational Regulation of Caveolin by Small-Ubiquitin-like Modifier (SUMO) Proteins.

by

Stephen Rush Fuhs

Doctor of Philosophy in Biomedical Sciences

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Professor Paul A. Insel, Chair

In this dissertation, I identify the covalent modification of caveolin-3 with small-ubiquitin-like modifier (SUMO) proteins and its interactions with components of the sumoylation machinery. Caveolins are the principal protein components of caveolae membrane microdomains and function as scaffolds that compartmentalize and regulate multiple signaling proteins, their downstream effectors and associated proteins. Caveolin-

3 is a muscle-specific caveolin family member that is highly expressed in skeletal muscle and cardiac myocytes. Beta 1- and beta 2-adrenergic receptors (βARs) use distinct pathways to regulate contraction in cardiac myocytes. Caveolar localization and interaction with caveolin-3 is required for signaling by the β_2AR but not the β_1AR. β_2ARs and β_1ARs also desensitize through distinct pathways in response to agonist stimulation. Evidence presented here suggests that sumoylation of caveolin-3 is involved in the regulation of agonist-induced desensitization of β_2ARs.

Sumoylation is a highly dynamic and reversible posttranslational modification that regulates substrates involved in many aspects of cellular function. SUMO E1, E2 and E3 enzymes catalyze the formation of covalent, isopeptide bonds with lysine side chains on target substrates while SUMO-specific proteases (SENPs) remove SUMO proteins from substrates. Experiments in this thesis show that caveolin-3 is covalently modified by SUMO and poly-SUMO chains in multiple cell types. *In vitro* and *in vivo* sumoylation assays demonstrate that modification of caveolin-3 by poly-SUMO-3 chains is enhanced by co-expression of the SUMO E3 ligase PIASy in a dose-dependent manner. Co-expression of SENP1 or SENP2 dramatically reduced modification of caveolin-3 by SUMO-3.

Site-directed mutagenesis was used to identify the preferred site of sumoylation and create a sumoylation-deficient mutant to probe the biological function of SUMO modification. Co-expression of caveolin-3 or the sumoylation-deficient mutant with βARs showed differential effects on the stability of β_2AR, but not β_1AR, expression levels in the presence of prolonged agonist stimulation. This effect on β_2ARs was attenuated by the βAR antagonist (-)-propranolol.

Taken together, the data in this dissertation identify sumoylation as a novel mechanism for the regulation of caveolin and its interactions with its signaling partners, including effects on the agonist-induced desensitization of β_2ARs.

CHAPTER 1: Introduction

1.1 Caveolins and Caveolae

Caveolins are the principal protein components of caveolae, a type of membrane microdomain enriched in cholesterol, sphingolipids and numerous proteins. Caveolae are 50-100 nm, flask-shaped invaginations of the plasma membrane that are found in most cell types and are particularly abundant in adipocytes, endothelial and muscle cells. Caveolae ("little caves") were discovered over 50 years ago due to their striking morphology in electron micrographs and were first referred to as "plasmalemmal vesicles" before being named "caveolae intracellulares" (Palade 1953; Yamada 1955). Electron microscopy was the sole means to study caveolae until caveolin-1, the first

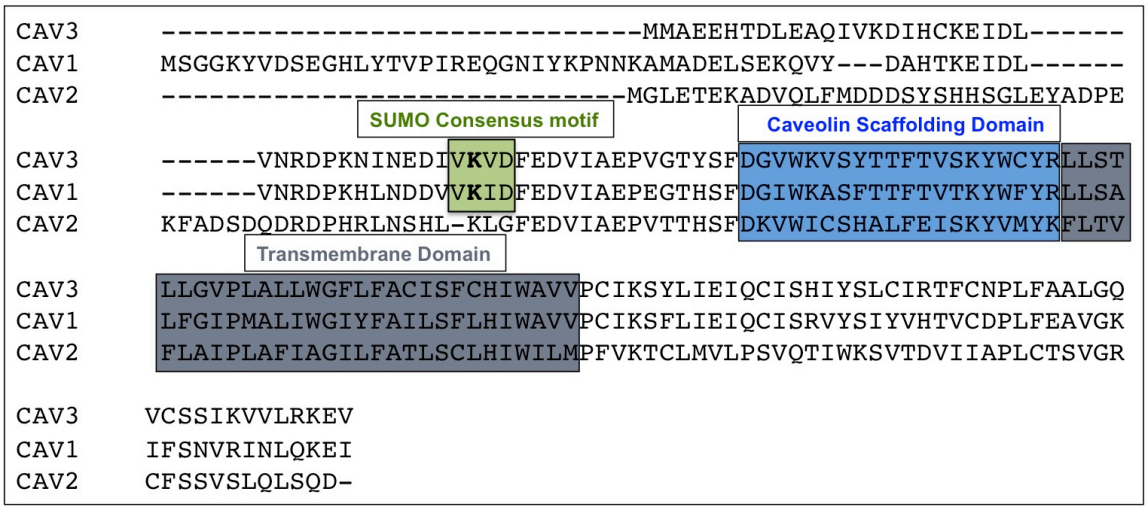

Figure 1.1 Alignment of the Caveolin Protein Family
Caveolin-3 shares 65% sequence identity and is 85% similar to caveolin-1 and 40% similar to caveolin-2. Caveolin-1 and -3, but not caveolin-2 have a core SUMO consensus motif (Ψ-K-X-[D/E], Ψ=VIL) in their N-termini. This motif also conforms to the extended, "negatively charged amino acid-dependent sumoylation motif" (NDSM, Chapter 1.6). The caveolin scaffolding domain (CSD; aa55-74) and transmembrane domain (aa75-104) however are highly conserved between all three family members.

protein marker of caveolae, was discovered forty years later (Rothberg et al., 1992).

With the discovery of caveolin-1, two related proteins were soon discovered through "*microsequencing of adipocyte-derived caveolin-enriched membranes*" and by protein sequence homology (Tang et al., 1996; Scherer et al., 1996). Thus, the caveolin family was shown to have three members: caveolin-1 and -2, co-expressed in most cell types, and caveolin-3, expressed primarily in cardiac, skeletal and smooth muscle cells (Song et al., 1996). Caveolin-1 has two isoforms: caveolin-1α with residues 1-178 and caveolin-1β having only residues 32-178. Caveolin-3 shares 65% sequence identity and is 85% similar to caveolin-1 (primarily due to caveolin-1's extended N-terminus) and 40% similar to caveolin-2. Caveolins are 22-24 kDa integral membrane proteins that form a hairpin loop in the plasma membrane so that both the N- and C-termini face the cytoplasm (Figure 1.2). Formation of high molecular mass homo- and hetero-oligomeric complexes (composed of ~14-16 caveolin monomers) is thought to contribute to their ability to deform the plasma membrane into caveolae invaginations (Das et al., 1999, Song et al., 1997). Caveolins-1 and -3 homo-oligomerize and caveolins-1 and -2 and caveolins-1 and -3 hetero-oligomerize (Volonte et al., 2007). Cholesterol is necessary to stabilize caveolin oligomers and caveolae can be disrupted by cholesterol-depleting agents such as methyl-β-cyclodextrin or filipin. Caveolae are also enriched in sphingolipids (e.g. sphingomyelin, ceramide and gangliosides) that occur primarily in the exoplasmic leaflet of the plasma membrane bilayer (Stan, 2005).

Expression of caveolin-1 or caveolin-3 in cells lacking caveolae and caveolin protein expression induces caveolae formation (Fra et al., 1995). Expression of caveolin-2 alone does not lead to formation of caveolae because caveolin-2 does not exit the Golgi

unless caveolin-1 is co-expressed. Thus, caveolins-1 and -3 are essential for formation of caveolae in their respective cell types; however, a recently recognized family of proteins called cavins (cavin-1, -2, -3 and -4) are also critical for the formation, regulation and morphology of caveolae (Hill et al., 2008; Bastiani et al., 2009; Chidlow et al., 2010).

Knockout mice have been created for each caveolin; such mice display a variety

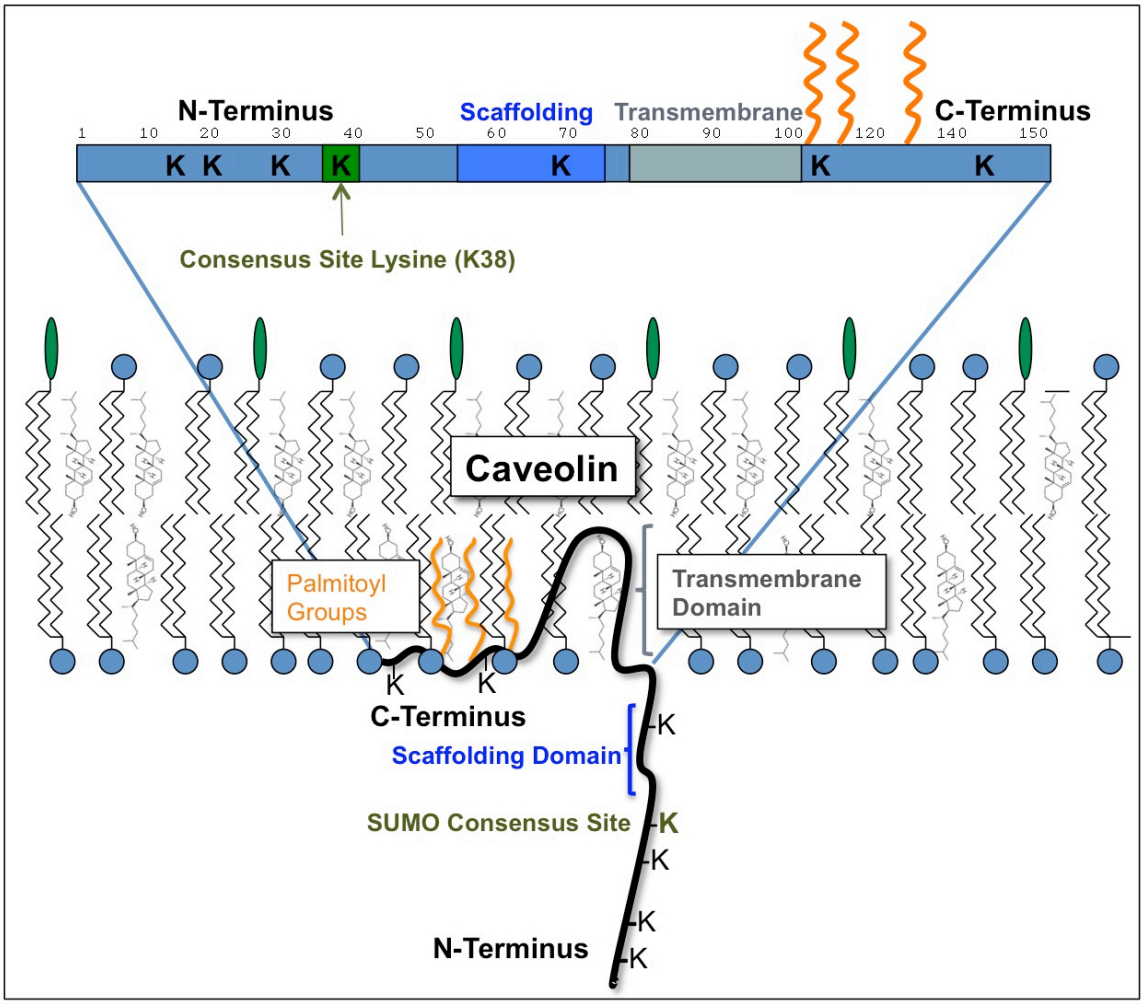

Figure 1.2 Caveolin Membrane Topology
Caveolins form a hairpin loop in the plasma membrane such that both the N- and C-termini face the cytoplasm. Caveolin-3 (P51638 [CAV3_RAT]; see also Figure S2.1 for evolutionary conservation of lysines) has seven lysine residues that are potential sumoylation sites. A putative SUMO consensus site at K38 in the N-terminus lies near the caveolin scaffolding domain. A hydrophobic transmembrane domain, cholesterol binding and palmitoylation at three cysteine residues in the C-terminus helps anchor caveolin-1 and -3 in the plasma membrane and stabilize caveolin oligomers.

of phenotypes, thereby implicating caveolins in numerous cellular functions including; signal transduction, endocytosis, vesicular trafficking, and cholesterol homeostasis. Phenotypes of caveolin knockout mice include: cardiac and pulmonary disease, skeletal muscle myopathy, adipose tissue abnormalities (lipodystrophies), reduction of atherosclerosis, disruption of eNOS signaling, and altered angiogenesis and tumorigenesis (Patel et al., 2008). Despite the lack of understanding of the precise molecular and biological mechanisms that cause these phenotypes, such findings suggest that caveolins may be an important therapeutic target for a variety of diseases.

Mutations in the muscle-specific caveolin-3 gene (CAV3) cause a group of diseases known as "caveolinopathies" that include a sub-type of limb-girdle muscular dystrophy (LGMD-1C), rippling muscle disease, cardiomyopathy, hyperCKemia and lipodystrophy (Woodman et al., 2004). Caveolin-3 localizes to the muscle cell plasma membrane (sarcolemma) and associates with dystrophin (another muscular dystrophy-associated protein) and members of the dystrophin complex (Song et al., 1996). Interestingly, this dissertation identifies a specific SUMO E3 ligase (PIASy) as a regulator of caveolin-3 sumoylation that is regulated by an E3 ubiquitin ligase called TRIM32 (Albor et al., 2006). Mutations in TRIM32 cause another subtype of muscular dystrophy (LGMD-2H), thus implying that sumoylation of caveolin-3 may contribute to the pathophysiology of muscular dystrophy.

The identification of an enrichment of a variety of receptors and signaling proteins in caveolae led to the proposal of a "caveolin signaling hypothesis" (Lisanti et al., 1994) in which caveolin functions as a scaffold that compartmentalizes, modulates and integrates signaling events (Shaul and Anderson, 1998; Insel et al., 2005). Binding to

caveolins often inhibits the activity of signaling molecules (e.g. eNOS) such that they are ready for the activation of signal transduction cascades (Couet et al., 1997).

Proteomic studies using subcellular fractionation, immunoprecipitation and mass spectrometry have revealed that greater than 300 proteins are enriched in caveolae, many bound to and regulated by interaction with caveolins (Sprenger et. al., 2007, Foster et. al., 2003). Among these proteins are numerous receptors and downstream signaling proteins that are enriched or found exclusively in caveolae membranes such as the beta 2-adrenergic receptor (β_2AR) and endothelial nitric oxide synthase ([eNOS], Xiang et al., 2002; Feron et al., 1998). Proteins can also undergo rapid translocation into or out of caveolae in response to specific stimuli (e.g., bradykinin receptors, muscarinic cholinergic receptors and the glucose transporter GLUT-4 (Fecchi et al., 2006; Sabourin et al., 2002)). Many proteins that bind caveolins do so via a "caveolin-binding motif" (ΦxΦxxxxΦ and ΦxxxxΦxxΦ: Φ = aromatic residue; W, F or Y). This sequence of aromatic residues binds the caveolin-scaffolding domain (CSD) in the N-termini of caveolins (Couet et al., 1997; Figure 1.1 and Figure 1.2).

Despite this long history of inquiry and recent advances, many questions about the function of caveolae and their primary component, caveolin, remain unanswered. For example, the mechanisms by which proteins bind to caveolins and do so in a dynamic manner is poorly understood. How are proteins targeted to caveolae, and, once bound to caveolin, how are they released? Are the CSD and caveolin-binding motifs the primary determinants of protein-protein interactions with caveolin, or are other mechanisms involved? Because of their rapid kinetics and regulated reversibility, regulation by posttranslational modifications may provide an answer to these questions. This

dissertation shows, for the first time, that caveolin-3 undergoes posttranslational modification by SUMO proteins. Modification by SUMO can both create and mask binding surfaces on target proteins, thereby altering their protein-protein interactions (Gocke et al., 2005). As such, sumoylation is a novel mechanism for the regulation of caveolin and its interactions with its signaling partners. This dissertation focuses on the posttranslational regulation of caveolin-3 by sumoylation. Of note, motifs important for covalent and non-covalent interaction with SUMO are also present in caveolin-1 (Figure 1.1 and 1.6). Therefore, the data and conclusions presented here may have implications for the regulation of the more widely expressed caveolin-1 as well as caveolin-3.

1.2 Posttranslational Modifications of Caveolins

Posttranslational modifications (PTMs) of proteins are alterations of the chemical nature of its constituent amino acids following translation. PTMs expand the functional and regulatory capability of a protein by covalently modifying it with functional groups such as phosphate, lipid, carbohydrate, nitrosyl groups (S-nitrosylation) as well as protein modifiers, such as ubiquitin. Phosphorylation is an important and well-studied PTM involved in a variety of cellular processes (Graves and Krebs, 1999; Hunter, 2009). Phosphorylation of caveolins-1 and -2 regulate their oligomerization (Lee et al., 2002) as well as caveolin-dependent caveolae formation (Sowa et al., 2003). Several kinases phosphorylate caveolin-1 on tyrosine including epidermal growth factor receptor (EGFR [Guha et al., 2008]), insulin receptor (IR [Kimura et al., 2002]), insulin-like growth factor receptor (IGF-IR [Maggi et al., 2002]), Src and Fyn (Labrecque et al., 2004). To date, no phosphorylation sites have been identified on caveolin-3, although as many as 8

phosphorylation sites have been documented on caveolin-1 (Table 1.1). Of these sites, phosphorylation at Y14 appears to be the primary regulatory site (Shajahan et al., 2007, Orlichenko et al., 2006, Labrecque et al, 2004). Multiple phosphorylation sites have been documented on caveolin-2 as well (Table 1.1). Phosphorylation at S36 has a role in modulating mitosis in endothelial cells (Sowa et al., 2003). Tyrosine phosphorylated caveolin-2 does not heterooligomerize; the Y19-phosphorylated form can exist as a monomer or dimer while the Y27 form is only a monomer (Lee et al., 2002).

Several disease-causing mutants of caveolin-3 are retained in the Golgi or ER and undergo ubiquitination and proteasomal degradation in response to protein misfolding (Galbiati et al., 2000; Galbiati et al., 1999). Otherwise, there is no evidence that wild-type caveolin-3 is regulated by modification with ubiquitin or other ubiquitin-like modifiers.

Caveolin-1 and -3, but not caveolin-2, are palmitoylated on three cysteine residues in the C-terminal region (Figure 1.2, Table 1.1) but palmitoylation is not

Table 1.1 Posttranslational Modifications of Caveolins

		Modification	Kinase/Enzyme	Reference
Caveolin-1	K5	N6-acetyllysine		
	Y6	Phosphotyrosine		
	S9	Phosphoserine		
	Y14	Phosphotyrosine	IR, IGF-IR, Src, Fyn, Abl	Maggi et al., 2002; Sanguinetti et al., 2003; Labrecque et al., 2004; Cao et al., 2002
	T15	Phosphothreonine		
	Y25	Phosphotyrosine		
	S37	Phosphoserine	PKC	
	Y42	Phosphotyrosine		
	S80	Phosphoserine		
	C133	S-palmitoyl cysteine		Dietzen et al., 1995; Uittenbogaard et al., 2000
	C143	S-palmitoyl cysteine		
	C156	S-palmitoyl cysteine		
Caveolin-2	S18	Phosphoserine		
	Y19	Phosphotyrosine	Src	Lee et al., 2002
	S20	Phosphoserine		
	S23	Phosphoserine	CK-2	Sowa et al., 2003
	Y27	Phosphotyrosine	Src	Wang et al., 2004
	S36	Phosphoserine	CK-2	Sowa et al., 2003
	S135	Phosphoserine		
Caveolin-3	C106	S-palmitoyl cysteine		Dietzen et al., 1995; Uittenbogaard et al., 2000
	C116	S-palmitoyl cysteine		
	C129	S-palmitoyl cysteine		

necessary for targeting caveolin to caveolae (Dietzen et al., 1995). Palmitoylation of caveolin can take place prior to its transport to the cell surface. For most acylated proteins palmitoylation is reversible and regulatable but palmitoylation of caveolin is irreversible (Parat et al., 2001). Palmitoylation seems to be important for stabilization of caveolin oligomers and assists the hydrophobic, transmembrane domain in anchoring caveolin proteins in the plasma membrane (Monier et al., 1996).

Given the dynamic nature of proteins that traffic in and out of caveolae and bind to caveolin, one can presume that caveolin proteins are dynamically regulated by a variety of PTMs, some of which have not-yet been discovered or identified. In this regard, NEDD8 and the NEDD8-specific E2 conjugating enzyme (Ubc12) interact with caveolin-1 and have been localized to caveolae (Foster et al., 2003; Ewing et al., 2007).

1.3 Sumoylation, SUMO and the Ubiquitin-Like Protein (Ubl) Family

Sumoylation is the reversible PTM of proteins by small ubiquitin-like modifier (SUMO) proteins. A growing number of substrates are known to be modified by SUMO and sumoylation plays a key role in a variety of biological processes; these include signal transduction, cell cycle regulation, gene transcription, pathogen/viral infection and cellular localization (Wilkinson and Henley, 2010). Sumoylation is related to, but distinct from, the well-studied process of ubiquitination. Ubiquitin and ubiquitin-like modifiers (Ubls) are ~100 amino acid proteins that modulate protein function through covalent attachment to lysine residues on target proteins. Ubiquitin, so named for its universal expression in cells, was originally identified for its role in an ATP-dependent proteolytic system present in cellular extracts. The function and components of the ubiquitination

pathway were discovered in the early 1980s (Hershko et al., 1983). The SUMO proteins were not discovered until almost two decades later due to their relatively low sequence homology with ubiquitin and difficulty of detection. SUMO modifications are often transient and many sumoylated substrates are expressed in cells at low abundance.

Sumoylation was first identified by Frauke Melchior as a 20 kDa electrophoretic mobility shift of the Ran GTPase–activating protein, RanGAP1 (Mahajan et al., 1997). Modification by SUMO-1 is necessary for targeting RanGAP1 to the nuclear pore where it forms a stable complex with RanBP2. This discovery was aided by the unique stability of SUMO-1-modified RanGAP1. The high stoichiometry of sumoylated to non-sumoylated RanGAP1 is due to its unique resistance to de-sumoylation by SUMO-specific proteases (SENPs) (Zhu et al., 2009; Chapter 1.8). Of note, the likely reason that sumoylation was not discovered until rather recently is because these proteases rapidly cleave SUMO from substrates upon cell lysis and are not inhibited by classic protease inhibitors. Therefore, special care must be taken to preserve sumoylated substrates when making cell lysates by adding a cysteine alkylating agent (e.g. N-ethylmaleimide) or high concentrations of denaturing detergents (e.g. 1-2% SDS) to inhibit SENP activity.

Since SUMO's discovery, other structurally similar Ubl family members have been identified. Ubls modify protein targets through a biochemical pathway that is similar to that of ubiquitin (Chapter 1.4). Ubls share varying degrees of amino acid similarity with ubiquitin, making their relationship not immediately obvious, however they all adopt a highly similar and distinctive β-Grasp structural fold ("ubiquitin fold", Figure 1.3). Other examples of Ubls include; FAT10, NEDD8, ISG15, Urm1, ATG8 and

ATG12. Modification by ubiquitin and Ubls help regulate nearly every aspect of cellular function (Hochstrasser, 2009; Gocke et al., 2005).

Ubiquitination occurs as either mono- or multi-ubiquitination of adjacent lysines or addition of poly-ubiquitin chains that are assembled by a variety of linkages via each lysine residue present in ubiquitin (e.g. K11, K48, K63). These different chain linkages have distinct functional consequences due to the ability of different ubiquitin-binding domains to distinguish between them (Dikic et al., 2009B). This variety of modifications has diverse effects on substrates, ranging from proteasome-dependent degradation to modulation of protein localization, function, structure, and complex formation.

Akin to ubiquitin, both SUMO-2 and SUMO-3 (SUMO-2/3) proteins can form polymeric chains as a consequence of an exposed, internal SUMO consensus motif near their N-termini (VKTE, Figure 1.3). SUMO-1 lacks this motif; however, it may act as a chain terminator for poly-SUMO-2/3 chains (Geoffroy and Hay, 2009). The role of poly-SUMO chains remains to be established; however, evidence for their role in proteasomal degradation has been shown by the identification of RNF4, a poly-SUMO-specific ubiquitin E3 ligase (Sun et al., 2007; Hay, 2007; Geoffroy and Hay, 2009). RNF4 has four tandem SUMO-Interacting Motifs (SIMs, Chapter 1.7) that allow it to selectively recognize and ubiquitinate poly-sumoylated proteins, targeting them for proteasomal degradation. Caveolin-1 and -3 have two tandem SIMs that resemble the SIMs of RNF4 (Figure 1.6; Figure S2.7), suggesting that caveolins may interact non-covalently with SUMO proteins.

Lower eukaryotes express a single SUMO-like modifier (Smt3 or sentrin) while higher eukaryotes express three conjugatable SUMO paralogs. Despite their structural

similarity, SUMO-1 is only 18% identical and 48% homologous to ubiquitin, while SUMO-2 and -3 (which are 97% identical), are 46% identical and 66% homologous (Figure 1.3). While almost all SUMO-1 in a cell is involved in conjugates, a free pool of SUMO-2/3 exists that becomes conjugated in response to stress or stimuli such as heat shock, oxidative stress or ethanol exposure (Manza et al., 2004). A fourth SUMO gene has been identified, however a unique proline residue (P90) inhibits its maturation

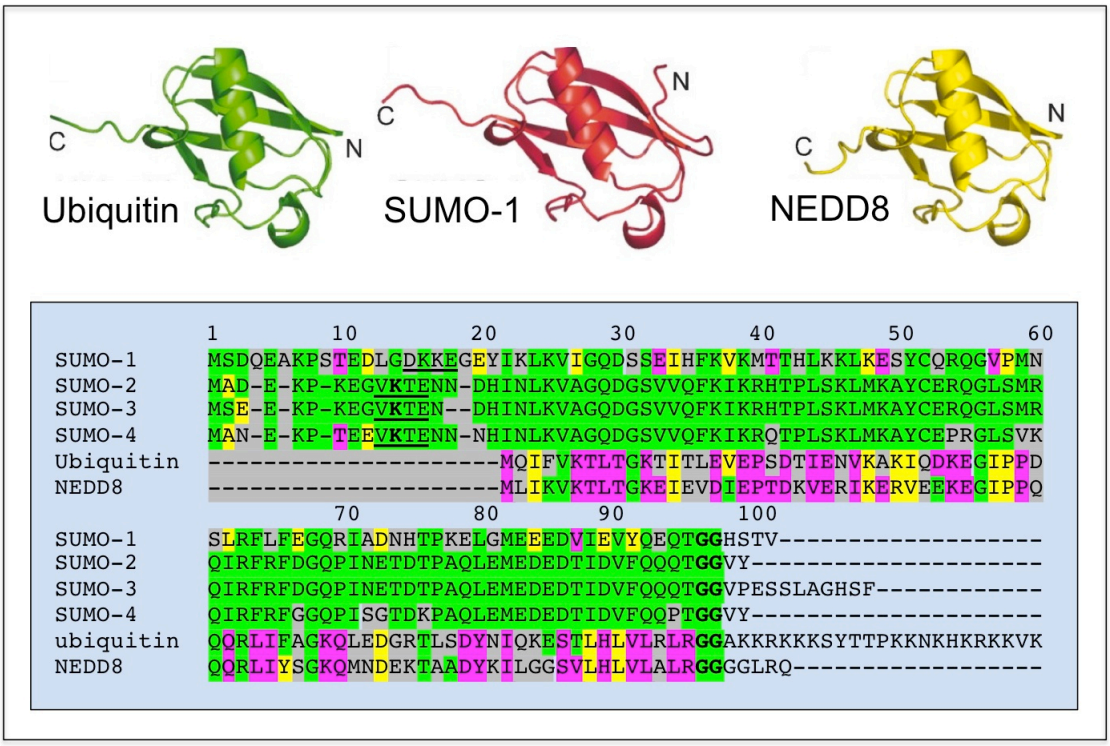

Figure 1.3 3D Structures and Amino Acid Sequence Alignment of SUMO Proteins with Ubiquitin and the Ubiquitin-like protein (Ubl) NEDD8
Despite a relatively low degree of sequence homology, ubiquitin, SUMO and all other Ubls (e.g. NEDD8) adopt a highly similar, β-grasp structural fold (images adapted from Dye and Schulman, 2007). Ubiquitin and Ubls also share a conserved C-terminal Gly-Gly motif that is exposed after proteolytic processing of the full-length precursor protein and is necessary for isopeptide bond formation with lysine residues on target proteins. SUMO-2 and -3 are almost identical, differing by only 3 residues after cleavage of their C-tails. A major difference between SUMO-1 and SUMO-2/3 is the presence of a SUMO consensus motif (VKTE) that, like ubiquitin, allows SUMO-2/3 to polymerize and form poly-SUMO chains. SUMO-4's biological role is unknown. It cannot be processed to an activated form due to a kink caused by a unique proline residue adjacent to the Gly-Gly (PTGG) and therefore cannot be conjugated to other proteins.

(Figure 1.3). Thus, SUMO-4 is unable to form covalent isopeptide bonds, though it may play a role in non-covalent interactions (Owerbach et al., 2005).

Sumoylation regulates protein-protein interactions by masking or creating additional binding surfaces on target proteins (Wilkinson and Henley, 2010; Gocke et al., 2005). Target proteins are selectively modified by SUMO-1 or SUMO-2/3 with differing consequences, presumably due to the ability of different SIMs (Chapter 1.7) that bind SUMO non-covalently to distinguish between SUMO paralogs (Hay, 2007).

Sumoylation was initially thought to occur primarily in the nuclear or perinuclear compartments where it helps regulate nucleo-cytoplasmic transport, gene expression and genome integrity (Muller et al., 2004). It is now apparent that sumoylation also regulates cytoplasmic and plasma membrane proteins. (Seeler et al., 2003; Bossis and Melchior, 2006A). A growing list of integral plasma membrane proteins have been identified as targets of sumoylation, such as the K^+ channel Kv1.5 (Benson et al., 2007, Scheschonka et al., 2007), the glucose transporters GLUT1 and GLUT4 (Giorgino et al., 2000; Martin et al., 2007B), and several G-protein-coupled receptors (GPCRs, e.g. kainate and metabotropic glutamate receptors (Martin et. al., 2007A). TGFβ induces sumoylation of the Type I TGFβ receptors (TβRI [Kang et al., 2008]), which localize to caveolae and interact with caveolin (Santibanez et al., 2008, Schwartz et al., 2005). Interestingly, several sumoylated proteins involved in signal transduction also localize to caveolae and interact with caveolin including; Kv1.5, PTP1B and RGS-Rz (Foster et al., 2003; Lee et al., 2006; Martens et al., 2007; Rodriguez-Munoz et al., 2007; Dadke et al., 2007). Thus, modification by SUMO may contribute to the ability of caveolin to dynamically bind and regulate numerous, diverse proteins.

1.4 Mechanism of Sumoylation

All ubiquitin-like modifications are carried out by a similar biochemical mechanism. Each Ubl has a specific set of E1, E2 and E3 enzymes responsible for conjugation to targets, as well as specific isopeptidases responsible for removal from targets (e.g. DUBs, SENPs). Ubl proteins are expressed as inactive precursors that require proteolytic processing to expose conserved, C-terminal di-glycine residues. In the case of sumoylation, inactive SUMO precursors are processed by SENPs that also remove SUMO from modified proteins (Figure 1.4, Chapter 1.8). This action exposes the glycine residue that forms isopeptide bonds with the ε-amino group of target lysines (Figure 1.4). The catalytic cysteine of the SUMO E1 activating enzyme forms a thioester bond with SUMO in a Mg^{2+}-ATP-dependent manner. The SUMO E1 is a heterodimer of SAE1 (Aos1) and SAE2 (Uba2) with sequence homology to the N- and C-termini, respectively, of ubiquitin E1 enzymes (Johnson et al., 1997). The SUMO~E1 thioester is then transferred to the catalytic cysteine of the SUMO E2 conjugating enzyme (Ubc9), which can directly modify substrates (Figure 1.4). Many SUMO targets have a consensus motif (Ψ-K-X-[D/E]) that is recognized by Ubc9 (Chapter 1.6, Sternsdorf et al., 1999, Sampson et al., 2001). Consequently, the SUMO E1 and E2 are sufficient for sumoylation of many substrates *in vitro*; however, *in vivo* an E3 ligase (e.g. PIAS family of SUMO E3s) is usually required for efficient sumoylation (Bohren et al., 2007). Sumoylation is regulated at both the substrate and the enzyme levels by diverse mechanisms. Other PTMs of a given substrate on or near the target lysine (e.g. ubiquitination, phosphorylation or acetylation) can prevent, promote or compete with sumoylation for modification of the

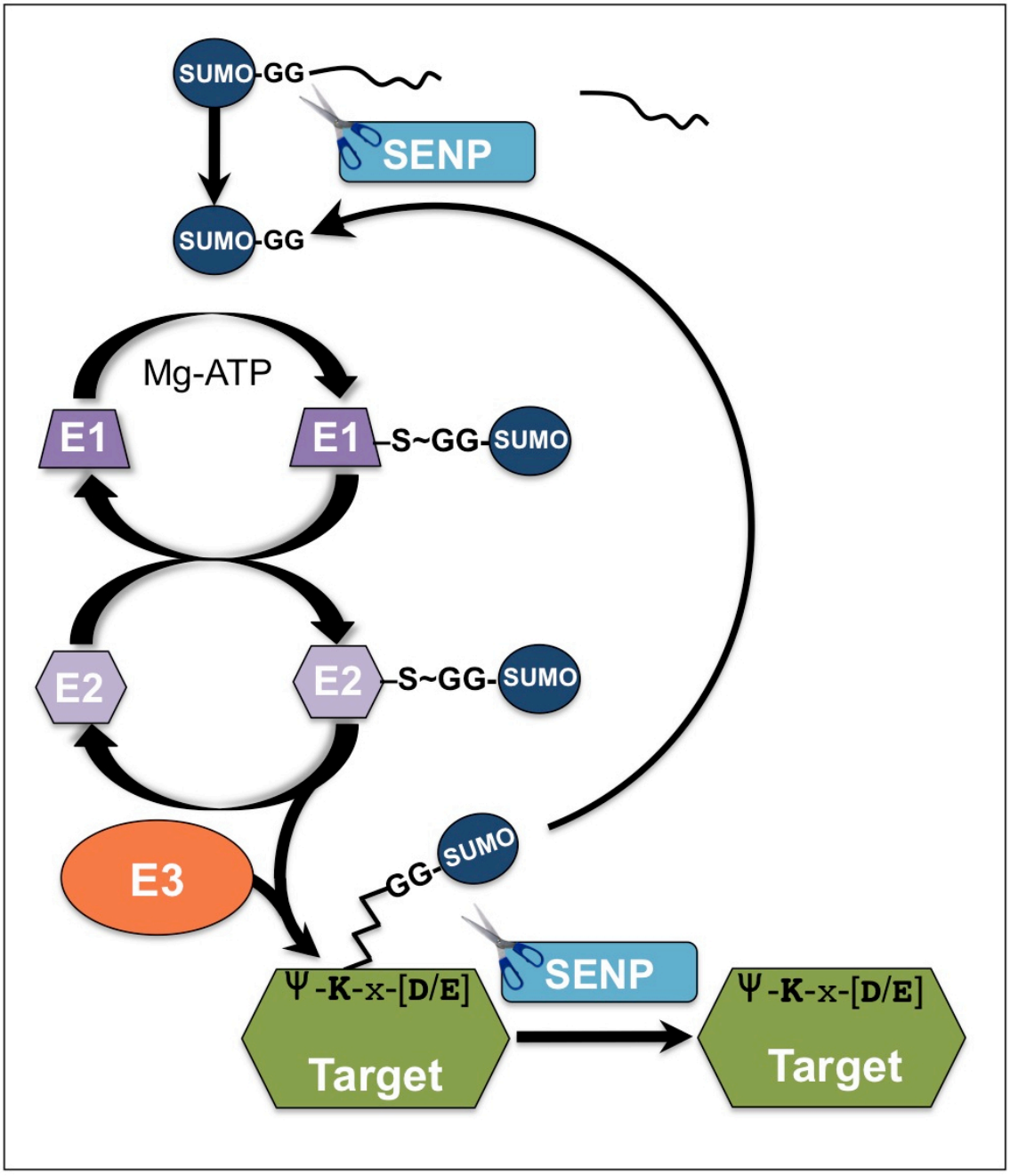

Figure 1.4 Mechanism of Sumoylation
SUMO precursors are processed by SUMO-specific proteases (SENPs) to reveal the c-terminal Gly-Gly that forms isopeptide bonds with target Lys residues. The SUMO E1 activating enzyme (SAE1/SAE2) forms a thioester with SUMO in a Mg^{2+}/ATP-dependent manner. Activated SUMO is transferred to the catalytic Cys residue on Ubc9, the SUMO E2 conjugating enzyme. SUMO targets often have a consensus motif, Ψ-K-X-[D/E], which interacts directly with Ubc9. The SUMO E1 and E2 enzymes are sufficient to modify targets *in vitro*, however an E3 ligase is usually required *in vivo* for efficient sumoylation. SUMO modification of targets is reversed by SENPs that cleave isopeptide bonds, readying the cycle of modification to begin again.

target lysine. The enzymes involved in sumoylation and de-sumoylation are regulated as well (Chapter 1.5, Chapter 1.8). Oxidative stress appears to be an important regulatory mechanism since it alters and increases SUMO-2/3 conjugates and leads to the formation of reversible disulfide bonds between the catalytic cysteine residues of the SUMO E2 and E1 enzymes (Bossis and Melchior, 2006B).

The SUMO E1, E2 and E3 enzymes are targets of auto- and cross-sumoylation. Auto-sumoylation of Ubc9 at K14 alters its target discrimination; activity toward histone deacetylase 4 (HDAC4) and acute promyelocytic leukaemia protein (PML) is unaltered but sumoylation impairs the activity of Ubc9 toward RanGAP1 and activates sumoylation of Sp100 (Knipscheer et al., 2008). This enhancement is dependent on a SIM in Sp100 that creates an additional binding surface with the isopeptide-linked SUMO-Ubc9 conjugate (a mechanism distinct from Ubc9~SUMO thioester recruitment) thereby providing an increase in affinity that is usually provided by a SUMO E3 ligase.

Recently, a novel regulation of the SUMO E1 and E2 enzymes by 'cross-sumoylation' has been identified (Subramaniam et al., 2010). The small G-protein Rhes has SUMO E3 ligase-like properties and also enhances E1-E2 cross-sumoylation. Rhes directly binds to both the SUMO E1 and E2 and enhances transfer of SUMO from E1~thioesters to formation of isopeptide linkages lysine residues on Ubc9. Likewise, sumoylation of E1 is enhanced through transfer of SUMO from Ubc9~thioesters.

1.5 SUMO E3 Ligases – PIASy

E3 ligases provide specificity to ubiquitin-like modifications by recognizing targets and mediating transfer of the Ubl from an E2 conjugating enzyme to the substrate.

E3 ubiquitin ligases consist of a wide variety of monomeric proteins and multi-protein complexes (e.g. Skp, Cullin, F-box (SCF) containing complexes). HECT (Homologous to E6-AP Carboxyl Terminus) domain E3 ubiquitin ligases have catalytic capabilities while RING (Really Interesting New Gene) domain and U-Box E3 ligases serve as adaptors that orient the E2~Ubl thioesters with target lysine residues on the substrate (Joazeiro et al., 2000). The first SUMO E3 ligases, Siz1 and Siz2, were identified in S. cerevisiae (Johnson et al., 2001) and are required for most sumoylation activity in yeast. While hundreds of E3 ubiquitin ligases have been identified, only a handful of mammalian proteins are known to act as E3 SUMO ligases; these include RanBP2, Pc2, Topors, and the mitochondrial-specific MAPL (Braschi et al., 2009). The most prominent E3 SUMO

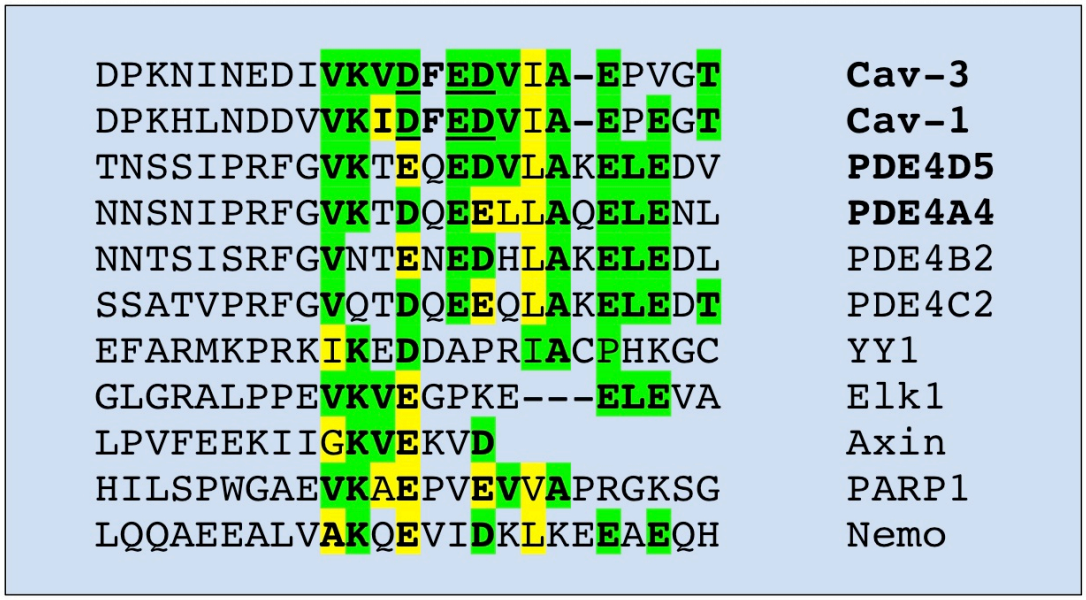

Figure 1.5 Alignment of Caveolin-1 and Caveolin-3 with PIASy-Mediated SUMO Targets

Sequence alignment of caveolin-1, caveolin-3 and PDE4 subtypes with other known PIASy mediated SUMO targets reveals a high degree of similarity in hydrophobic and acidic residues downstream of the core SUMO consensus motif Ψ-K-X-[D/E]. Interestingly, PDE4D5 and PDE4A, but not PDE4B2 and PDE4C2 are sumoylated. While PDE4B2 and PDE4C2 have almost identical amino acid sequence, they lack the necessary lysine residue for sumoylation (Li et al., 2010).

ligases belong to the PIAS (Protein Inhibitor of Activated STAT Signaling) family and possess a "RING related motif" (Johnson et al., 2001). PIAS proteins are eukaryotic homologs of the yeast Siz1 and Siz2 SUMO E3s and were originally identified as cofactors that inhibit DNA binding and transcriptional activation by STATs. There are five mammalian PIAS proteins: PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy (Schmidt and Muller, 2002).

This dissertation identifies PIASy as a SUMO E3 ligase specific for caveolin-3. It was recently shown that PIASy also targets PDE4D5 (Li et al., 2010), a β_2AR-specific phosphodiesterase (Baillie et al., 2003, Lynch et al., 2005, Li et al., 2009). A comparison of the primary sequence surrounding the sumoylation sites of caveolin-3 and PDE4 reveal a high degree of similarity of the residues downstream and within their core SUMO consensus motifs (Figure 1.5). Other PIASy targets include; p53, HIF1α, YY1, Elk1, Ets1, NEMO, Axin and Topoisomerase-II (Carter et al., 2007; Deng et al., 2007; Nishida et al., 2007; Kang et al., 2010; Mabb et al., 2006; Azuma et al., 2005; Rui et al., 2002). Of note, PIASy interacts with the transforming growth factor-beta type I receptor (TβRI), which localizes to caveolae and interacts with caveolin-1 via the CSD (Razani et al., 2001, Conrotto et al., 2007). TβRI is modified by SUMO-1 in response to TGF-β and sumoylation of the activated receptor enhances its signaling through enhanced recruitment and activation of Smads (Kang et al., 2008).

1.6 The Core SUMO Consensus Motif and Extended Motifs

Many SUMO targets have a consensus motif that surrounds the target lysine, Ψ-K-X-[D/E] (Ψ = IVL). The SUMO E2 conjugating enzyme (Ubc9) interacts directly with the residues in this core motif (Bernier-Villamor et al, 2002). One can identify potential SUMO substrates by searching for this motif; however, many substrates are modified on sites that do not conform to this minimal motif (Blomster et al., 2009). The core motif is found in ~1/3 of all proteins and since not all SUMO consensus sites are modified, other factors must play a role. Attempts have been made to expand the core motif to better predict SUMO substrates. Extended motifs have been identified by examining residues upstream or downstream of the core motif in validated SUMO targets.

A '<u>p</u>hosphorylation-<u>d</u>ependent <u>s</u>umoylation <u>m</u>otif' (PDSM) that consists of a downstream serine residue followed by proline, ΨKxExxSP, has been identified in a few substrates, many of which are transcription factors (e.g. HSFs, GATA-1, and MEF2; Hietakangas et al, 2006). Phosphorylation of the serine residue within this motif plays an important role in regulating the sumoylation status. Analysis of validated SUMO sites in transcription factors revealed an over-representation of acidic residues in the tail region downstream of the core motif compared to randomly selected proteins containing potential core SUMO motifs (Yang et al., 2006B). Thus, an extended sumoylation motif, called the '<u>n</u>egatively charged amino acid-<u>d</u>ependent <u>s</u>umoylation <u>m</u>otif' (NDSM) was identified and demonstrated to predict targets modified by SUMO (Blomster et al., 2009).

Caveolin-1 and -3 both have a conserved NDSM in their N-termini that is followed by a conserved proline residue (**VKID**F**<u>ED</u>**VIA**<u>E</u>P** and **VKVD**F**<u>ED</u>**VIA**<u>E</u>P** respectively, Figure 1.1 and Figure 1.6), suggesting that they are potential SUMO substrates. This eight amino acid sequence immediately downstream of the core SUMO

motif, F**ED**VIA**EP** that conforms to the NDSM, is known as the "caveolin signature motif" (Tang et al., 1996) as it is found in all 3 caveolin family members. In the NDSM, a sequence of several acidic residues is clustered within the 10 amino acids directly downstream of the core motif. The catalytic cleft of Ubc9 interacts with the core SUMO motif and the acidic residues are proposed to enhance modification by acting as a secondary binding site with a basic patch on the surface of Ubc9 (Mohideen et al., 2009).

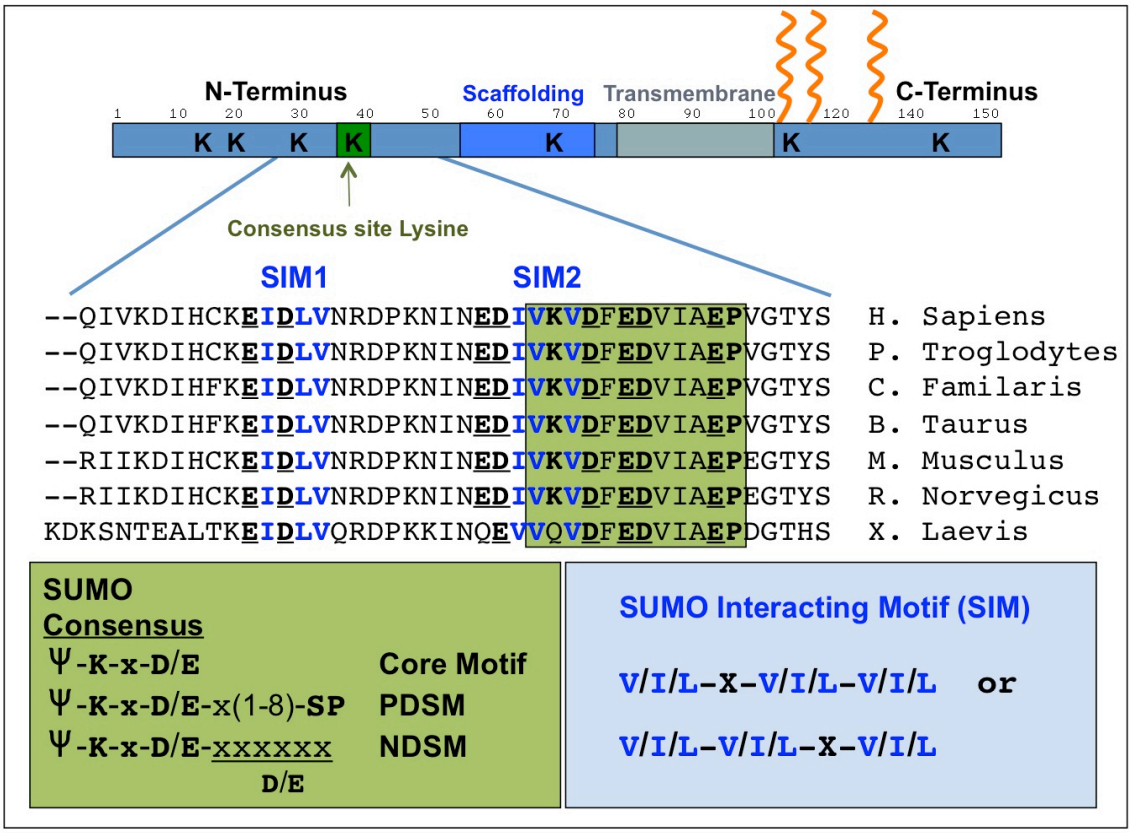

Figure 1.6 Caveolin-3 has an Evolutionarily Conserved SUMO Consensus Motif and Tandem SUMO Interacting Motifs (SIMs) in its N-Terminus

The evolutionarily conserved core consensus motif in caveolin-3 (VKVD) also conforms to an extended 'negatively charged amino acid-dependent sumoylation motif' (NDSM). SUMO-Interacting Motifs (SIMs) regulate non-covalent interactions with SUMO. Caveolin-3 has two tandem SIMs that are highly conserved and similar to SIMs found in several proteins known to bind SUMO non-covalently (see also Figure S2.7). The flanking acidic residues (underlined in bold: **D**/**E**) enhance the interaction (Song et al., 2004). Of note, SIM2 overlaps with caveolin-3's SUMO consensus site.

The majority of acidic residues in SUMO targets lie in an acidic patch 3-6 residues downstream of the core motif. This is consistent with the location of the phospho-serine residue in the PDSM, suggesting it is the negative charge that is important. Thus, insertion of an acidic residue in place of a phosphorylated serine residue in a PDSM is sufficient for efficient sumoylation (Yang et al., 2006B).

A search for proteins that share a similar NDSM with caveolin-1 and -3 (VK[VIL]DxED; www.expasy.org/tools/scanprosite/) revealed that SAE1, part of the SUMO E1 heterodimer, shares a strikingly similar sequence of amino acids (aa96-131, Figure 1.7). SAE1 is modified by SUMO-3 at K108 within this motif, which is almost identical to that in caveolins-1 and -3 (Manza et al., 2004). SAE1 has a second region (aa128-161) that is less similar, but still shares an identical core SUMO motif (VKVD) as well as a few upstream, hydrophobic residues. Other proteins with similar motifs include AKAP9, ZN513, calnexin, cardiomyopathy-associated protein 5 (CMYA5), Early endosome antigen 1 (EEA1), glucocorticoid receptor, potassium voltage-gated channel

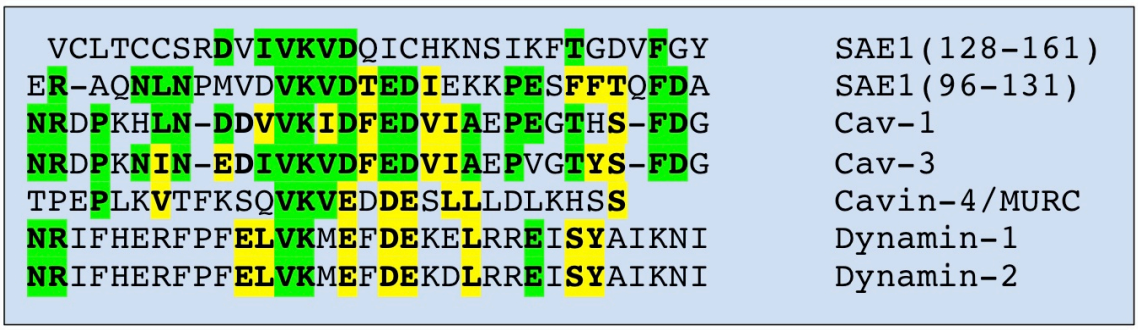

Figure 1.7 Caveolin-1 and -3 Share a Similar NDSM with the SUMO Activating Enzyme (SAE1)
SAE1 is modified by SUMO-3 at K108, which lies in a SUMO consensus motif almost identical to that in caveolin-1 and -3. Similar motifs are found in cavin-4/MURC, dynamin-1 and dynamin-2 suggesting that that they may also be modified by SUMO.

subfamily H member 1 and 5 (Kv10.1 and Kv10.2), PDE4A, PDE4D, PDE6A, dynamin-1 and dynamin-2 (Figure 1.7). Dynamin is responsible for pinching off the neck of caveolar vesicles during endocytosis (Razani et al., 2002) and interacts non-covalently with the sumoylation machinery (SUMO-1, Ubc9 and PIAS-1; Mishra et al., 2004), although it has not yet been identified as a target of covalent SUMO conjugation, it is possible that sumoylation has a role in regulation of endocytosis of caveolar vesicles. Notably, another protein that shares this motif is muscle-related coiled-coil protein (MURC or cavin-4; Figure 1.7), which is the muscle-specific member of the cavin family (Bastiani et al., 2009).

1.7 SUMO Interacting Motifs (SIMs) – Non-Covalent Interactions

Proteins interact non-covalently with ubiquitin through ubiquitin binding domains (UBDs); to date over 15 UBDs have been identified (Dikic et al., 2009A). The different UBDs can distinguish between various ubiquitin chain linkages to mediate a wide variety of functional outcomes (Dikic et al., 2009B). SUMO proteins play biological roles distinct from ubiquitin, thus they require recognition by a distinct, SUMO-specific binding domain. Proteins interact non-covalently with SUMO via a SUMO Interacting Motif (SIM). In contrast to the variety of UBDs, only one SIM has been identified (Song et al, 2004), which consists of a core sequence of hydrophobic residues (V/L/I, X, V/L/I, V/L/I or V/L/I, V/L/I, X, V/L/I; Figure 1.6) and flanking acidic residues that enhance affinity (Song et al., 2004 and 2005; Hecker et al., 2006; Tatham et al., 2008). SIMs can regulate paralog specificity of covalent modification (Meulmeester et al., 2008) and likely regulate sumoylation-dependent protein-protein interactions.

The presence of tandem SIMs in caveolin-1 and -3 suggests that they could bind non-covalently with mono- or poly-sumoylated proteins (Figure 1.6 and Figure S2.7). Alternatively, due to the proximity of these SIMs to caveolin's SUMO consensus motif, an interesting possibility is that they help recruit and orient specific Ubc9~SUMO thioesters, thereby influencing the type of sumoylation (e.g. di- or poly-SUMO-2/3 or mixed SUMO-2/3~SUMO-1 chains). In addition, caveolin forms homo- and hetero-oligomers, so these SIMs could help direct the sumoylation of an adjacent caveolin monomer intermolecularly rather than intramolecularly.

1.8 SUMO Specific Proteases (SENPs)

SUMO-specific proteases (sentrin-specific protease, SENPs) belong to the cysteine protease clan and have a conserved, C-terminal catalytic domain with unique N-terminal regulatory domains (Yeh, 2009; Figure 3.1 A). SENPs carry out several functions in SUMO metabolism. They have endopeptidase (C-terminal hydrolase) activity that allows them to cleave inactive SUMO precursors to produce the conserved Gly-Gly motif necessary for conjugation to lysine residues on target proteins (Hay, 2007). SENPs also possess SUMO-specific isopeptidase activity that deconjugates SUMO from targets and de-polymerizes or 'edits' poly-SUMO chains (Figure 1.4).

Yeast possesses two Smt3-specific (the yeast SUMO homolog) proteases (Ulp1 & Ulp2) that both can remove Smt3 from proteins and process precursors via a homologous domain of about 200 amino acids. Sequence homology has identified 7 human genes with potential SUMO protease activity (SENP1, 2 , 3, 5, 6, 7 & 8); however, SENP8 was later shown to be specific for the Ubl NEDD8 rather than SUMO. Human SENPs can be

divided into two main groups based on their evolutionary relationships; SENP1, 2, 3 and 5 are more closely related to Ulp1, while SENP6 and 7 are more closely related to Ulp2 (Hay, 2007). The expansion of this protease family from yeast to humans likely reflects their evolution to perform specific cellular functions. Different SENPs have distinct subcellular localizations as well as substrate and SUMO paralog specificities.

The Ulp1-related SENPs can be divided into two subfamilies; SENP1 and 2, and SENP3 and 5. SENP1 and 2 are of most interest to this dissertation, since only they showed deconjugation activity towards sumoylated caveolin-3 (Figure 3.2). SENP1 displays paralog specificity in precursor processing but not in isopeptidase activity. Cells lacking SENP1 accumulate SUMO-1 conjugates and unprocessed SUMO-1 precursors, but SUMO-2/3 deconjugation and processing are not disrupted. SENP1 undergoes nucleo-cytoplasmic shuttling due to a N-terminal NLS and NES (Figure 3.1 A; Kim and Baek, 2005). SENP2 is most similar to SENP1 (Figure 3.1 A) and does not discriminate between SUMO paralogs in deconjugation, but does so during processing of SUMO precursors. SENP2 also undergoes nucleo-cytoplasmic shuttling due to a N-terminal NLS and NES. It is found primarily in the nucleus and its cytosolic localization is regulated by ubiquitination and proteasomal degradation (Itahana et al., 2006).

It has recently been demonstrated that SENP2 expression is induced in a cAMP-dependent manner in adipocytes and plays an essential role in the control of adipogenesis (Chung et al., 2010). Chung et al. demonstrated that elevated cAMP induces SENP2 expression through phospho-CREB binding to a functional CRE (*cis*-acting cAMP response element) in SENP2's promoter region. The finding that SENP2 expression is induced by elevated cAMP levels complements other recent findings regarding the

PIASy dependent-sumoylation of the β_2AR-specific phosphodiesterase subtype, PDE4D5 (Li et al., 2010) and evidence presented in this dissertation regarding PIASy-dependent sumoylation of caveolin-3 and its effects on desensitization of the β_2AR. Such findings suggest that reversible sumoylation may provide a feedback mechanism that contributes to regulation and compartmentation of the cAMP signaling pathway in caveolae.

Members of a second subfamily, SENP3 and SENP5, the other members of the Ulp1 branch of the SENP tree, are closely related to one other. They both preferentially process and deconjugate SUMO-2/3 vs. SUMO-1 and contain N-terminal sequences that target them to the nucleolus. The Ulp2-related SENP6 and SENP7 subfamily members prefer poly-SUMO-2/3 as a substrate and are both found in the nucleoplasm (Kolli et al., 2010). In general, SUMO-2/3 conjugation and deconjugation appears to be more dynamic than that of SUMO-1 because most SENPs prefer SUMO-2/3 (Kolli et al., 2010), which exists in a "free" un-conjugated pool.

1.9 *In vitro* Sumoylation Assays

Sumoylation is a highly dynamic posttranslational modification and *in vivo;* sumoylated proteins are notoriously difficult to detect due to a typically low stoichiometry of modification (with the exception of RanGAP1) and the highly active SENPs. *In vitro* sumoylation assays have therefore become a valuable tool to identify new targets of sumoylation or validate targets identified by other methods, such as two-hybrid or proteomic studies. Assays are performed using small volumes of purified, recombinant proteins in the presence of buffer and Mg^{2+}-ATP (Werner et al., 2009). The reactions are then run out by SDS-PAGE and immunoblotted to detect characteristic

electrophoretic mobility shifts due to modification by SUMO (~15-20 kDa). Another type of *in vitro* sumoylation assay has been validated (Saitoh et al., 2009; Mencia and Lorenzo, 2004; Uchimura et al., 2004) and involves the co-transformation of E. coli with plasmids containing the sumoylation machinery and the substrate of interest. This system has advantages compared to expression in mammalian cells or *in vitro* studies with purified proteins. Unlike mammalian cells, E. coli do not express SENPs, so the higher stoichiometry of modification makes SUMO targets easier to identify. Such assays are also less expensive and time consuming than expressing and purifying each component individually. Only the substrate needs to be purified prior to downstream assays (e.g. SDS-PAGE, Mass Spectrometry, X-Ray Crystallography etc).

1.10 Beta 2-Adrenergic Receptor – Agonist-Induced Desensitization and Downregulation

The seven-transmembrane, G protein-coupled receptors (GPCRs) are the largest family of signaling molecules in the human genome as well as the most prominent class of drug targets (Drake et al., 2006). β1- and β2-adrenergic receptors (βARs) regulate the intrinsic contraction rate in myocytes in response to catecholamines (i.e. epinephrine and norepinephrine). β_1ARs and β_2ARs signal through distinct pathways. Caveolar localization and interaction with caveolin-3 is required for signaling by the β_2AR, but not the β_1AR (Xiang et al., 2002). β_1ARs and β_2ARs are highly homologous both structurally and functionally, sharing 52% identity overall and 76% identity in the transmembrane domains (Shcherbakova et al., 2007). β_1ARs and β_2ARs desensitize through distinct

pathways in response to agonist stimulation (Liang et al., 2003). Like β_2ARs, β_1ARs can localize to caveolae and interact with caveolin-3, but distribute among other compartments (Rybin et al., 2000). β_1ARs are resistant to agonist-induced ubiquitination and down-regulation (Liang et al., 2003; Liang et al., 2004). This difference is mediated by differential PTMs of their C-termini. Domain swapping of the β_2AR C-terminus into the C-terminus of B_1AR allows it to undergo agonist-induced ubiquitination and down-regulation and vice versa (Liang et al., 2003).

Agonist stimulation of GPCRs often leads to rapid desensitization mediated by phosphorylation by a G protein-coupled receptor kinase-(GRK) of serine and threonine residues of the receptors' C-termini. β-arrestin proteins then bind to phosphorylated receptors and act as adaptors for recruitment of the endocytic machinery which internalizes receptors through clathrin-coated pits (Shenoy et al., 2008). Agonist-induced conformational changes and β-arrestin binding to receptors terminates signaling by uncoupling receptors from heterotrimeric G-proteins and turning off their respective signaling cascades; however, some receptors are still able to signal after internalization (Ostrom et al., 2004; Marchese et al., 2008). This rapid desensitization occurs in response to acute stimulation by agonist while long-term desensitization requires removal of receptors from the plasma membrane and a decrease in the number of receptors. These events occur by two mechanisms: degradation of receptor protein in lysosomes and by a decrease in receptor mRNA levels (Shenoy et al., 2008).

The rates of agonist-induced receptor internalization, recycling, and lysosomal degradation vary widely among GPCRs, suggesting that distinct mechanisms control trafficking of different of receptors (Marchese et al., 2008). These mechanisms have been

best characterized for β_2ARs (Drake et al., 2006; Marchese et al., 2008). Activated β_2ARs are phosphorylated by GRK2 and preferentially interact with β-arrestin-2. The β_2AR-β-arrestin-2 complex is then internalized through clathrin-coated pits and sorted into early or late endosomes. Receptors can be either be resensitized by recycling back to the plasma membrane (by dephosphoylation in early endosomes in response to acute agonist stimulation), or targeted to lysosomes (from late endosomes in response to prolonged agonist stimulation) and degraded to terminate signaling. Thus, trafficking of β_2ARs is critical for signal termination and receptor resensitization.

For β_2ARs, receptor fate and the initial step of β-arrestin-2-mediated internalization are regulated by ubiquitination of both proteins by E3 ubiquitin ligases (Shenoy et al., 2001). Agonist-induced ubiquitination of β-arrestin-2 by the E3 ubiquitin ligase Mdm2 is critical for rapid β_2AR internalization (Shenoy et al., 2009). The deubiquitinating enzyme (DUB), ubiquitin-specific protease 33 (USP33), selectively removes ubiquitin from β-arrestin-2; the kinetics of β-arrestin-2 deubiquitination correlates with the dissociation of β-arrestin-2 from activated receptors (Shenoy et al., 2008). β-arrestin-2 also recruits the E3 ubiquitin ligase Nedd4 which ubiquitinates activated β_2ARs (Shenoy et al., 2008). USP20 and USP33 are DUBs that regulate the deubiquitination of β_2AR (Berthouze et al., 2009). Knockdown studies have shown that deubiquitination of receptors inhibits lysosomal trafficking and promotes recycling and resensitization from the late-endosomes back to the plasma membrane (Berthouze et al., 2009). USP20 and USP33 are constitutively bound to β_2AR and immediately dissociate upon agonist stimulation, allowing it to become ubiquitinated. Berthouze et al. speculate

that USP33 switches from constitutive association with β_2AR to association with β-arrestin-2 when it translocates to activated receptor. siRNA knockdown or dominant-negative expression of Nedd4 in HEK 293 cells blocks β-agonist-induced ubiquitination and lysosomal degradation of β_2ARs (Shenoy et al., 2008), suggesting that receptor fate is regulated by the opposing forces of E3 ligase- and DUB-mediated ubiquitination and deubiquitination of β_2ARs (Shenoy et al., 2009). Interestingly, proteomic studies have localized USP20 to caveolae (MacLellen et al., 2005) and β_2ARs are found almost exclusively in caveolae (Xiang et al., 2002).

Recent studies also indicate that GRK2's ability to phosphorylate β_2AR is regulated by S-nitrosylation, the covalent attachment of nitric oxide (nitrosyl groups) to cysteine residues. It has also been shown that eNOS mediated S-nitrosylation of

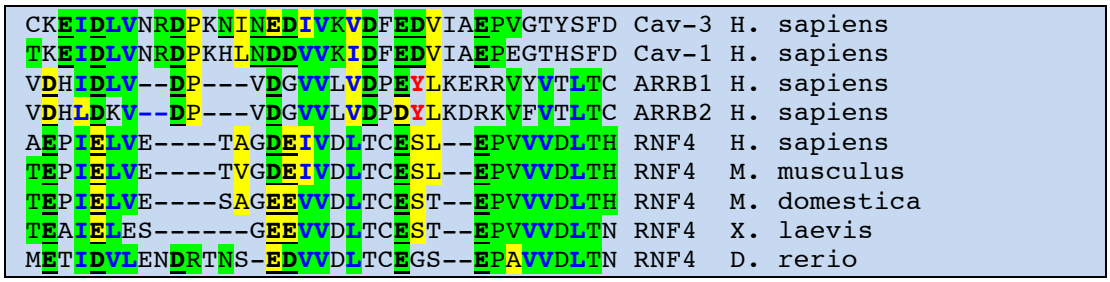

Figure 1.8 β-arrestin-1(ARRB1) and β-arrestin-2(ARRB2) contain tandem SIMs that align with the SIMs in caveolin-1, caveolin-3 and RNF4 proteins

RNF4 is an E3 ubiquitin ligase that selectively binds poly-sumoylated proteins via four tandem SIMs. Ubiquitination and proteasomal degradation of poly-sumoylated proteins represents a novel form of cross-talk between the ubiquitin and SUMO pathways. Comparison of RNF4's SIMs with caveolin-1 and -3 shows significant similarity between the hydrophobic (**VIL**) and acidic residues (**DE**) that define the SIM. A search for similar tandem motifs using expasy.org/tools/scanprosite revealed that β-arrestin-1(ARRB1) and β-arrestin-2(ARRB2) contained similar tandem SIMs in their N-termini (aa28-59). Interestingly, both β-arrestin-1 and β-arrestin-2 are tyrosine phosphorylated (**Y**) at positions that align with either acidic residues in caveolins or phosphorylatable serine residues in RNF4 (S) suggesting that tyrosine phosphorylation of β-arrestins may enhance or regulate their ability to bind SUMO proteins non-covalently.

β-arrestin-2 at a single cysteine residue regulates β_2AR trafficking. S-nitrosylation of β-arrestin-2 promotes the transition from β-arrestin-2's interaction with eNOS to interaction with clathrin and β-adaptin, which accelerates agonist-induced receptor internalization (Ozawa et al., 2008). Since caveolin-3 is a primary regulator of eNOS activity in myocytes, the findings presented in this dissertation are likely important for this process as well (Ostrom et al., 2004).

β-arrestins have multiple, critical functions in GPCR signaling and are both regulated by multiple PTMs and respond to PTMs of their signaling partners, including the phosphorylation and ubiquitination events described here. Caveolin-1 and -3 have tandem SIMs that align with SIMs found in RNF4, a poly-SUMO specific E3 ubiquitin ligase. A bioinformatic search for similar motifs (expasy.org/tools/scanprosite) identified sequences in both β-arrestin-1 (ARRB1) and β-arrestin-2 (ARRB2) that share a high degree of similarity of hydrophobic and acidic residues that define the SIM (Figure 1.8; Song et al., 2004), suggesting that β-arrestins may interact non-covalently with sumoylated proteins. Interestingly, Mdm2 also interacts with the SUMO E3 ligase PIASy (Meek and Knippschild, 2003; Carter et al., 2007). Mdm2 is a target of sumoylation and β-arrestin-2 preferentially interacts with the sumoylated form of Mdm2 (p90Mdm2, Shenoy et al., 2001). The presence of SIMs in the N-terminus of β-arrestin-2 suggests that Mdm2 preferentially binds p90Mdm2 in a SUMO-dependent manner. Therefore sumoylation and interaction with caveolin-3 may be an important, as-yet unappreciated contributors to agonist-induced trafficking of β_2ARs in addition to other known mechanisms (e.g. phosphorylation, ubiquitination and S-nitrosylation).

CHAPTER 2: Covalent Modification of Caveolin-3 by SUMO-3 and Effects on Desensitization of the Beta 2-Adrenergic Receptor (β_2AR)

2.1 Summary

Assays performed *in vitro* and *in vivo* reveal, for the first time, that caveolin-3 is a target of sumoylation. Modification of caveolin-3 by poly-SUMO-3 chains was enhanced by interaction with the SUMO E3 ligase PIASy. Site-directed mutagenesis revealed that the SUMO consensus site lysine, K38, is the preferred site of sumoylation, however mutation of this site alone was not sufficient to abolish sumoylation. Mutation of all 7 lysine residues to arginine was necessary to create a "sumoylation-deficient" caveolin-3 mutant (K7R). Caveolin-3 scaffolds many GPCRs and other proteins in caveolae membrane microdomains. The preferred sumoylation site on caveolin-3 (K38) is near the caveolin scaffolding domain (CSD, aa54-73) that binds caveolae-targeted proteins which contain a "caveolin-binding motif" (ΦxΦxxxxΦ and ΦxxxxΦxxΦ: Φ = aromatic residue; W, F or Y). Co-transfection of HEK 293 cells with FLAG-β_2AR alone or with wild-type (WT) Cav-3-V5 or K7R, revealed that WT stabilizes while the K7R mutant destabilizes FLAG-β_2AR protein expression levels. β_1ARs also localize to caveolae and interact with caveolin-3 but are resistant to agonist-induced ubiquitination and down-regulation. The stability of FLAG-β_1AR or the canonical binding partner of caveolin, eNOS (NOS3), was not affected by co-transfection of WT Cav-3-V5 or K7R, suggesting that sumoylation of caveolin-3 regulates the function of specific binding partners including β_2ARs. These

data show that caveolin-3 undergoes sumoylation, which contributes to the agonist-induced desensitization and trafficking of β_2ARs but not β_1ARs.

2.2 Introduction

Caveolins are the principal protein component of caveolae, a subset of lipid raft, membrane microdomains that are enriched in cholesterol, sphingolipids and signaling proteins. Caveolins are both necessary and sufficient for the formation of caveolae (Fra et al., 1995), although a recently recognized family of proteins, cavins (cavin-1, -2, -3 and -4), are also critical for the formation, regulation and morphology of caveolae (Bastiani et al., 2009, Chidlow et al., 2010). Caveolae are found in many cell types and are abundant in endothelial, adipose and muscle cells. In electron micrographs, caveolae ("little caves") are visible as 50-100 nm in diameter flask-shaped invaginations of the plasma membrane. The caveolin gene family has three members: caveolin-1 and -2 are co-expressed in most cell types, while caveolin-3 is expressed primarily in cardiac, skeletal and smooth muscle cells (Song et al., 1996). Caveolins are 22-24 kDa integral membrane proteins that form a hairpin loop in the plasma membrane so that both the N- and C-termini face the cytoplasm (Figure 1.2).

A hydrophobic transmembrane domain, cholesterol binding and palmitoylation at three C-terminal cysteine residues anchor caveolin in the membrane and stabilize caveolin oligomers (Figure 1.2; Dietzen et al., 1995; Uittenbogaard et al., 2000). Caveolin-3 shares 65% sequence identity with caveolin-1 and 40% with caveolin-2. Caveolins form homo- and hetero-oligomeric complexes made up of ~14-16 caveolin

monomers (Das et al., 1999, Song et al., 1997). Caveolins-1 and -3 homooligomerize and Caveolins-1 and -2 and Caveolins-1 and-3 hetero-oligomerize (Volonte et al., 2007).

Proteomic studies using subcellular fractionation, immunoprecipitation and mass spectrometry have revealed that >300 proteins are enriched in caveolae, many of which are regulated by direct interaction with caveolins (Sprenger et. al., 2007, Foster et. al., 2003). Among these proteins are numerous receptors, channels, transporters and signaling proteins that are enriched or found exclusively in caveolar membranes (e.g. G-proteins, GPCRs, eNOS, EGFR, TβRI, IR, adenylyl cyclase, PLCβ etc.). Proteins can also undergo rapid translocation to or from caveolae in response to specific stimuli (e.g. bradykinin receptors, muscarinic cholinergic receptors and the glucose transporter GLUT-4 (Feron et al., 1998; Lamb et al., 2002; Sabourin et al., 2002; Fecchi et al., 2006)). Many proteins that bind caveolins do so via a caveolin-binding motif (ΦxΦxxxxΦ and ΦxxxxΦxxΦ: Φ = aromatic residue; W, F or Y) which, via hydrophobic interactions, interacts with the caveolin-scaffolding domain (CSD) in the N-terminal region of caveolins (aa55-74 in caveolin-3; Figures 1.1 and 1.2). How this binding to caveolins is dynamically regulated is poorly understood. How are proteins targeted to caveolae and once bound to caveolin, how are they released? Because of their rapid kinetics and regulated reversibility, control by posttranslational modification is a likely mechanism. Are the CSD and caveolin-binding motifs the primary determinants of protein-protein interactions with caveolin, or are there other mechanisms at work?

Caveolin-1 and -2 are regulated by posttranslational modifications (PTMs) including phosphorylation; however, no phosphorylation sites have been identified in the muscle-specific caveolin-3. Caveolin-1 can be phosphorylated at multiple sites but Tyr14

seems to be the major site of regulation and several tyrosine kinases can phosphorylate it (Maggi et al., 2002; Sanguinetti et al., 2003; Labrecque et al., 2004; Cao et al., 2002). Based on the lack of knowledge regarding the posttranslational regulation of caveolin-3 and its high degree of similarity to caveolin-1, we used a bioinformatic approach to search for motifs important for PTMs in the amino acid sequence of caveolin-3. This approach revealed the presence of a conserved, SUMO consensus motif in caveolin-1 and caveolin-3, but not caveolin-2 (Figure 1.1). Based on the presence of this SUMO motif and the lack of information on the posttranslational regulation of caveolin-3 vs. caveolin-1, we investigated the ability of caveolin-3 to undergo sumoylation.

Sumoylation is a reversible PTM that targets numerous substrates and alters their activity, localization and protein-protein interactions. The biochemical mechanism of sumoylation is similar to that of ubiquitination. SUMO has its own set of E1, E2 and E3 enzymes responsible for conjugation to targets, as well as specific isopeptidases that remove SUMO from targets (Figure 1.4). Inactive SUMO precursors are processed by SUMO-specific proteases (SENPs) that also remove SUMO from modified proteins (Chapter 1.8). This exposes a conserved glycine residue that eventually forms isopeptide bonds with the ε-amino group of lysines on target proteins. The catalytic cysteine of the E1 activating enzyme (a heterodimer of SAE1 and SAE2) forms a thioester bond with SUMO in a Mg^{2+}-ATP dependent manner. The SUMO~E1 thioester is then transferred to the catalytic cysteine of the single SUMO E2 conjugating enzyme (Ubc9), which can directly modify substrates (Figure 1.4). Many SUMO targets have a consensus motif (Ψ-K-X-[D/E], Ψ=VIL) that is recognized by Ubc9 (Chapter 1.6; Sternsdorf et al., 1999; Sampson et al., 2001; Johnson and Blobel, 1997)). Consequently, the SUMO E1 and E2

are sufficient for sumoylation of many substrates *in vitro*; however, *in vivo* an E3 ligase (e.g. PIAS family) is usually required for efficient sumoylation (Bohren et al., 2007).

This chapter shows, that caveolin-3 undergoes posttranslational modification by SUMO proteins. This novel regulation of caveolin influences its interactions with signaling partners, including the agonist-induced desensitization of β_2ARs.

2.3 Results

2.3.1 Caveolin-1 and Caveolin-3, But Not Caveolin-2, Have Conserved SUMO Consensus Sites in Their Cytosol-exposed N-Termini

Amino acid sequence alignment of the caveolin family members reveals that caveolin-1 and caveolin-3 contain conserved, core SUMO consensus motifs, VKID and VKVD, respectively (Figures 1.1 and 1.6), which are near the CSD in their cytosol-exposed N-termini. Two versions of an extended SUMO consensus motif have been identified; the 'phosphorylation-dependent sumoylation motif' (PDSM, e.g. ΨKxExxSP) and the 'negatively charged amino acid-dependent sumoylation motif' (NDSM) (Blomster et al., 2009, Hietakangas et al, 2006, Yang et al., 2006B). Both extended motifs specify the presence of negatively charged amino acids downstream of the core SUMO motif (Chapter 1.6). Caveolins-1 and -3 both have a conserved NDSM in their N-termini that is followed by a conserved proline residue (VKIDFEDVIAEP and VKVDFEDVIAEP respectively; Figures 1.1 and 1.6), suggesting that they are potential SUMO substrates. This eight amino acid sequence immediately downstream of the core SUMO motif, FEDVIAEP that conforms to the NDSM, is known as the 'caveolin

signature motif' that is invariant among the three caveolin family members and is conserved in the evolution of caveolin proteins (Tang et al., 1996).

2.3.2 *In vitro* Sumoylation Assay Using Recombinant Cav-3-V5 as a Substrate Reveals that Caveoin-3 is Modified by Both SUMO-1 and SUMO-3 *in vitro*

In vitro, the SUMO E1 (SAE1/SAE2) and E2 (Ubc9) enzymes are sufficient to modify SUMO targets in the presence of Mg^{2+}/ATP (Werner et al., 2009). I performed an *in vitro* sumoylation assay using a dual, affinity-tagged caveolin-3 construct [Cav-3-V5/His, referred to as Cav-3-V5] expressed in a transcription/translation system to determine if caveolin-3 can undergo modification by SUMO. Aliquots of the Cav-3-V5 expression reaction were included in 20 µl reactions that contained: sumoylation buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM ATP), SAE1/SAE2, Ubc9 and SUMO. Reactions were incubated for 1 h at 37°C and analyzed by immunoblotting with anti-V5-HRP antibodies. Modification by SUMO results in a characteristic electrophoretic mobility shift of ~15-20 kDa (Mahajan et al., 1997). Cav-3-V5 migrates at ~25 kDa but a ~40 kDa band was observed when Cav-3-V5 was incubated with SUMO-1 or SUMO-3 (Figure 2.1 A, lanes 1 and 5). This was not observed if Ubc9, SUMO or recombinant Cav-3-V5 were omitted from the reaction suggesting that the 40 kDa band in V5-HRP immunoblots is the sumoylated form of caveolin-3 (Figure 2.1 A, lanes 2-4).

2.3.3 Production of Sumoylated Caveolin-3 in E. coli

An alternative *in vitro* strategy for identification, expression and purification of sumoylated proteins in E. coli has been developed and validated (Saitoh et al., 2009;

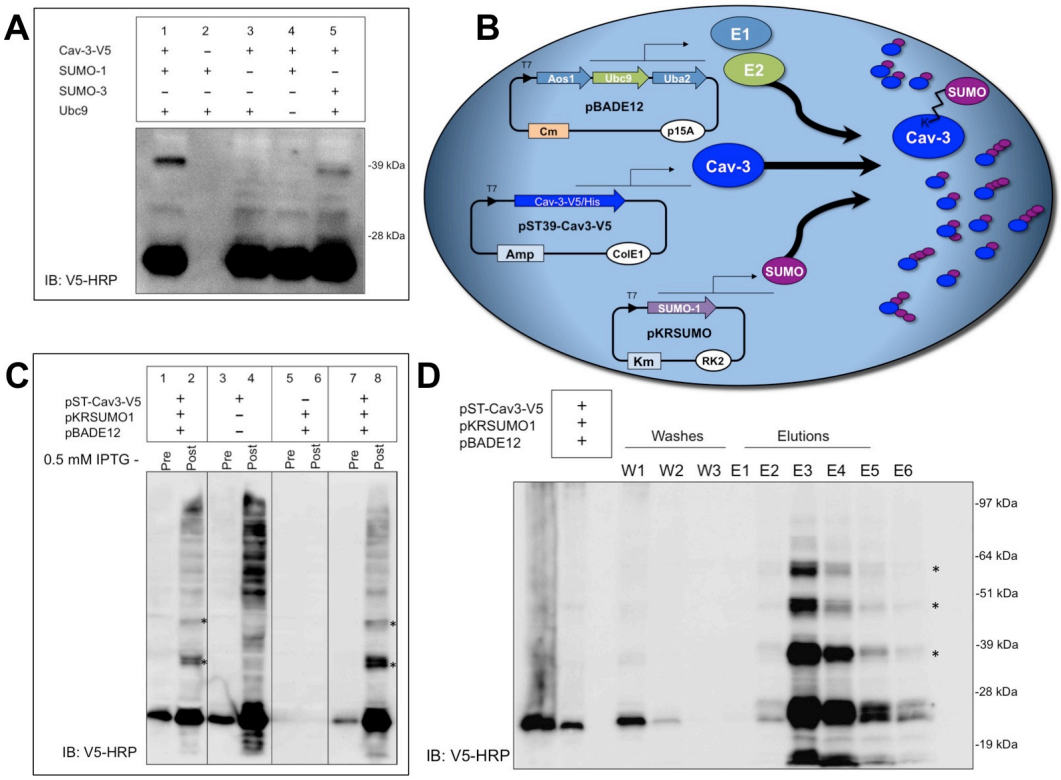

Figure 2.1 (A-D). *In Vitro* Sumoylation of Caveolin-3

(A) *Sumoylation assay with recombinant Cav-3-V5*. Reactions included sumoylation buffer, Mg^{2+}/ATP, and recombinant SUMO E1 and E2 enzymes and were incubated at 37°C for 1 h with either SUMO-1 (lanes 1, 2 and 4) or SUMO-3 (lane 5). Reactions omitting Cav-3-V5, SUMO or Ubc9 were negative controls (lanes 2, 3 and 4, respectively). Reactions were analyzed by immunoblotting with anti-V5-HRP antibodies.

(B) *Strategy for expression of sumoylated Cav-3-V5 in E. coli*. BL21(DE3) cells were sequentially transformed with 3 plasmids with compatible origins of replication and selection markers: pKRSUMO, pBADE12 and pST39-Cav-3-V5, which allow inducible expression by IPTG of SUMO-1, the SUMO E1 and E2 enzymes and Cav-3-V5 respectively (plasmid maps adapted from Mencia and Lorenzo, 2004).

(C) *Small scale expression to determine solubility of Cav-3-V5 and validate expression of each plasmid*. BL21(DE3) cells transformed with all three plasmids (lanes 1-2 and 7-8) were lysed pre- and post-IPTG induction of protein expression. Cells transformed with pST39-Cav-3-V5 alone (lanes 3-4) or just pKRSUMO and pBADE12 (lanes 5-6) were negative controls. The soluble supernatant and insoluble pellet of cell lysates were analyzed by immunoblotting with anti-V5-HRP antibodies (Suppl. Fig S2.3 A).

(D) *Cells expressing all three plasmids were scaled up for Ni-NTA purification* of *sumoylated Cav-3-V5*. Cav-3-V5 was eluted with 250 mM imidazole. Cells transformed with pST39-Cav-3-V5 alone or with pKRSUMO and pBADE12 were negative controls (Figure S2.3 D and E) (IN=Input, SN=Supernatant, W=Wash, E=Elution).

Mencia and Lorenzo, 2004; Uchimura et al., 2004). This strategy is an attractive alternative to small scale, *in vitro* sumoylation reactions (Chapter 1.9). Its advantages include scalability, reduced cost, long-term storage of bacterial cultures as glycerol stocks and the lack of SENPs that make it difficult to detect endogenously sumoylated proteins.

This E. coli-based strategy was used to express and purify sumoylated caveolin-3 (Figure 2.1 B; for complete protocol see Supplemental Methods). The rat caveolin-3 gene was subcloned from the pcDNA3.1-V5/His mammalian expression vector (Cav-3-V5) into a bacterial expression vector (pST39; Tan, 2001) to create pST39-Cav-3-V5. Plasmids for expression of SUMO-1 (pKRSUMO) and the SUMO E1 and E2 enzymes (pBADE12) were previously described (Mencia and Lorenzo, 2004). In order to validate this strategy for production of sumoylated caveolin-3, small-scale expression reactions were set up by sequentially transforming BL21(DE3) cells with all three plasmids (Figure 2.1 C, lanes 1-2 and 7-8), pST39-Cav-3-V5 alone (lanes 3 and 4) or pKRSUMO and pBADE12 (lanes 5 and 6). Maintenance of bacteria co-transformed with multiple plasmids required selection with multiple antibiotics (i.e. Ampicillin [Amp], Chloramphenicol [Cm] and Kanamycin [Kan]; Figure 2.1 B) and compatible origins of replication (i.e. p15a, ColE1, RK2). Transformed cells were grown at 37°C to an OD_{600} of 0.5 - 0.6 and plasmid expression was induced with 0.5 mM IPTG for 3 h. The presence of Cav-3-V5 in the pre-IPTG lanes suggests “leakiness” of the inducible plasmid; however, IPTG induction greatly amplified protein expression (Figure 2.1 C).

Because caveolin is an integral membrane protein, the solubility of Cav-3-V5 and SUMO-conjugated Cav-3-V5 was investigated. Crude E. coli lysates were prepared in

lysis buffer supplemented with octyl-β-D-glucopyranoside, a detergent that helps solubilize membrane proteins (Li et al., 1996). Both the soluble supernatant (Figure 2.1 C) and insoluble pellet (Figure S2.3 A) were analyzed by immunoblotting with anti-V5-HRP antibodies. The majority of sumoylated Cav-3-V5 was soluble, indicating that sumoylation of caveolin-3 increases its solubility-a result consistent with the growing use of "SUMO tagging" to aid in the purification of difficult-to-express proteins in E. coli.

BL21(DE3) cells transformed with the three plasmids (Figure 2.1 C, lanes 1-2 with duplicates grown from a second, single colony in lanes 7-8; see also Figure S2.3 C) showed two slower migrating bands at 40 and 50 kDa not present when Cav-3-V5 was expressed alone (*, Figure 2.1 C, lanes 3-4). Incubation of these lysates with the catalytic domain of SENP1 ($SENP1_{CD}$) caused the bands at 40 kDa and 50 kDa to disappear; confirming that they correspond to SUMO-1 conjugates (Figure S2.3 B). Bands of higher molecular mass (>50 – 200 kDa) observed in addition to those at 40 and 50 kDa are not unique when compared with Cav-3-V5 expressed alone (Figure 2.1 C lanes 3-4) and are likely due to SDS-resistant oligomers of caveolin-3. When expressed alone, Cav-3-V5 (Figure 2.1 C, lane 2 vs. lane 4) was expressed at a higher level than when co-expressed with SUMO-1, E1 and E2. The greater intensity of the 25 kDa band and the higher molecular weight bands when Cav-3-V5 is expressed alone suggests that these bands are oligomers and not additional SUMO conjugates. The relatively lower expression level of Cav-3-V5 when co-expressed with SUMO-1, Ubc9 and SAE1/SAE2 is likely due to the competition among the multiple plasmids for replication and the protein expression machinery. The band at 40 kDa is consistent with results from the *in vitro* sumoylation assays (Figure 2.1 A) for the size of Cav-3-V5-SUMO conjugates. The additional band at

50 kDa indicates that either multiple lysines can be modified or a single lysine is modified by poly-SUMO chains.

After showing that caveolin-3 is sumoylated in this system and soluble under native, non-denaturing conditions, the expression cultures were scaled up for nickel-nitrilotriacetic acid (Ni-NTA) purification of sumoylated Cav-3-V5. BL21(DE3) cells transformed with pST39-Cav-3-V5, pKRSUMO and pBADE12 were induced with IPTG for 3 h, pelleted, lysed and incubated overnight with Ni-NTA resin. The resin was washed and Cav-3-V5 was eluted with 250 mM imidazole. Negative controls included cells transformed with pST39-Cav-3-V5 *alone* (Figure S2.3 D) or pKRSUMO and pBADE12 *without* pST39-Cav-3-V5 (Figure S2.3 E). When all three plasmids were expressed, elutions (E3-E5) contained Cav-3-V5 and multiple slower migrating bands that corresponded to sumoylated caveolin-3 (Figure 2.1 D). Additional bands (60, 70 and 80 kDa etc…) in these purified fractions were not visible when the crude lysates were analyzed (Figure 2.1 C) and may be low-abundance, poly-sumoylated Cav-3-V5 or SDS-resistant dimers and multimers of Cav-3-V5 and sumoylated Cav-3-V5. Based on immunoblots of purified Cav-3-V5 with anti-SUMO-1 antibodies, I infer that these additional bands are SUMO-1 conjugates (Figure S2.3 C and Figure S2.4 A and B).

2.3.4 Two-Step Purification of Cav-3-V5-SUMO-1 Conjugates

Coomassie staining of the Ni-NTA purification fractions failed to show distinct bands corresponding to Cav-3-V5 or sumoylated Cav-3-V5 and revealed many non-specific bands in the eluted fractions (data not shown). Caveolin is known to be difficult to stain with Coomassie and silver stains (Scherer et al., 1997), which may explain why

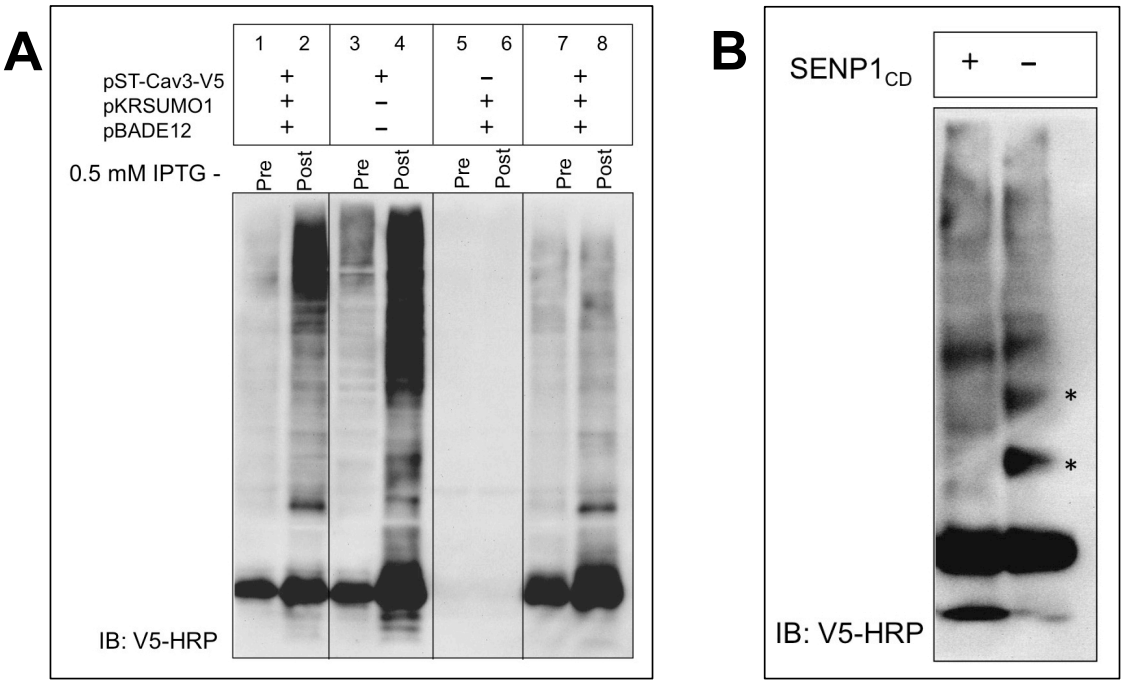

Figure S2.3 (A-B). ***In Vitro*** **Sumoylation of Caveolin-3: Control Experiments**

(A) *Small scale expression of Cav-3-V5*: *analysis of the insoluble pellet.* BL21(DE3) cells transformed with all 3 plasmids (lanes 1-2 and 7-8) were lysed pre- and post-IPTG induction of protein expression. Cells transformed with just pST39-Cav-3-V5 (lanes 3-4) or pKRSUMO and pBADE12 (lanes 5-6) were negative controls. After clearing lysates by centrifugation, the insoluble pellet was resuspended and analyzed by immunoblotting with anti-V5-HRP antibodies. The soluble supernatant was analyzed in Figure 2.1 C.

(B) *Incubation of E. coli lysates with the catalytic domain of SENP1.* Lysates from BL21(DE3) cells transformed with all 3 plasmids (Figure 2.1 C) were incubated for 1 h at 37°C with and without the catalytic domain of SENP1 to validate that electrophoretic mobility shifts in anti-V5-HRP immunoblots were caused by covalent modification of Cav-3-V5 by SUMO-1.

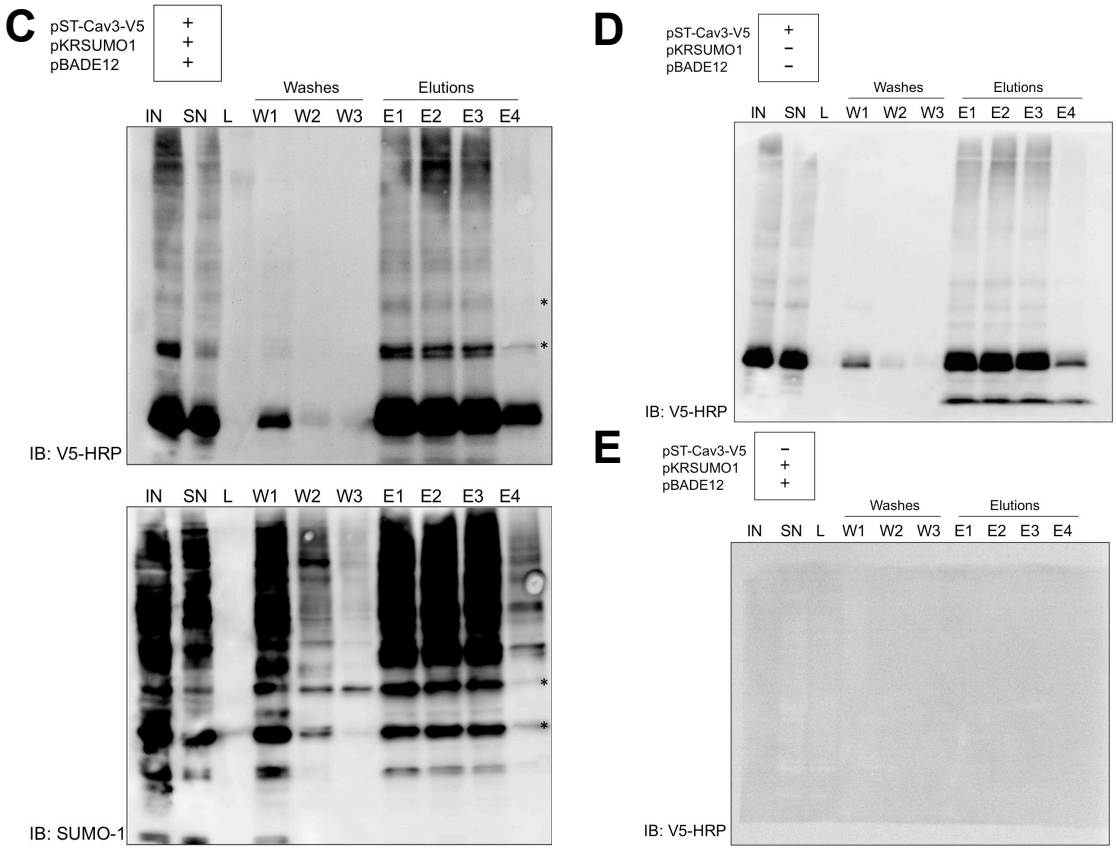

Figure S2.3 (C-E). ***In Vitro*** **Sumoylation of Caveolin-3: Control Experiments**

(C-E) *Small scale Ni-NTA purification of sumoylated caveolin-3 was performed in parallel with negative controls.* **(C)** Lysates from BL21(DE3) cells transformed with all 3 plasmids were purified under native conditions using Ni-NTA resin. The resin was washed 3 times and Cav-3-V5 and Cav-3-V5-SUMO-1 conjugates (*, 40 and 50 kDa) were eluted with 250 mM imidazole. The purification fractions were analyzed by immunoblotting with anti-V5-HRP antibodies. The membrane was stripped and re-probed with anti-SUMO-1 antibodies.

(D-E) Cells transformed with pST39Cav-3-V5 alone **(D)** or with pKRSUMO and pBADE12 **(E)** were processed in parallel with cells expressing all three plasmids **(C)**. Overexposure of the immunoblot in **(E)** demonstrates the specificity of the anti-V5-HRP antibody (IN=Input, SN=Supernatant, W=Wash, E=Elution, L=Ladder).

Coomassie bands were not distinguishable despite strong signals in immunoblots. I developed a two-step purification scheme utilizing Ni-NTA purification followed by anti-V5-agarose immunoprecipitation to try and obtain a sample sufficient for identification of the site(s) of sumoylation by mass spectrometry (MS; Supplemental Figure S2.4 A-B) but I encountered several difficulties. Trypsin digestion of purified Cav-3-V5-SUMO was unsuitable for MS analysis due to the large tryptic peptide fragment of SUMO-1 ((K.ELGMEEEDVIEVYQEQTGG) left attached to target lysines and the poor *in silico* digestion of Cav-3-V5, especially surrounding the likely sumoylation site in the SUMO consensus motif (Figure 1.1; K.NINEDIV**K**VDFEDVIAEPEGTYSFDGVWR). This ruled out some useful MS sample preparation methods such as in-gel digestion and the ability to analyze gel slices by MS. Purified Cav-3-V5-SUMO-1 (IP samples shown in Figure S2.4 B) was instead digested with GluC for MS analysis (data not shown). Despite the addition of new, custom parameters to the Protein Pilot analysis software (Applied Biosystems) to search for the unique mass shifts caused by GluC-digested SUMO-1 (QTGG), definitive detection of Cav-3-V5-SUMO-1 conjugates was not achieved. Despite these difficulties, the two-step purification was useful in the identification of Cav-3-V5-SUMO-1 conjugates by immunoblotting.

Before attempting the two-step protocol, the second step was validated by pulldown of Cav-3-V5 from crude E. coli lysates with anti-V5-agarose beads. E. coli whole cell lysates (WCL; Figure S2.4 A) were made with cells expressing all three plasmids or pST39-Cav-3-V5 alone. SUMO-1 expression was confirmed in the WCL by immunoblotting (Figure S2.4 A, left panel). Cell lysates were incubated with anti-V5-agarose beads, eluted with 2x LDS buffer, and analyzed by immunoblotting with anti-V5-

HRP and anti-SUMO-1 antibodies (Figure S2.4 A, middle and right panels). In cells expressing all three plasmids, bands at 40 and 50 kDa in both blots confirmed that Cav-3-V5-SUMO-1 conjugates were pulled down. The presence of additional SUMO-1 bands in the purification suggests the presence of contaminating proteins including SUMO-1 modified E. coli proteins or poly-SUMO-1 chains, which may co-purify with caveolin-3 by non-covalent interaction of caveolin's tandem SIMs with SUMO-1 and/or SUMO-1 modified proteins.

After validation of both steps, the two-step purification was carried out by an initial Ni-NTA purification (Supplemental Methods). Briefly, elution fractions E2 - E4 (Figure 2.1 D) from the Ni-NTA resin were pooled, concentrated (Viva Spin, 30,000 MWCO) and incubated with anti-V5-agarose beads. After extensive washing, the anti-V5-agarose beads were eluted with low pH (glycine buffer pH 2.6, for elution without disruption of immobilized anti-V5 IgG) followed by 2x LDS buffer with 100 mM DTT (Figure S2.4 B). The supernatants, washes and elution fractions were analyzed by immunoblotting with anti-V5-HRP and anti-SUMO-1 antibodies. The bands at 40 kDa, 50 kDa, 60 kDa and larger in the V5-HRP immunoblots correspond to bands in the anti-SUMO-1 immunoblots (*, Figure S2.4 B, upper and lower panels respectively).

Based on these *in vitro* assays, caveolin-3 is likely an *in vivo* target of sumoylation provided it co-localizes with the sumoylation machinery in cells. It is unclear from these experiments whether the multiple slower migrating bands are indicative of multiple sites of sumoylation or modification by poly-SUMO chains. To validate these results and better understand the determinants of caveolin-3 sumoylation, I performed a series of "*in vivo* sumoylation assays".

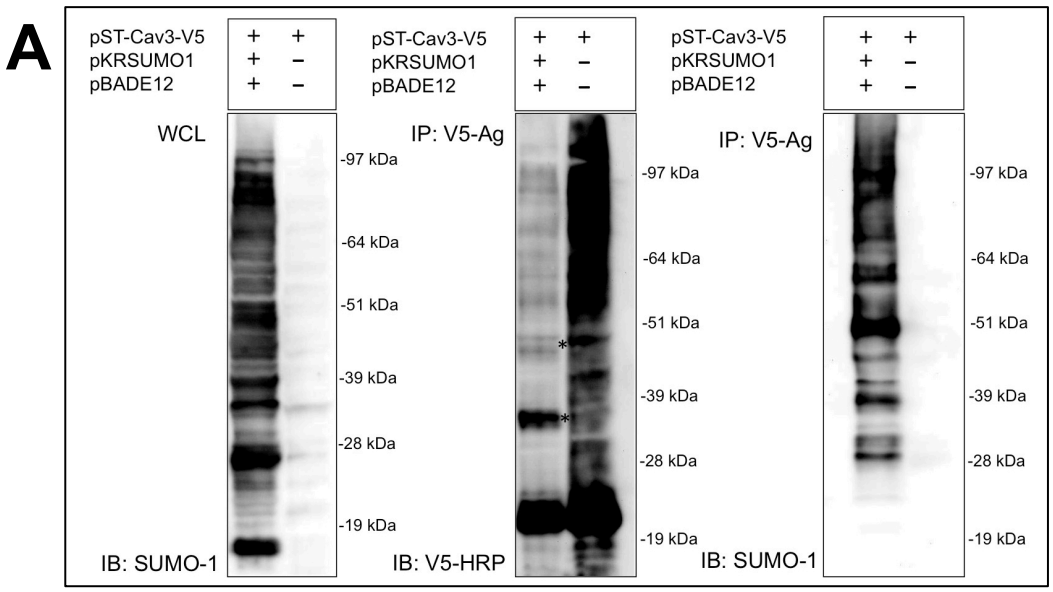

Figure S2.4 (A). ***In Vitro*** **Sumoylation of Cav-3-V5 in E. coli: Validation of a Two-Step Purification Protocol**

(A) *Purification with anti-V5-agarose*. BL21(DE3) cells transformed with either all 3 plasmids (left lanes) or pST39-Cav-3-V5 alone (right lanes) were grown to an OD_{600} of 0.6 and protein expression was induced with 0.5 mM IPTG for 3 h. Whole cell lysates (WCL) were either separated by SDS-PAGE and immunoblotted with anti-SUMO-1 antibodies or incubated overnight with anti-V5-agarose beads (IP: V5-Ag), washed and eluted with 2x LDS buffer + 100mM DTT. Eluted immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-V5-HRP and anti-SUMO-1 antibodies.

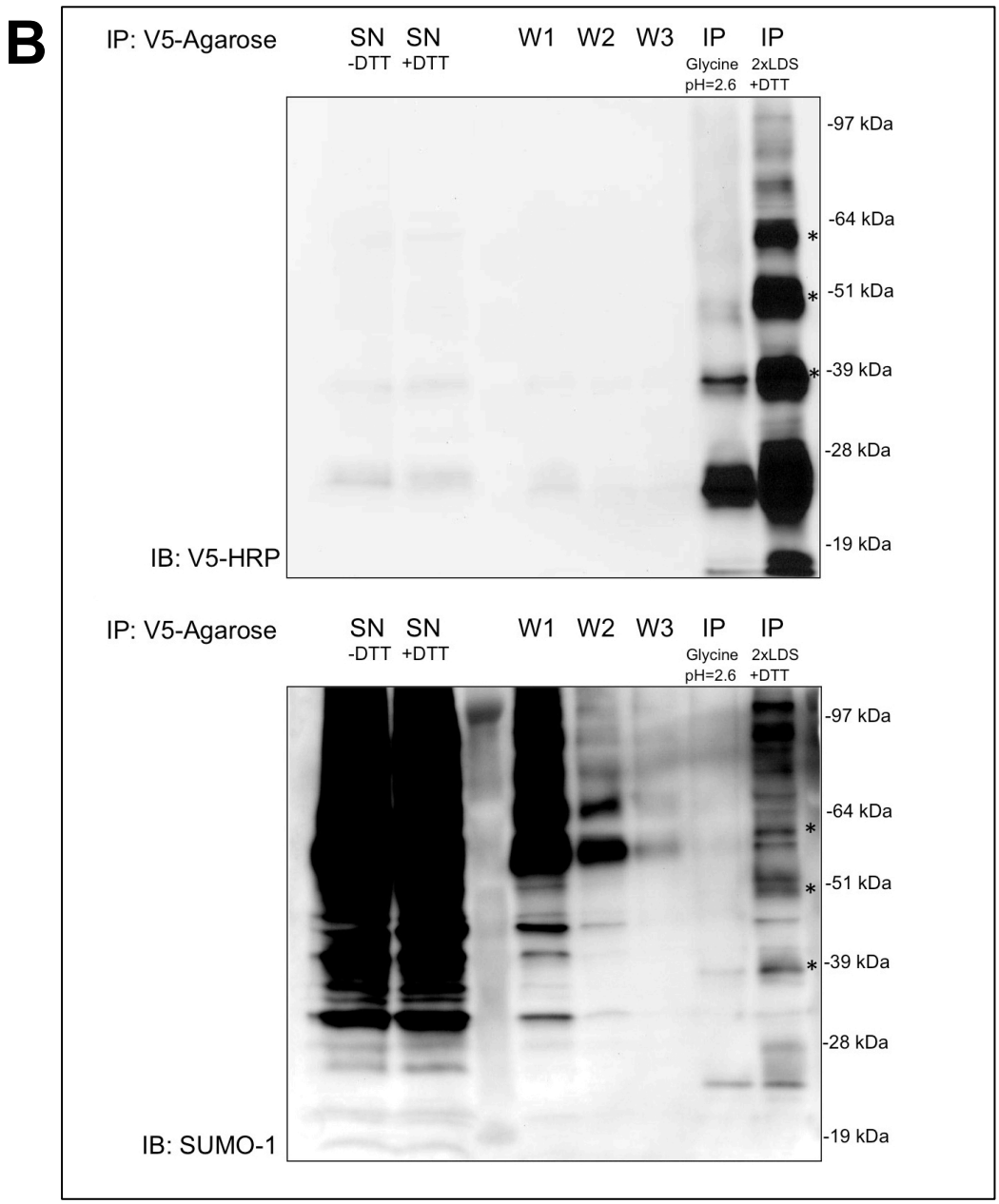

Figure S2.4 (B). ***In Vitro*** **Sumoylation of Cav-3-V5 in E. coli: Validation of a Two-Step Purification Protocol**

(B) *The two-step purification was performed using lysates from BL21(DE3) cells transformed with pKRSUMO, pBADE12 and pST39-Cav-3-V5.* Elutions from the Ni-NTA purification were pooled, concentrated (Viva Spin 30k MWCO) and incubated with anti-V5-agarose beads. The beads were washed and eluted with glycine buffer (pH 2.6) to remove immunoprecipitated proteins without denaturing anti-V5 IgG and then eluted with 2x LDS buffer + 100 mM DTT to free any protein still bound to the beads. Fractions from the purification were analyzed by immunoblotting with anti-V5-HRP and anti-SUMO-1 antibodies (SN=Supernatant, W=Wash, IP=Immunoprecipitate).

2.3.5 *In Vivo* Sumoylation Assay: Covalent Modification of Caveolin-3 by SUMO is Enhanced by the SUMO E3 Ligase PIASy

The SUMO E1 and E2 enzymes (SAE1/2 and Ubc9) sumoylate caveolin-3 *in vitro*; however, *in vivo* a specific SUMO E3 ligase is often required. While both SUMO-1 and SUMO-3 covalently modified caveolin-3 *in vitro*, only SUMO-3 proteins were able to modify Cav-3-V5 when co-expressed in HEK 293 cells (Figure 2.3 B and unpublished data). In order to identify an E3 ligase specific for caveolin-3, I performed *in vivo* sumoylation assays in HEK 293 cells by co-transfecting several SUMO E3 ligases (PIAS1, PIAS3 and PIASy) with Cav-3-V5, Ubc9 and Myc-SUMO-3 (Figure 2.2 A, top panels). Immunoblots with anti-V5-HRP and anti-Myc antibodies show bands at 40 kDa and 50 kDa, corresponding to sumoylated caveolin-3 only when PIASy was co-expressed. Of note, the ~45 kDa band visible in many of the anti-V5-HRP blots in between the Cav-3-V5-SUMO conjugates (which migrate at 40 and 50 kDa) is likely dimerized Cav-3-V5 as it does not vary with SUMO, Ubc9 or PIASy co-expression or correspond to the expected size of SUMO-modified Cav-3-V5 (Figure 2.2 A-D). Immunoblotting with anti-PIASy antibodies shows that endogenous expression of PIASy in HEK cells is low (Figure 2.2 A, bottom panel).

2.3.6 PIASy Dose-Dependently Enhances Modification of Caveolin-3 by SUMO-3

To further investigate the role of PIASy in stimulating sumoylation of caveolin-3, HEK 293 cells were transfected with increasing amounts of PIASy (Figure 2.2 B, lanes 3-10; 0 - 1000 ng plasmid) to determine if there is a dose-dependent effect. Indeed, up to a certain amount of plasmid, increased expression of PIASy produced an increase in

modification of Cav-3-V5 by SUMO-3. A ladder of bands, likely corresponding to caveolin-3 modified at multiple lysine residues or by poly-SUMO-3 chains, can be seen (*, Figure 2.2 B) at 65 kDa and higher in addition to the bands at 40 kDa and 50 kDa previously observed *in vitro*. These bands are specific for Cav-3-V5-SUMO-3 conjugates because there is no cross-reactivity of the anti-V5-HRP antibody if PIASy is expressed alone (Figure 2.2 B, lane 2). Expressing >400 ng PIASy per well did not further enhance Cav-3-V5 sumoylation and had toxic effects on the cells, a result consistent with previous findings (Bischof et al., 2006). Thus, in subsequent experiments in which PIASy was overexpressed in HEK 293 cells, I used 200 ng or less (or 5% of total DNA transfected, i.e. the smallest amount needed to achieve a maximal level of sumoylated Cav-3-V5).

2.3.7 PIASy Co-Immunoprecipitates with Caveolin-3 in HEK 293 Cells

HEK 293 cells transfected with Cav-3-V5, SUMO-3, Ubc9 and PIASy were lysed and immunoprecipitated with anti-V5 and anti-Cav-3 antibodies. Immunoblotting with anti-V5-HRP and anti-PIASy antibodies revealed that Cav-3-V5 pulled down PIASy (Figure 2.2 C). The reciprocal immunoprecipitation, performed with anti-PIASy antibodies and immunoblotting with anti-Cav-3 and anti-V5-HRP antibodies, showed that PIASy pulled down the 25 kDa, unmodified Cav-3-V5 as well as sumoylated Cav-3-V5 (*, Figure 2.2 D). Thus, it appears that PIASy is a SUMO E3 ligase that functionally interacts with caveolin-3 and stimulates its modification at either multiple lysine residues or by poly-SUMO-3 chains.

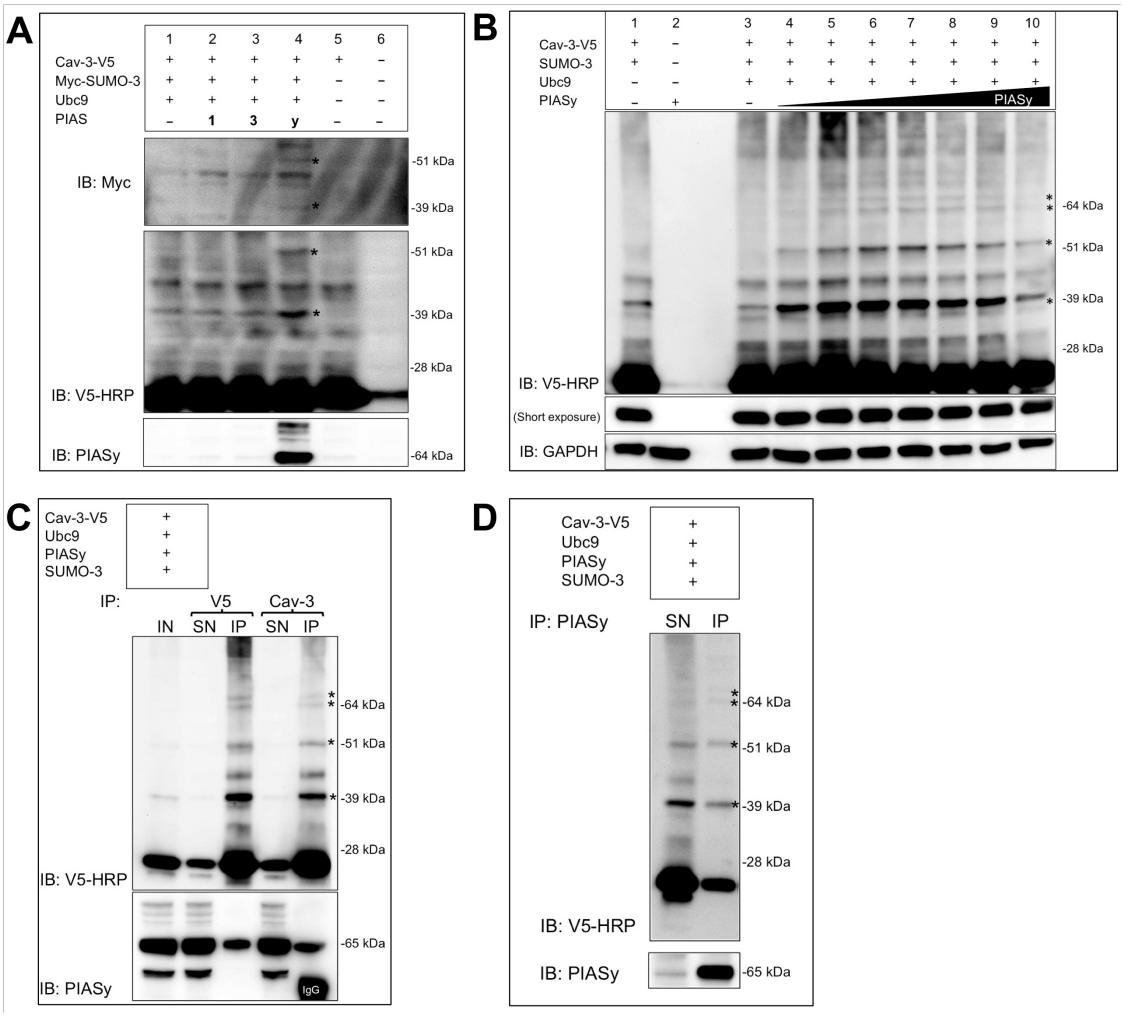

Figure 2.2 (A-D). *In Vivo* Sumoylation Assays: Sumoylation of Caveolin-3 is Enhanced by Interaction with the SUMO E3 Ligase PIASy

(A) *PIASy Stimulates Sumoylation of Caveolin-3.* HEK 293 cells were co-transfected with Cav-3-V5 (lanes 1-5), Myc-SUMO-3 and Ubc9 (lanes 1-4) and either PIAS1, PIAS3 or PIASy (lanes 2, 3 and 4 respectively). Cells transfected with empty vector DNA were included as a negative control (lane 6). Lysates were analyzed by immunoblotting with anti-Myc, anti-V5-HRP and anti-PIASy antibodies.

(B) *PIASy Dose-Dependently Enhances Caveolin-3 Sumoylation*. HEK 293 cells were co-transfected with Cav-3-V5, SUMO-3 and Ubc9 and increasing doses of PIASy (lanes 3-10; 0, 40, 100, 200, 400, 600, 800 or 1000 ng plasmid DNA). Cells transfected with Cav-3-V5 and SUMO-3 or PIASy alone were negative controls (lanes 1 and 2). Lysates were analyzed by immunoblotting with anti-V5-HRP and anti-GAPDH antibodies.

(C-D) *PIASy Co-Immunoprecipitates with Caveolin-3.* **(C)** Lysates from HEK 293 cells co-transfected with Cav-3-V5, Ubc9, SUMO-3 and PIASy were immunoprecipitated with anti-V5 and anti-Cav-3 antibodies and immunoblotted with anti-V5-HRP and anti-PIASy antibodies. **(D)** The reciprocal immunoprecipitation was performed with anti-PIASy antibodies and immunoblotted with anti-V5-HRP and anti-Cav-3 antibodies.

2.3.8 The SUMO Consensus Site Lysine Mutant (K38R) Reduced, but Did Not Abolish, Sumoylation of Caveolin-3

PIASy stimulates modification of Cav-3-V5 by SUMO-3 in HEK 293 cells and immunoblots of adult rat cardiac myocytes show that unlike HEK 293 cells, PIASy is highly expressed in myocytes (Figure S2.2 A). Rat cardiac myocyte lysates were immunoprecipitated with anti-Cav-3 or anti-SUMO antibodies and bands corresponding to sumoylated caveolin-3 were visible (Figure S2.2 B and C), indicating that endogenous caveolin-3 is an *in vivo* target of sumoylation as well as *in vitro*. Given this, I next sought to determine the biological significance of sumoylation by first identifying the specific lysine residues modified. I reasoned that mutation of the sumoylation site lysine(s) to arginine (i.e. a "sumoylation-deficient" mutant) should facilitate identification of the biological functions regulated by sumoylation of caveolins.

Caveolin-3 has seven potential sumoylation sites; K15, K20, K30, K38, K69, K108 and K144 (Figure 2.3 A; see also Figure S2.1 for conservation of lysine residues among caveolin-3 homologs). Located in the cytosol-exposed, N-terminus of caveolin-3, K38 lies within a 'negatively charged amino acid-dependent sumoylation motif' (NDSM), in which acidic amino acids downstream of the consensus tetrapeptide motif aid SUMO conjugation by binding to a positively charged region on Ubc9 (Chapter 1.6, Blomster et al., 2009, Yang et al., 2006B). I used site-directed mutagenesis to mutate this consensus site lysine to arginine (K38R). HEK 293 cells were co-transfected with WT Cav-3-V5 or K38R with Ubc9, PIASy and SUMO-3 (Figure 2.3 B). Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies. I found that the K38R mutation reduced, but did not abolish sumoylation of Cav-3-V5, indicating that:

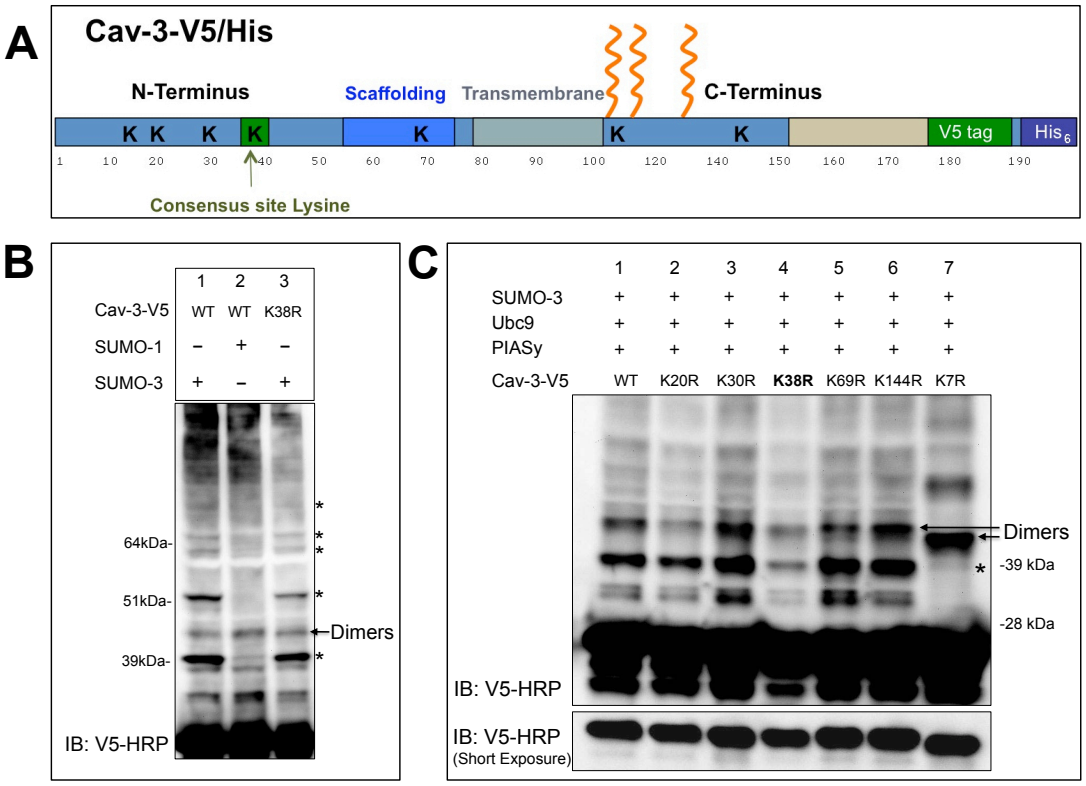

Figure 2.3 (A-C). Modification of Cav-3-V5 by SUMO-3 is *reduced* by mutation of the SUMO consensus Site Lys-38 to Arg (K38R), and *abolished* by mutating all of Caveolin-3's Lys to Arg (Cav-3-V5-K7R)

(A) *Schematic of the Cav-3-V5 construct.* Positions of each lysine residue (K15, K20, K30, K38, K69, K108, K144) in context with the N-terminus, scaffolding domain, transmembrane domain, C-terminus and V5/His affinity tags.

(B) *Caveolin-3 sumoylation is reduced, but not abolished by mutation of consensus site lysine.* Wild-type Cav-3-V5 (WT, lanes 1-2) or the consensus site, Lys to Arg mutant (K38R, lane 3) were co-transfected into HEK 293 cells with Ubc9, PIASy (lanes 1-3) and either SUMO-1 (lane 1) or SUMO-3 (lanes 2 and 3). Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies.

(C) *Sumoylation of 'K to R' mutants.* HEK 293 cells were co-transfected with Cav-3-V5 'K to R' mutants; K20R, K30R, K38R, K69R and K144R (lanes 2-6) and the sumoylation machinery; SUMO-3, Ubc9 and PIASy (lanes 1-7). Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies (long exposure (top panel) and a short exposure (bottom panel)). The WT (lane 1) and Cav-3-V5-K7R mutant (lane 7) are included as positive and negative controls for Cav-3-V5-SUMO-3 conjugates.

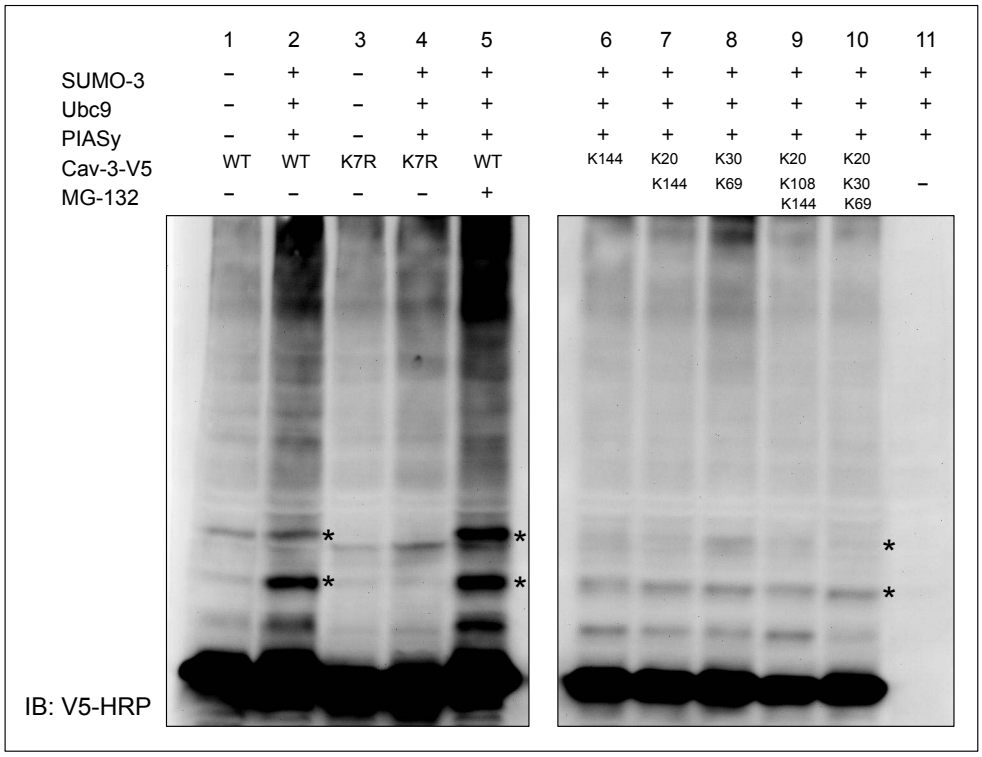

Figure 2.3 (D). Modification of Cav-3-V5 by SUMO-3 is *reduced* by mutation of the SUMO consensus Site Lys-38 to Arg (K38R), and *abolished* by mutating all of Caveolin-3's Lys to Arg (Cav-3-V5-K7R)

(D) *The lysine-less K7R Cav-3-V5 mutant cannot be sumoylated.* WT or the K7R mutant were transfected with and without the sumoylation machinery (lanes 1-5). MG-132 was pre-incubated with cells 3 h prior to lysis (lane 5). Multiple 'K to R' mutants (Table 2.2) were also co-transfected with the sumoylation machinery (lanes 6-11). Labels indicate residues *not* mutated (i.e. K144 has only a single Lys at position 144).

1) multiple lysine residues are modified or 2) mutation of the consensus site allows a different lysine to be modified.

2.3.9 Identification of a Sumoylation-Deficient Mutant of Caveolin-3: Systematic Mutagenesis of Caveolin-3's Lysine Residues to Arginine ('K to R' Mutants)

Mutation of caveolin-3's consensus site lysine reduced, but failed to abolish, sumoylation. The slower migrating bands corresponding to sumoylated caveolin-3 (*, 40, 50 and 65 kDa; Figures 2.2 and 2.3) are potentially due to mono-sumoylation at multiple sites, modification by poly-SUMO chains or a combination of both. Therefore, I undertook a systematic approach to mutate each of caveolin's seven lysines to arginine one a time ('K to R' mutants; Table 2.1). HEK 293 cells transfected with SUMO-3, Ubc9 and PIASy were co-transfected with WT Cav-3-V5 or the series of 'K to R' mutants (Figure 2.3 C and unpublished data). Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies and long exposure (Figure 2.3 C, top panel) and short exposure (bottom panel)) so as to visualize the Cav-3-V5-SUMO-3 conjugates and the relative expression level of each mutant. The 40 kDa band corresponding to Cav-3-V5-SUMO-3 conjugates was reduced in K38R relative to WT Cav-3-V5 (Figure 2.3 C, lane 4 vs. 1), while the other 'K to R' mutants had equal or greater signal at 40 kDa relative to WT. It is unclear whether this is due to increased stoichiometry of sumoylation or variability in the stability and/or expression level of those mutants (Figure 2.3 C, lanes 3, 5 and 6).

K38 thus appeared to be the major site of sumoylation; however, other sites could be modified as well. This suggested that two or more lysines may need to be mutated to generate a sumoylation-deficient mutant for definitive functional studies. To this end, I

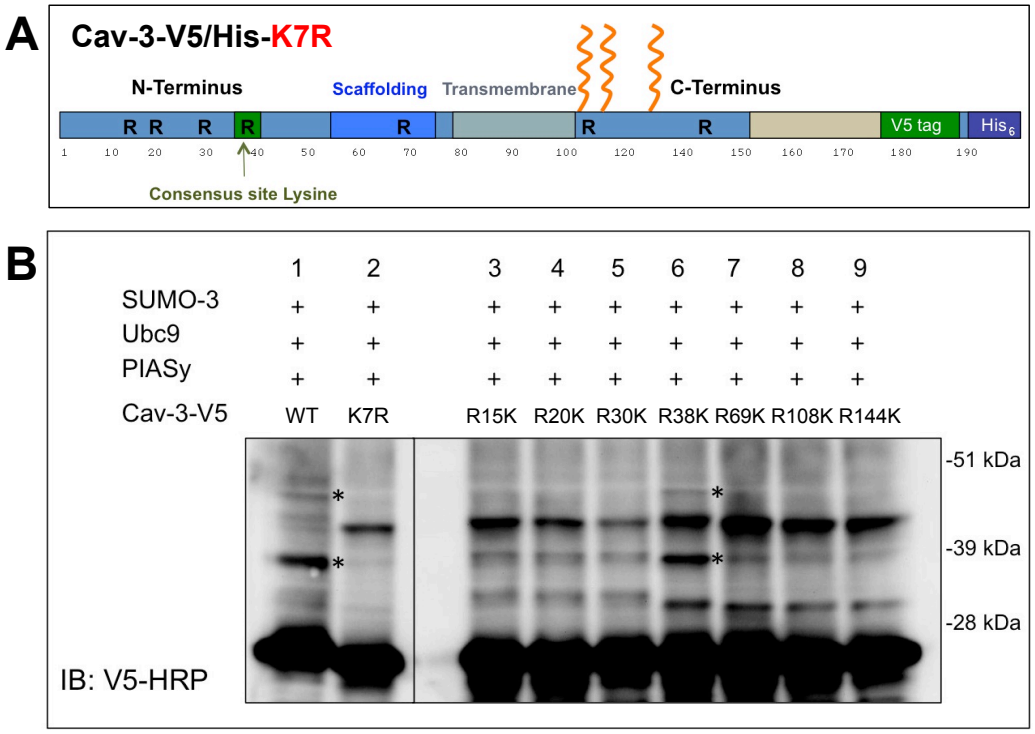

Figure 2.4 (A-B). Reversal of Each Arg Mutation in Cav-3-V5-K7R Back to Lys One at a Time Reveals That K38 is the Preferred Sumoylation Site When PIASy is Co-Expressed

(A) *Schematic of the Cav-3-V5-K7R construct*. Positions of each mutated arginine residue (R15, R20, R30, R38, R69, R108, R144) in context with the N-terminus, scaffolding domain, transmembrane domain, C-terminus and V5/His affinity tags.

(B) *Sumoylation of 'R to K' mutants.* HEK 293 cells were co-transfected with each of the seven 'R to K' mutants along with the sumoylation machinery (SUMO-3, Ubc9 and PIASy). WT and K7R (lanes 1 and 2 respectively) served as positive and negative controls for Cav-3-V5-SUMO-3 conjugates. Lysates were analyzed by immunoblotting with anti-V5-HRP antibodies.

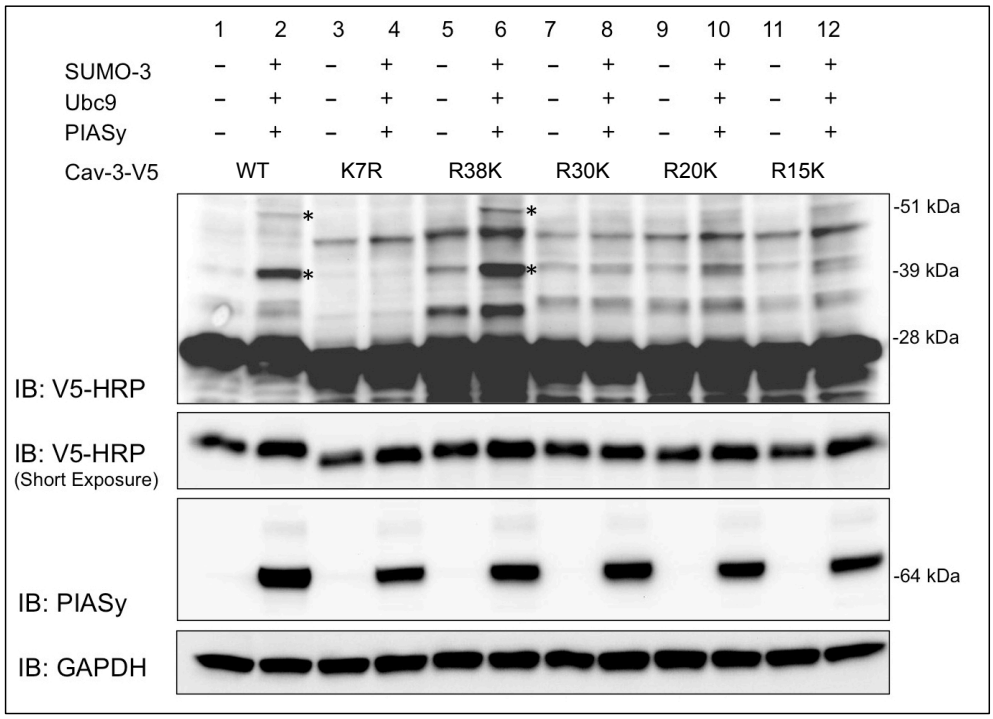

Figure 2.4 (C). Reversal of Each Arg Mutation in Cav-3-V5-K7R Back to Lys One at a Time Reveals That K38 is the Preferred Sumoylation Site When PIASy is Co-Expressed

(C) *PIASy-dependent sumoylation of R38K***.** The four N-terminal 'R to K' mutants; R15K, R20K, R30K and R38K (lanes 5-12) were transfected with and without the sumoylation machinery. WT and Cav-3-V5-K7R (lanes 1-4) were included as positive and negative controls for Cav-3-V5-SUMO-3 conjugates. Lysates were analyzed by immunoblotting with anti-V5-HRP, anti-PIASy and anti-GAPDH antibodies.

performed two rounds of multisite-mutagenesis to generate multiple ‘K to R’ mutants for screening in HEK 293 cells (Table 2.2). None of the multiple ‘K to R’ mutants completely prevented sumoylation of caveolin-3 (Figure 2.3 D, lanes 6-11 and unpublished data). Sumoylation was abolished when all seven of the lysines in caveolin-3 were mutated to arginine (Cav-3-V5-K7R) (Figure 2.3 C, lane 7; Figure 2.3 D, lanes 1-4), implying that if the preferred sumoylation site(s) are mutated, remaining lysines have the potential to be sumoylated.

2.3.10 K38 is the Preferred Sumoylation Site: Systematic Reversal of Lys Mutations in Cav-3-V5-K7R from Arg back to Lys (‘R to K’ mutants)

In order to define precisely which lysine residues are *potential* vs. *preferred* sumoylation sites (and thus the minimal lysine mutations necessary to create a sumoylation-deficient mutant) the K7R mutant was used to create a series of seven Cav-3-V5 ‘R to K’ mutants, each with a single available lysine (Figure 2.4 A, Table 2.3). To determine the relative ability of each of the 7 lysines of caveolin-3 to be sumoylated, the ‘R to K’ mutants were transfected in HEK 293 cells in parallel with WT and the Cav-3-V5- K7R mutant as positive and negative controls respectively. Immunoblotting with anti-V5-HRP antibodies showed that while sumoylation was detectable at each lysine residue, re-introduction of the consensus site lysine in the R38K mutant was much more effective at rescuing Cav-3-V5’s ability to undergo sumoylation (Figure 2.4 B, lane 6). Moreover, R38K was the only mutant that, akin to WT Cav-3-V5, was poly-sumoylated (*, Figure 2.5 B, lane 1 vs. 6). Since R38K has a single lysine, I conclude that PIASy stimulates modification of caveolin-3 by poly-SUMO-3 chains on K38.

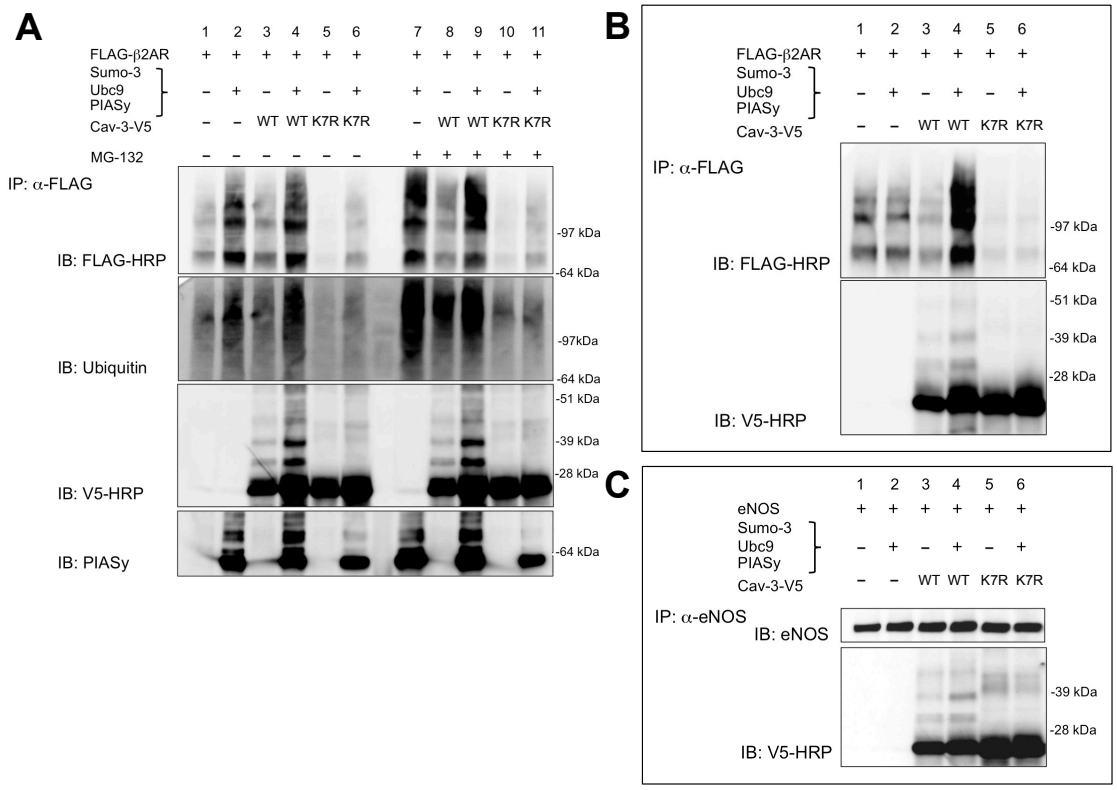

Figure 2.5 (A-C). Wild-Type Cav-3-V5 Stabilizes While The Sumoylation-Deficient K7R Mutant Destabilizes FLAG-β_2AR

(A) - (B) *Co-expression of Cav-3-V5-K7R dramatically reduces the level of FLAG-β_2AR protein and WT has an opposite effect when co-transfected with the sumoylation machinery.* HEK 293 cells were co-transfected with FLAG-β_2AR (lanes 1-11) and either WT Cav-3-V5 (lanes 3-4 and 8-9) or the K7R mutant (lanes 5-6 and 10-11), both with and without the sumoylation machinery; SUMO-3, Ubc9 and PIASy (even lanes vs. odd lanes respectively). The proteasomal inhibitor MG-132 was pre-incubated with cells 3 h prior to lysis (lanes 7-11). 48 h post-transfection, lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG-HRP, anti-ubiquitin, anti-V5-HRP and anti-PIASy antibodies.

(B) HEK 293 cells were transfected identical to those in **(A)** lanes 1-6 however cells were harvested at 24 h post-transfection rather than at 48 h. Lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG-HRP and anti-V5-HRP antibodies.

(C) *eNOS expression is not effected by co-transfection WT or Cav-3-V5.* HEK 293 cells were co-transfected with eNOS (lanes 1-6) and either WT Cav-3-V5 (lanes 3-4) or the K7R mutant (lanes 5-6) with and without the sumoylation machinery; SUMO-3, Ubc9 and PIASy. Cells were harvested 24 h post-transfection and anti-eNOS immunoprecipitates were analyzed by immunoblotting with anti-eNOS and anti-V5-HRP antibodies.

In order to confirm that co-expression of PIASy was required and specific for poly-sumoylation at K38, the four N-terminal R to K mutants (R15K, R20K, R30K and R38K) were transfected with and without co-expression of the sumoylation machinery (SUMO-3, Ubc9 and PIASy). Immunoblotting with anti-V5-HRP, anti-PIASy and anti-GAPDH revealed that co-expression of the sumoylation machinery had no effect on the relatively low level of modification of R15K, R20K or R30K, but strongly enhanced modification of R38K (Figure 2.4 C, lane 6). The modification of R38K closely resembled WT Cav-3-V5 (*, Figure 2.4 C, lane 2 vs. lane 6). Thus, expression of PIASy is necessary and specific for modification of K38 on caveolin-3 by poly-SUMO-3 chains.

2.3.11 Wild-type Cav-3-V5 Stabilizes While the Sumoylation-Deficient K7R Mutant Destabilizes β_2ARs in HEK 293 Cells

Sumoylation is known to modulate protein-protein interactions. The sumoylation-deficient mutant of caveolin-3 was used to probe the effect of SUMO modification on protein-protein interactions of caveolin with a well-known GPCR binding partner, the β2-adrenergic receptor (β_2AR). β_2ARs reside predominantly in caveolar microdomains in cardiac myocytes and directly interact with caveolin-3 (Ostrom et al., 2001; Xiang et al., 2002; Shcherbakova et al., 2007). In order to determine whether sumoylation of caveolin-3 modulates its interaction with β_2ARs, a FLAG-tagged β_2AR construct was transfected into HEK 293 cells either alone, or with WT or Cav-3-V5-K7R. Transfected cells were grown in DMEM supplemented with 10 % FBS, harvested 48 h post-transfection, lysed and immunoprecipitated with anti-FLAG-agarose beads. Immunoprecipitates were analyzed by immunoblotting with anti-FLAG-HRP, ubiquitin, V5-HRP and PIASy

antibodies (Figure 2.5 A). The supernatants of each anti-FLAG immunoprecipitation were also analyzed by immunoblotting and FLAG-β_2AR was not detectable (data not shown). It was anticipated that there might be a sumoylation-dependent increase or decrease in FLAG-β_2AR's ability to pull down Cav-3-V5, perhaps through interference of binding to the CSD. Surprisingly, the level of FLAG-β_2AR was either increased or decreased dependent on co-expression of WT or Cav-3-V5-K7R respectively (Figure 2.5 A, lanes 1, 3 and 5). Co-expression of the sumoylation machinery (SUMO-3, Ubc9 and PIASy) had a stabilizing effect on the level of FLAG-β_2AR (Figure 2.5 A, lanes 2, 4 and 6) independent of Cav-3-V5 expression.

Unstimulated β_2ARs have a half-life of ~24 h (Berthouze et al., 2009). I thus conducted studies at 24 vs. 48 h post-transfection to determine if a similar change in receptor levels occurred at an earlier time point. WT Cav-3-V5 significantly stabilized the expression of FLAG-β_2AR when the sumoylation machinery was co-expressed (Figure 2.5 B, lanes 1-2 vs. 3-4) while the K7R mutant destabilized FLAG-β_2AR (lanes 1-2 vs. 5-6). At this time point, there was no effect of co-expression of the sumoylation machinery independent of Cav-3-V5. Thus, co-expression of the K7R mutant consistently decreases expression levels of FLAG-β_2ARs.

The mechanisms of agonist-induced internalization, β-arrestin-mediated Mdm2 recruitment, ubiquitination and lysosomal downregulation of the β_2AR have been extensively studied in HEK 293 cells (Shenoy et al., 2001; Liang et al., 2003; Chapter 1.10). β-adrenergic agonists (norepinephrine and epinephrine) are present in fetal bovine serum (FBS) used to supplement cell culture media (Dibner and Insel, 1981). Therefore,

the FLAG-β_2AR transfected HEK cells were unintentionally exposed to agonist stimulation during these experiments through the supplementation of culture media with FBS. The data shown thus far suggest that modification of caveolin-3 by SUMO-3 inhibits, while the sumoylation-deficient caveolin-3 mutant may promote agonist-dependent desensitization of β_2ARs.

In order to determine whether the effect of the K7R mutant of caveolin-3 was specific for β_2AR, a similar experiment was performed with eNOS (NOS3), an unrelated, but well-known, caveolin-regulated protein. Binding of caveolin to eNOS inhibits eNOS activity (Ostrom et al., 2004; García-Cardeña et al., 1997). We thus transfected HEK 293 cells with eNOS alone or with WT or Cav-3-V5-K7R. Cells were co-transfected both with and without the sumoylation machinery (Figure 2.6 C, lanes 1, 3 and 5 vs. 2, 4 and 6). 24 h post-transfection, cells were lysed, immunoprecipitated with anti-eNOS antibodies and analyzed by immunoblotting with anti-eNOS and anti-V5-HRP antibodies. The expression level of eNOS was unaffected by expression of either WT Cav-3 or Cav-3-V5-K7R, suggesting that the effect on FLAG-β_2AR is not non-specific inhibition or interference of protein expression caused by the Cav-3-V5-K7R mutant.

2.3.12 Cav-3-V5-K7R Does Not Destabilize FLAG-β_1AR in HEK 293 Cells

To test if the Cav-3-V5-K7R mutant might affect a protein more closely related to β_2AR, impact of the mutant on β_1ARs was examined. β_1ARs and β_2ARs are highly homologous both structurally and functionally, sharing 52% identity overall and 76% identity in the transmembrane domains (Shcherbakova et al., 2007). A portion of β_1ARs

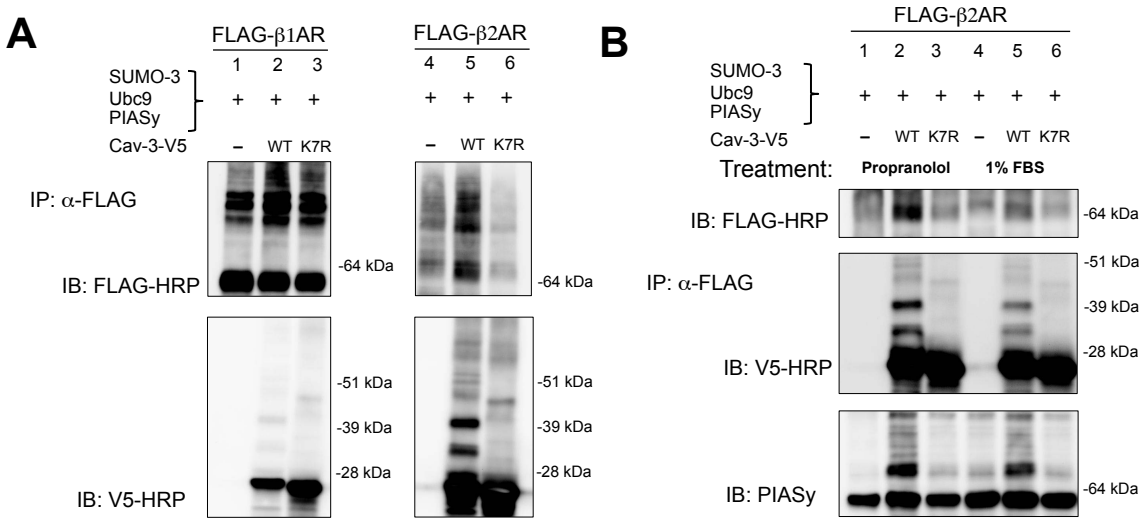

Figure 2.6 (A-B). The Sumoylation-Deficient K7R Mutant Does Not Affect the Stability of FLAG-β_1AR and Destabilization of β_2AR by K7R is Blocked by a β-Adrenergic Antagonist (-)-Propranolol

(A) *FLAG-β_1AR is not destabilized by the K7R mutant*. HEK 293 cells were co-transfected with FLAG-β_1AR (lanes 1-3) or FLAG-β_2AR (lanes 4-6), the sumoylation machinery (lanes 1-6) and either WT Cav-3-V5 (lanes 2 and 5) or the K7R mutant (lanes 3 and 6). Cell lysates were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG-HRP and anti-V5-HRP antibodies.

(B) *The destabilization of FLAG-β_2AR by Cav-3-V5-K7R is blocked by (-)-propranolol and reduced serum.* HEK 293 cells were co-transfected with FLAG-β_2AR and the sumoylation machinery (lanes 1-9) with either WT Cav-3-V5 (lanes 2, 5 and 8) or the K7R mutant (lanes 3, 6 and 9). Cells were cultured in media supplemented with 2 μM or 20 μM (-)-propranolol for 48 hours (lanes 1-3 and 7-9 respectively). Cells were also cultured with reduced serum for 24 h prior to harvest (lanes 4-6). Anti-FLAG immunoprecipitates were analyzed by immunoblotting with anti-FLAG-HRP and anti-V5-HRP antibodies.

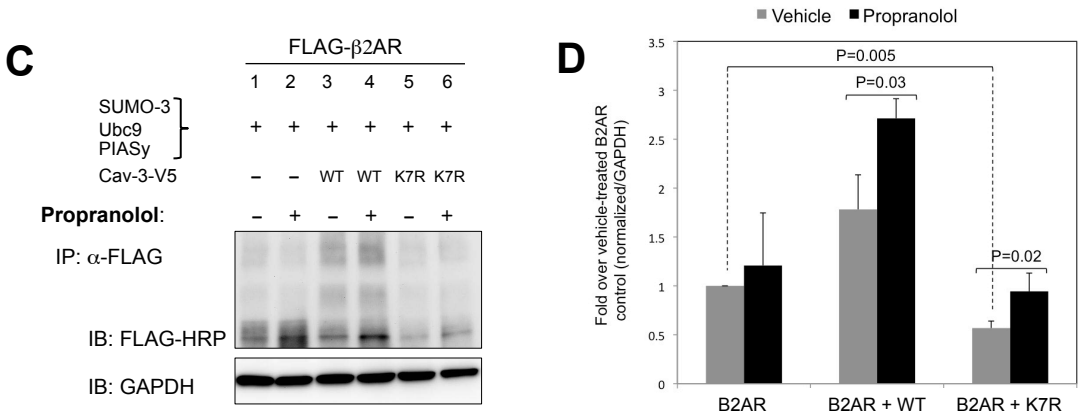

Figure 2.6 (C-D). The Sumoylation-Deficient K7R Mutant Does Not Affect the Stability of FLAG-β_1AR and Destabilization of β_2AR by K7R is Blocked by a β-Adrenergic Antagonist (-)-Propranolol

(C) HEK 293 cells were transfected as in A and B and were treated or not with 2 μM (-)-propranolol for 48 hours. As before, lysates were immunoprecipitated with anti-FLAG antibodies, however proteins were *not* heated prior to SDS-PAGE to reduce formation of FLAG-β_2AR aggregates. Immunoprecipitates and cell lysates were analyzed by immunoblotting with anti-FLAG-HRP and anti-GAPDH antibodies respectively.

(D) FLAG-HRP immunoblots performed in triplicate were quantified by densitometry, normalized to GAPDH and expressed as fold over vehicle-treated control (lane 1). A representative blot is shown in **(C)**. Mean values and standard deviations are shown. P values were calculated using Student's *t*-test (Graphpad).

also localize to caveolae and interact with caveolin-3 but do not undergo agonist-induced ubiquitination, internalization and desensitization (Liang et al., 2003; Liang et al., 2004).Studies with β_1ARs were designed to test the generality of sumoylation of caveolin in terms of regulation of βARs and GPCRs. FLAG-tagged β_1AR or β_2AR were co-transfected into HEK 293 cells either alone or with WT or Cav-3-V5-K7R. Unlike FLAG-β_2ARs, FLAG-β_1ARs were not destabilized by co-transfection of the Cav-3-V5-K7R mutant (Figure 2.6 A, lanes 1 and 3 vs. 4 and 6), suggesting that this destabilization is specific for β_2ARs.

2.3.13 The Beta Adrenergic Antagonist (-)-Propranolol Prevents the Destabilization of β_2AR by Cav-3-V5-K7R

To determine whether the destabilization FLAG-β_2AR by Cav-3-V5-K7R depends upon β-agonist stimulation by catecholamines present in the culture media, I used the β-adrenergic antagonist (-)-propranolol to block stimulation of FLAG-β_2AR. HEK 293 cells transfected with the sumoylation machinery (SUMO-3, Ubc9 and PIASy) were co-transfected with either; FLAG-β_2AR alone, WT Cav-3-V5 or the K7R mutant in the absence or presence of (-)-propranolol. Treatment with (-)-propranolol prevented the destabilization of β_2AR by the K7R mutant (Figure 2.6 B, lanes 1-3 and 7-9). Moreover, overnight incubation of cells in media supplemented with reduced serum (1% vs. 10% FBS) also prevented desensitization of FLAG-β_2AR (Figure 2.6 B, lanes 4-6). In each case, WT Cav-3-V5 stabilized FLAG-β_2AR expression levels (Figure 2.6 B).

This result was confirmed in a subsequent experiment in which HEK 293 cells were co-transfected as before with FLAG-β_2AR alone, WT Cav-3-V5 or the K7R mutant, and treated with or without 2 μM (-)-propranolol (Figure 2.6 C). FLAG-HRP immunoblots of anti-FLAG immunoprecipitates were quantified by densitometry, normalized to GAPDH and expressed as fold over vehicle-treated control (Figure 2.6 D). Immunoprecipitates and protein samples are typically heated prior to analysis by SDS-PAGE; however, this can cause self-aggregation of membrane proteins such as GPCRs (Ren et al., 2009). For the purpose of accurate quantification, anti-FLAG immunoprecipitates were *not* heated prior to separation by SDS-PAGE (as they were in previous experiments) to reduce the formation of FLAG-β_2AR aggregates (i.e. Figure 2.6 A and B, >100 kDa). I found that K7R reduced FLAG-β_2AR expression levels by about 50% (P=0.005) in vehicle-treated cells and treatment with (-)-propranolol increased the expression level back up to control levels (Figure 2.6 C). Again, WT Cav-3-V5 increased FLAG-β_2AR expression by ~2 fold and expression was further stabilized by (-)-propranolol up to 2.5 – 3.0 fold. These data indicate that caveolin-3 and its ability to undergo sumoylation modulates the stability of β_2ARs, and agonists, including those present in FBS, contribute to the destabilization that occurs in cells expressing K7R.

2.4 Discussion

The identification of a core SUMO consensus motif and extended, NDSM in its N-terminus prompted the investigation of caveolin-3 as a potential sumoylated substrate. These studies demonstrate, for the first time, that caveolin-3 is post-translationally

modified by SUMO proteins both *in vitro* and *in vivo*. The functional importance of sumoylation of caveolin-3 includes the regulation of specific caveolin partners, such as β_2AR, whose agonist-induced desensitization is influenced by such sumoylation.

In vitro sumoylation assays and co-expression of recombinant caveolin-3 in E. coli with the sumoylation machinery showed that conjugation with SUMO-1 or SUMO-3 is possible, however in mammalian cells, conjugation was specific for SUMO-3. In E. coli transformed with the sumoylation machinery, caveolin-3 was modified at multiple sites or by poly-SUMO-1 (Figure 2.1 C and D; Figure S2.4 B). There is debate regarding the formation of SUMO-1 chains *in vivo* (Yang et al., 2006A); however, such formation is favored in the *in vitro* assay because the sumoylation machinery and SUMO-1 proteins are present at higher levels than they are when expressed endogenously. The absence in E. coli of SENPs, which reverse SUMO conjugation and edit poly-SUMO chains, may also contribute to the formation of poly-SUMO-1 chains *in vitro*. In addition, the presence of tandem SIMs in caveolin-3's N-terminus may stimulate its modification by poly-SUMO chains by recruitment of Ubc9 thioesters containing polymerized SUMO-1 (i.e. Ubc9~(Su-1)-(Su-1)-(Su-1)). The specificity for modification by SUMO-3 in HEK 293 cells may result from the greater cytosolic localization of SUMO-3 vs. SUMO-1 or the preferential binding of the SIMs in caveolin-3 to SUMO-3 rather than SUMO-1 (Su et al., 2002; Hay, 2007; Meulmeester et al., 2008).

The current studies show that the SUMO E3 ligase PIASy enhances modification of caveolin-3 by SUMO-3 when co-expressed in HEK 293 cells and that K38 is the preferred sumoylation site. This specificity for K38, which lies in the tetrapeptide core SUMO motif, was identified through the systematic use of site-directed mutagenesis and

transient expression in HEK 293 cells. The identification was complicated by the fact that mutation of K38 reduced, but did not abolish, sumoylation of caveolin-3, suggesting the possibility that multiple lysines were modified. Analysis of the 'R to K' mutants allowed me to conclude that K38 was modified by SUMO-3 and poly-SUMO-3 chains. This was demonstrated by the fact that multiple, slower migrating bands in V5-HRP immunoblots only occurred with the R38K mutant (Figure 2.4 B and C).

It is unclear why mutation of the preferred sumoylation site failed to abolish sumoylation of caveolin-3, however it may be due to the structure of caveolin and the accessibility of alternate lysines for modification. The secondary structure of caveolin's N-terminus has not been well defined, but it appears to be very flexible. Circular dichroism studies of amino acids 79–96 in caveolin-1 that make up the CSD (aa55-72 in caveolin-3) reveal that they form an α-helix while the remainder of the N-terminus lacks secondary structure (Fernandez et al., 2002). This lack of secondary structure could help explain why non-consensus site lysines are accessible when K38 is mutated. Fernandez et al. proposed that this N-terminal tail region (aa1-54 in caveolin-3) wraps back around the α-helical region (Figure 2.7 A). They showed that mutation of caveolin-1 aa66–70 (^{66}IDFED^{70}) to alanine dramatically affected oligomerization and speculated that this acidic patch binds to basic residues in the α-helix, thereby causing the tail to wrap around the helix (i.e. the CSD). This acidic region in caveolin-1 corresponds to aa39-43 in caveolin-3 (^{39}VDFED^{43}) which is next to the sumoylated K38 (Figure 2.7 A; EDIV**K(**Su)VDFED). A helical wheel projection illustrates clustering of basic, polar and non-polar residues on different sides of the α-helical scaffolding domain (Figure 2.7 B).

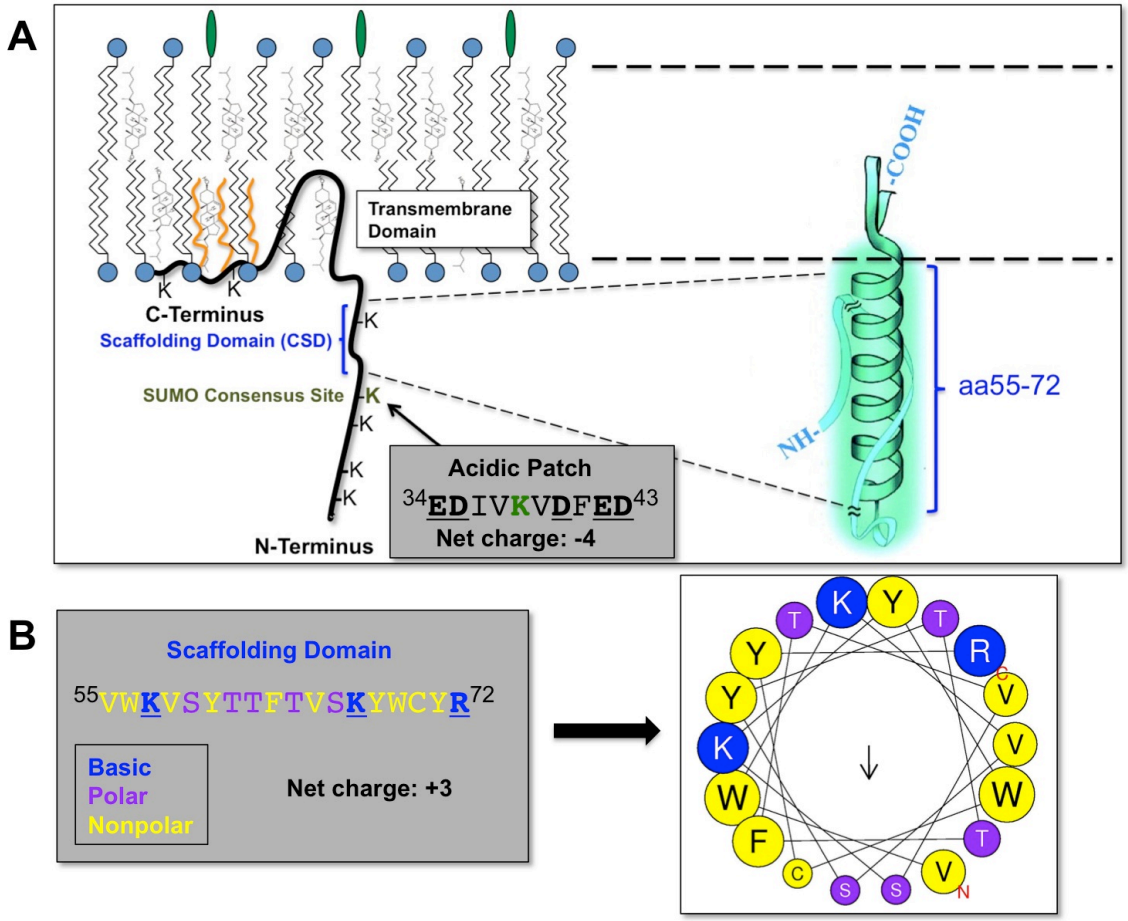

Figure 2.7 A "SUMO Switch" for Regulation of CSD Protein-Protein Interactions: Proposed Interaction of Basic Residues in the α-Helical Scaffolding Domain with an 'Acidic Patch' Surrounding Caveolin-3's Sumoylation Site.

(A) *Caveolin-3's membrane topology and α-helical secondary structure of the scaffolding domain (CSD)*. The CSD (aa55-72) was shown to form an α-helix while the remainder of the N-terminus lacks secondary structure (Fernandez et al. 2002). It was proposed that the N-terminal tail region (aa1-54) wraps back around the α-helical CSD. Interaction of the N-terminal tail with the CSD might occur through electrostatic interactions of an acidic patch (^{34}**ED**IVKV**D**F**ED**^{43}) with basic residues in the CSD. (α-helix image adapted from Fernandez et al.)

(B) *Helical wheel projection of caveolin-3's scaffolding domain.* The amino acid sequence of the CSD (^{55}VW**K**VSYTTFTVS**K**YWCY**R**^{72}) is color coded by each residue's side chain properties and represented linearly and as a helical wheel projection to illustrate the clustering of basic (blue), polar (purple) and non-polar (yellow) residues along different sides of the helix. (Gautier et al, 2008; http://heliquest.ipmc.cnrs.fr/). The single letter amino acid codes in the helical wheel are proportional to amino acid volume.

Thus, modification by SUMO at this site could block the N-terminal tail region from wrapping around the α-helical CSD and act as a switch to regulate binding of other proteins to the CSD or oligomerization of caveolins.

The two tandem SIMs in caveolin-3's N-terminus (Figure 1.6 and Figure S2.7) and caveolin's ability to oligomerize may also contribute to the ability of other lysines to be sumoylated, albeit at a much lower level than WT or the R38K (Figure 2.4 C). Proteins can be sumoylated on non-consensus sites that are in close proximity to a SIM motif (e.g. USP25, Meulmeester et al., 2008). This suggests that stabilization of the binding between Ubc9~SUMO thioesters and the target by a SUMO-SIM interaction or by substrate-E3 ligase interactions may facilitate the interaction of Ubc9 with non-consensus site lysines. The presence of tandem SIMs in caveolin-3 may facilitate such interactions when K38 is mutated (Figure 1.6, Chapter 4). Also, given the proximity of these SIMs to caveolin's SUMO consensus motif, it is conceivable that they recruit and orient specific Ubc9~SUMO thioesters (e.g. mono-, di- or poly-SUMO-2/3 chains), thereby intramolecularly regulating the modification of caveolin-3. Since caveolin also forms homo- and hetero-oligomers, another possibility is that the SIMs help direct the sumoylation of an adjacent caveolin monomer, *inter*molecularly rather than *intra*molecularly.

As with most SUMO targets, only a fraction of caveolin-3 is sumoylated at any given time, thus complicating one's ability to assess how this modification is biologically relevant. This low stoichiometry may result from rapidly reversible cycles of sumoylation regulated by SENPs. It has been estimated that caveolin typically forms oligomeric complexes of 14-16 monomers (Scherer et al., 1996). Thus, sumoylation of just 6-7% of

the total caveolin protein pool potentially could result from each caveolin oligomer containing a sumoylated, caveolin monomer.

Results with the 'lysine-less' Cav-3-V5-K7R mutant show that the slower migrating bands observed in V5-HRP immunoblots result from lysine modifications of caveolin-3. The 40, 50 and 65 kDa bands are most likely due to modification of K38 by poly-SUMO-3 chains. This is supported by the evidence presented here as well as data to be presented in Chapter 3 that shows the SUMO-specific proteases, SENP1 and SENP2 are both able to dose-dependently decrease the Cav-3-V5-SUMO conjugates (i.e. the bands observed in V5-HRP immunoblots at 40, 50 and 65 kDa). However, there is a doublet of bands in V5-HRP immunoblots consistently seen between 40 kDa and 28 kDa that is not seen with the K7R mutant. Initially, these bands seemed to be too small to be SUMO conjugates and were attributed to smearing caused by protein overloading, lysis conditions or possible SDS-resistant non-covalent interactions that hindered a portion of Cav-3-V5's electrophoretic mobility. The Cav-3-V5-K7R and 'R to K' mutants brought these bands into focus although their identity remains poorly defined. Perhaps there are other types of lysine modifications occurring, for example, by ubiquitin or another Ubl protein. Ubiquitination can cause smaller shifts in SDS-PAGE migration (~10 kDa); however, immunoblotting with anti-ubiquitin antibodies did not reveal corresponding bands (unpublished data). Alternatively, since some degradation products are seen in V5-HRP immunoblots (multiple bands <25 kDa), this intermediate band may be a sumoylated version of a major degradation product.

The results presented here, as well as evidence in the literature, strongly suggest that PIASy interacts with caveolin-3 and is a key regulator of caveolin-3 sumoylation,

however it is not clear how or in which cellular compartment they interact. PIASy and other PIAS proteins have generally been considered nuclear proteins (Ihara et al., 2005; Albor et al., 2006) while caveolin-3 is primarily found in plasma membrane microdomains, with some cytosolic and perinuclear localization (Smythe et al., 2003). A study in PIASy $^{+/+}$ MEFs showed that while PIASy was predominantly found in the nucleus, it could also be detected in the cytoplasm (Martin et al., 2008). PIASy accumulates in the cytoplasm and co-localizes with the E3 ubiquitin ligase TRIM32 when MG-132 is used to inhibit proteasomal degradation (Albor et al., 2006), which may explain why it is normally detected at low levels or not at all in the cytoplasm. Consistent with this, treatment of HEK 293 cells with MG-132 for 3 h enhanced sumoylation of caveolin-3 relative to untreated cells (Figure 2.3 D, lane 5 vs. lane 2), presumably by increasing the cytosolic expression of PIASy.

These data are also consistent with a preliminary experiment I performed in HEK 293 cells that attempted to manipulate the endogenous, rather than exogenous, expression of PIASy. TGFβ treatment was shown to stimulate a 6-8 fold increase in PIASy mRNA expression that peaked after 12 h (Imoto et al., 2008). Thus, I used TGFβ to stimulate endogenous PIASy expression in HEK 293 cells in the presence or absence of MG-132. After 12 h, cells treated with MG-132 showed a 3-fold increase in PIASy protein expression (Figure S2.5 A) and a corresponding 50% increase in 40 kDa Cav-3-V5 (Figure S2.5 B).

PIASy appears to be highly expressed in cardiac myocytes (Supplemental Figure S2.2 A) and transfection of HEK 293 cells (which express low endogenous levels of PIASy (Figure 2.2 A)), with as little as 40 ng PIASy (1% of total DNA transfected)

greatly enhances sumoylation of caveolin-3 (Figure 2.2 B). PIASy co-immunoprecipitated with caveolin-3 from transfected HEK 293 cells (Figure 2.2 C and D). Preliminary immunofluorescence experiments performed on HEK 293 cells transfected with Cav-3-V5 alone or with the sumoylation machinery showed that the affinity tagged caveolin-3 construct was localized in cytosolic and plasma membrane puncta, similar to endogenous caveolin-3, indicating that PIASy co-localizes with caveolin-3 in these compartments (Figure S2.6).

PIASy interacts with the type I TGF-β (TβRI) receptor in MCF7 human breast cancer cells (Conrotto et al., 2007) and TβRI receptors localize to caveolae and interact with caveolin-1 and eNOS in endothelial cells (Schwartz et al., 2005); however, the extent to which caveolin-3 and PIASy co-localize in other cell types, such as cardiac myocytes and skeletal muscle has not been investigated. The E3 ubiquitin ligase TRIM32 can regulate cellular levels of PIASy by its ubiquitination in the cytoplasm. Akin to caveolin-3, TRIM32 is mutated in LGMD (type 2H) and the mutation prevents TRIM32 from binding to PIASy (Albor et al., 2006). Thus, the regulation of cytosolic sumoylation by PIASy may have physiological and pathophysiological effects.

Sumoylation is also regulated by SUMO-specific proteases. SENP2 has the ability to de-sumoylate caveolin-3 (Chapter 3) and, like PIASy, its cytosolic expression is regulated by ubiquitination and proteasomal degradation (Itahana et al., 2006). Therefore it is conceivable that MG-132 affects the level of caveolin sumoylation through SENP2 expression and/or localization. SENP2 expression can be induced in a cAMP-dependent manner in adipocytes (Chung et al., 2010) and elevated cAMP induces SENP2 expression through phospho-CREB binding to SENP2's promoter region. This finding complements

data in a recent study which showed PIASy-dependent sumoylation of phosphodiesterase 4D5 (PDE4D5), which is recruited to activated β_2ARs (Lynch et al., 2005; De Arcangelis et al., 2009; Li et al., 2010) as well as findings presented in this dissertation regarding the PIASy-dependent sumoylation of caveolin-3 and its effects on β_2AR desensitization. Together, such findings suggest existence of a sumoylation-regulated feedback loop in the regulation and compartmentation of GPCR-G_s-cAMP-PDE signaling pathways in cardiac myocyte caveolae microdomains. It is possible that caveolin-3, β_2AR, PDE4D5, PIASy and SENP2 exists as part of a signaling complex ("signalosome") localized in caveolae that specifically regulates cellular responses to agonist stimulation.

The precise mechanism by which sumoylation of caveolin affects desensitization of β_2ARs is not clear, but it adds a new feature to the desensitization process. Caveolin-3 might regulate β_2ARs by influencing agonist-induced internalization of the receptors. For example, β-arrestin-2 and Mdm2 are recruited to activated receptors after GRK2 phosphorylation of the β_2AR C-tail. Caveolin-1 interacts with Mdm2 following H_2O_2 treatment (Bartholomew et al., 2009) and oxidative stress caused by H_2O_2 can stimulate altered patterns and increases in SUMO-2/3 conjugation (Manza et al., 2004). Moreover, Mdm2 is a target of sumoylation (Meek and Knippschild, 2003; Shenoy et al., 2001), and PIASy co-operates with Mdm2 to regulate sumoylation of p53 (Carter et al., 2007). β-arrestin-2 preferentially interacts with the sumoylated form of Mdm2 (p90Mdm2, Shenoy et al., 2001). A search for SIMs similar to those in caveolin-1 and -3 revealed the presence of tandem SIMs in β-arrestin-1 and β-arrestin-2 (Figure 1.8), suggesting that these motifs may mediate the preferential binding of p90Mdm2. Destabilization of β_2AR

by the caveolin-3 K7R mutant might thus be attributable to sumoylation-dependent alterations of Mdm2-β-arrestin-2 interactions.

The mechanism of caveolin-3's effect on the stability of β_2AR could also be mediated though PIASy. Co-expression of Cav-3-V5-K7R appears to decrease the expression level of PIASy relative to WT (Figure 2.4 C, lanes 2 vs. 4, Figure 2.5 A, lanes 6 and 11) and Cav-3-V5-K7R may affect the posttranslational modification of PIASy, which is regulated by ubiquitination and sumoylation (Albor et al., 2006; Ihara et al., 2005). Consistent with this possibility, we find multiple bands in PIASy immunoblots, which likely correspond to modified forms of PIASy, that differ when WT Cav-3-V5 or K7R is co-expressed (Figure 2.6 B, bottom panel).

2.5 Methods

2.5.1 Materials and Chemicals

Reagents and their sources were as follows: Protein-G-agarose (Roche; Cat. 11243233001), MG-132 (Calbiochem; Cat. 474790), N-Ethylmaleimide (NEM) and (S)-(-)-Propranolol (Sigma; Cat. E3876 and P8688), Halt Protease Inhibitor Cocktail, EDTA-Free (Pierce; Cat. 87785), Ni-NTA agarose resin (Qiagen; Cat. 30210), goat anti-V5-agarose (Bethyl Labs; Cat. S190-119). Multi-Site and Lightning Mutagenesis kits (Stratagene; Cat. 200515 and 210518) and BL21(DE3) competent cells (Novagen; Cat. 70235-3), Isopropyl-β-D-1-thiogalactopyranoside (IPTG, Invitrogen; Cat. 15529019). 1-O-Octyl-β-D-glucopyranoside (A.G. Scientific; Cat. 0-1036). For SDS-PAGE, NuPAGE Bis-Tris 4-12% 12-well gels (Invitrogen) and PVDF membranes (Milliopore) were used.

SeeBlue Plus2 pre-stained standard (Invitrogen; Cat. LC5925) was used for visualization of protein molecular weights.

2.5.2 Antibodies

Antibodies and their sources were as follows: anti-FLAG M2 Affinity Gel and mouse monoclonal anti-FLAG M2 antibody (Sigma; Cat. A2220 and F1804), anti-FLAG-HRP and anti-Myc (Cell Signaling; Cat. 2044 and 2276), mouse monoclonal anti-V5 and anti-V5-HRP (Invitrogen; Cat. R960-25 and R961-25), rabbit polyclonal anti-PIASy, anti-Cav-3, anti-eNOS, anti-SUMO-1 and anti-rabbit secondary antibodies (Abcam; ab58416, ab2912, ab66127, ab11672 and ab6013), anti-SUMO-2/3, anti-Ubc9 and anti-ubiquitin (Santa Cruz; sc-32873, sc-10759 and sc-47721), rabbit polyclonal anti-SUMO-1 (Invitrogen; Cat. 38-1900).

2.5.3 Plasmids

The mammalian expression plasmids for eNOS, SUMO-1, SUMO-3, Ubc9, PIAS3 and PIASy were obtained from Origene (Cat. SC108440, SC118050, SC115792, SC109867, SC11632 and SC114580) and the FLAG-β_1AR and FLAG-β_2AR mammalian expression plasmids were obtained from Addgene (Cat. 14697 and 14698). Myc-SUMO-1, Myc-SUMO-3 and PIAS1 were provided by Dr. Serena Ghisletti. The E. coli expression plasmids pKRSUMO and pBADE12 were from Dr. Mario Mencia (Mencia and Lorenzo, 2004). The Cav-3-V5/His plasmid (referred as Cav-3-V5) was provided by Dr. Brian Head and was constructed by cloning the rat caveolin-3 gene (AC_NM019155) into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Cat. K4800). The E. coli

expression plasmid pST39-Cav-3-V5/His (referred as pST39-Cav-3-V5) was generated by subcloning the Cav-3-V5/His coding region from the pcDNA3.1-V5/His expression vector and inserting it into cassette 2 (EcoRI/HindIII) of the polycistronic vector pST39 (Tan, 2001). The following primers were used to amplify Cav-3-V5/His and introduce EcoRI and HindIII restriction sites by PCR: Cav3Fw 5'-GATCGAATTCA TGATGACCGAAGAGCACAC-3', Cav3Rv 5'-GATCAAGCTTTCAATGGTGATGGT GATGATG-3'.

2.5.4 Site-Directed Mutagenesis

The single and multiple 'K to R' mutations (Table 2.1 and 2.2) were introduced into WT Cav-3-V5 using Multi-Site and Lightning QuikChange Site-Directed Mutagenesis Kits (Stratagene). The 5' and 3'-primers designed for creation of the 'K to R' mutants were as follows: K15R 5'-CTGGAGGCACGGATCATCAGGGACATTCACTG C-3', K15R_antisense 5'-GCAGTGAATGTCCCTGATGATCCGTGCCTCCAG-3', K20R 5'-TCAAGGACATTCACTGCAGGGAGATAGACTTGG TG-3', K20R_antisense 5'-CACCAAGTCTATCTCCCTGCAGTGAATGTCCTTGA-3', K30R 5'-GGTGAACAGAGACCCCAGGAACATCAATGAGGACA-3', K30R_antisense 5'-TGTCCTCATTGATGTTCCTGGGGTCTCTGTTCACC-3', K38R 5'-CCAAGAACAT CAATGAGGACATTGTGAGGGTGGATTTCGAAG-3', K38R_antisense 5'-CTTCGAAATCCACCCTCACAATGTCCTCATTGATGTTCTTGG -3', K69R 5'-CTTTCACCGTCTCCAGGTACTGGTGCTACCG-3', K69R_antisense 5'-CGGTAGC ACCAGTACCTGGAGACGGTGAAAG-3', K108R 5'-CCGTGGTGCCCTGCATTAG

GAGCTACCTGATTG-3', K144R 5'-GCCAGGTCTGCAGCAACATTAGGGTGGTG CTGC-3'.

Cav-3-V5-K7R was used to create a series of Cav-3-V5 ‘R to K’ mutants (Table 2.3). The 5' and 3'-primers designed for creation of the ‘R to K’ mutants were as follows: R15K 5' CTGGAGGCACGGATCATCAAGGACATTCACTGC-3', R15K_antisense 5'-GCAGTGAATGTCCTTGATGATCCGTGCCTCCAG-3', R20K 5'-TCAGGGACATTCACTGCAAGGAGATAGACTTGGTG-3', R20K_antisense 5'-CACCAAGTCTATCTCCTTGCAGTGAATGTCCCTGA-3', R38K 5'-AGGAACATCA ATGAGGACATTGTGAAGGTGGATTTCGAA-3', R38K_antisense 5' TTCGAAATCC ACCTTCACAATGTCCTCATTGATGTTCCT-3', R69K 5'-CTTTCACCGTCTCCAAG TACTGGTGCTACCG-3', R69K_antisense 5'-CGGTAGCACCAGTACTTGGAGACG GTGAAAG-3', R108K 5'-CCGTGGTGCCCTGCATTAAGAGCTACCTGATTG-3', R108K_antisense 5'-CAATCAGGTAGCTCTTAATGCAGGGCACCACGG-3'. The R30K and R144K mutants were previously obtained during the second round of multisite mutagenesis of WT Cav-3-V5 (Table 2.2). All plasmids were sequenced to validate presence of desired mutations. Cav-3-V5 mutants were sequenced using the T7 promoter primer 5'-TAATACGACTCACTATAGG-3'.

2.5.5 Cell Culture and Transfection

Human embryonic kidney cells (HEK AD-293,) were obtained from Stratagene (Cat. 240085) and cultured in a 37°C, 5.0% CO2 incubator. Cells were grown in “HEK medium” (DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate, supplemented with 10% FBS) without antibiotics. The HEK AD-293 cell line is derived

from the HEK 293 cell line and has improved adherence properties. Confluent HEK AD-293 cells were transfected 2-3 days post-plating in 6-well plates. 4.0 μg plasmid DNA and 9 μl Lipofectamine 2000 (Invitrogen) were each diluted separately in 250 μl OptiMEM (Invitrogen) before being combined, incubated for 20 min at RT and diluted with 500 μl HEK medium. Transfection complexes (1 ml total volume) were pipetted onto cells in 6-well plates to which 2 ml fresh HEK medium had previously been added. Cells were incubated with transfection complexes overnight and medium was replaced with 3 ml fresh HEK medium the following day. Except where stated, cells were analyzed 48 h post-transfection. For co-expression of the sumoylation machinery with Cav-3-V5 the following ratios were used: Cav-3-V5 : SUMO-3 : Ubc9 : PIASy = 1 : 1 : 0.5 : ~0.15 - 0.25. FLAG-β_2AR, FLAG- β_1AR or eNOS were co-expressed in a 1:1 ratio with Cav-3-V5 using the same ratios of sumoylation machinery components. The total DNA for all transfections was normalized to 4.0 μg with empty pcDNA3.1 vector.

2.5.6 Immunoprecipitation and Immunoblotting

Transfected HEK AD-293 cells were washed with ice-cold PBS and scraped into 500 – 800 μl ice-cold "*in vivo* sumoylation" lysis buffer (20 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, 1 mM EDTA) supplemented with 20 mM NEM (prepared fresh by dissolving 125 mg NEM into 1 ml 90% EtOH) and Halt protease inhibitor cocktail (Pierce) and pipetted into pre-chilled 1.5 ml Eppendorf tubes. Cells were incubated on ice for 30 min and sonicated in an ice water bath (3 x 10 sec). Lysates were clarified by centrifugation (5,000 x g for 15 min at 4°C) and protein concentrations were normalized by dilution with lysis buffer after performing

BCA protein assays (Pierce). Clarified lysates were directly resolved by SDS-PAGE and immunoblotted with appropriate antibodies or subjected to immunoprecipitation. Lysates were diluted with 4x LDS sample loading buffer (Invitrogen) supplemented with 50 - 100 mM DTT and heated (except where stated) to 80°C for 5 min before being resolved by NuPAGE Bis-Tris 4-12% 12-well gels. Proteins were then transferred for 1.5 - 2.5 h at 55V onto PVDF membranes that were immediately blocked for 1 h at RT in 4% milk/0.1% TBST. In general, primary antibodies were diluted at 1:1000 in 4% milk/0.1% TBST except for V5-HRP (1:5000), Cav-3 (1:2000) and PIASy (1:2000). For anti-SUMO-1 and anti-SUMO-2/3 antibodies 5% BSA/0.1% TBST was used for blocking and dilution instead of milk to reduce background signal. PVDF membranes were incubated overnight at 4°C with primary antibodies and washed at least 3 x 10 min with 0.1% TBST before incubation with secondary antibodies for 45-50 min at RT. Membranes were then washed (4 x 10 min) with 0.1% TBST and detected by Amersham ECL (enhanced chemiluminescence, GE Heathcare; Cat. RPN2135V2) or SuperSignal West Dura (Pierce; Cat. 34075). Images were collected with a Sensicam QE High Performance CCD camera (Cooke Corporation) mounted on an Epi Chemi II Darkroom (UVP BioImaging Systems) and analyzed using LabWorks 4.0 image acquisition and analysis software (UVP BioImaging Systems).

For immunoprecipitations, 300-500 µl clarified lysates were pre-cleared with 40 µl Protein G-agarose (Roche), incubated overnight at 4°C with primary antibodies (1-2 µg) followed by incubation with 40 µl Protein G-agarose for 1 h. Complexes were centrifuged at 10,000 x g at 4°C for 1 min; supernatants were transferred to pre-chilled 1.5 ml Eppendorf tubes. The beads were washed 4 times with lysis buffer and eluted by

heating to 80-90°C for 5 min in 50 - 100 µl 2x LDS buffer with 100 mM DTT. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted with appropriate antibodies. Immunoprecipitations with anti-FLAG M2 and anti-FLAG M2-agarose (Sigma) were performed per manufacturers instructions with FLAG IP lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with 20 mM NEM, 10% glycerol and Halt protease inhibitor cocktail. Isolation of adult rat cardiac myocytes for immunoprecipitation with anti-Cav-3, anti-SUMO-1 and anti-SUMO-2/3 antibodies was performed as described previously (Head et al., 2006). Myocytes were washed with ice cold PBS and incubated on ice with lysis buffer (0.2% SDS, 0.5% NP-40, 15 mM $MgCl_2$, 1 mM DTT) supplemented with Halt protease inhibitor cocktail and 20 mM NEM. Lysates were sonicated, clarified by centrifugation and incubated overnight with primary antibodies at 4°C and processed by the Protein G-agarose method as described above.

2.5.7 *In Vitro* Sumoylation Assay

An *in vitro* protein synthesis kit (Qiagen EasyXpress Insect Kit II) was used to express recombinant Cav-3-V5 per manufactures instructions. 2 µl of protein synthesis reactions containing Cav-3-V5 were used directly in 20 µl *in vitro* sumoylation reactions (BioMol International) consisting of 2 µl of 10x sumoylation buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM ATP), 1 µl each of 20x SUMO E1 and SUMO E2 enzyme solutions and recombinant SUMO-1 or SUMO-3 proteins. Reactions were incubated at 37°C for 1 h and quenched with 2x LDS sample buffer with 100 mM DTT. 10 µl of the reaction product was analyzed by immunoblotting with anti-V5-HRP antibodies.

2.5.8 Expression and Purification of Sumoylated Caveolin-3 in Escherichia coli

For production of sumoylated caveolin-3, BL21(DE3) competent cells were sequentially transformed with three E. coli expression plasmids; pKRSUMO, pBADE12 and pST39-Cav-3-V5 as described (Mencia and Lorenzo, 2004; Saitoh et al., 2009). For negative controls, cells were transformed with pST39-Cav-3-V5 alone or with pKRSUMO and pBADE12. Transformed cells were stored as glycerol stocks at -80°C. For protein expression, transformed cells were grown at 37°C in LB supplemented with antibiotics (e.g. Amp, Cm and Kan). The OD_{600} was monitored and protein expression was induced at an OD_{600} of 0.5 - 0.6 for 3 h. Cultures were pelleted and resuspended in TNE buffer (25 mM Tris-HCL pH 7.5, 150 mM NaCl, 5 mM EDTA). Purifications were performed with Ni-NTA agarose (Qiagen) and anti-V5-agarose (Bethyl Labs) resins per manufacturer's instructions. For a detailed protocol see Supplemental Methods.

S2.5 Supplementary Methods

S2.5.1 Protocol for Expression and Native Purification of Sumoylated Cav-3-V5 in E. coli

A tandem affinity purification protocol was developed for the expression and purification of Cav-3-V5-SUMO conjugates. A single colony or glycerol stock of BL21(DE3) cells transformed with pKRSUMO, pBADE12 and pST39-Cav-3-V5 was used to inoculate a starter culture of 3-4 ml LB broth supplemented with ampicillin (Amp, 35mg/ml), chloramphenicol (Cm, 15mg/ml) and kanamycin (Kan, 15 mg/ml). The starter culture was incubated overnight (12-18 h) at 37°C with shaking (250 RPM) and 0.1 – 1.0 ml was used to inoculate a culture of 25-2,000 ml LB supplemented with Amp,

Cm and Kan. Expression cultures were incubated at 37°C with shaking (250 RPM) until the OD_{600} was 0.5 – 0.6 (~3-4 h). Aliquots were then removed for creation of glycerol stocks. For analysis of "Pre-IPTG" protein expression, 1-5 ml samples were pipetted into centrifuge tubes, pelleted (centrifuged at 2,000 x g for 10 min at 4°C) and resuspended in TNE buffer (25 mM Tris-HCL pH 7.5, 150 mM NaCl, 5 mM EDTA). Protein expression was induced with 0.5 mM IPTG for 3 h at 37°C. Expression cultures were pelleted (centrifuged at 4,000 x g for 10-20 min at 4°C), resuspended in ice-cold TNE buffer and stored overnight at -20°C. Bacteria were thawed on ice, pelleted (centrifuged at 2,000 x g for 10 - 20 min at 4°C), weighed and resuspended in Native Lysis Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) supplemented with 60 mM Octyl-β-D-Glucopyranoside (OG [A.G. Scientific; Cat. 0-1036]), Halt protease inhibitor cocktail and 0.1 mM EDTA. 3.0 ml lysis buffer was used per gram wet-weight of each pellet. Cells were incubated on ice for 30 min in lysis buffer and placed in an ice-water bath for sonication (6 x 10 sec, maximum setting). Lysates were passed through a 22 ½ gauge needle with a 10 ml syringe (BD#305156), aliquoted in to 2.0 ml centrifuge tubes and spun at 10,000 x g for 30 min to pellet insoluble material. Insoluble pellets were resuspended in Native Lysis Buffer with 2% Triton X-100, mixed with 4x LDS buffer plus 100 mM DTT and analyzed by immunoblotting with anti-V5-HRP antibodies. Supernatants (i.e. cleared lysates) were pooled and transferred to pre-chilled Poly-Prep Chromatography Columns (BioRad, Cat. 44285). The 50% slurry Ni-NTA resin (Qiagen) was equilibrated by rinsing once with 3 volumes ddH_2O followed by rinsing twice with 3 volumes 2X Native Lysis Buffer. Cleared lysates were combined with equilibrated Ni-NTA resin in a 4:1 ratio (e.g. 4 ml lysates per 1 ml resin). 200 µl of lysate was saved and

labeled as “Input” for SDS-PAGE and immunoblot analysis. Lysates and Ni-NTA resin were incubated for 1 h or overnight at 4°C with constant rotation. The plastic tab at the bottom of the chromatography column was removed and the flow through was collected in a 15 ml conical tube. A 200 µl aliquot was saved and labeled as “Supernatant” for SDS-PAGE and immunoblot analysis. The Ni-NTA resin was washed 3-4 times with 4-5 ml Native Wash Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, pH 8.0) supplemented with 60 mM OG and 0.1 mM EDTA. 200 µl of each wash was saved for analysis by SDS-PAGE and immunoblotting. The washed resin was eluted with Native Elution Buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) supplemented with 60 mM OG and 0.1 mM EDTA. 0.5 or 1.2 ml elution fractions were collected (for 250 or 2,000 mL starting cultures respectively) and adjusted to 20% glycerol for storage at -80°C. All purification fractions (Input, Supernatant, Washes and Elutions) were analyzed by immunoblotting with anti-V5-HRP and anti-SUMO-1 antibodies (Figure S2.3 C-E). Elutions containing Cav-3-V5 and Cav-3-V5-SUMO conjugates were pooled and concentration and buffer exchange was performed by rinsing 3 x with Desalting Buffer (50 mM NaH_2PO_4, 60 mM OG, pH 8.0) in Vivaspin 4 Polyethersulfone 30,000 MWCO ultrafiltration spin columns (Vivascience, Cat. VS0423).

The second step of the purification was performed by incubating the pooled and concentrated Ni-NTA elutions with anti-V5-agarose beads (Bethyl Labs) overnight at 4°C. The beads were washed 3 times with Desalting Buffer (supplemented with 100 mM NaCl and 2mM EDTA) and eluted with glycine buffer (0.1 M glycine pH 2.6) to avoid contamination with anti-V5 IgG proteins. The beads were eluted 3 x with 150 µl glycine

buffer, pooled and neutralized by adding 150 µl 2M Tris-HCl, pH 8.5. 2x LDS buffer with 100 mM DTT was used to elute remaining proteins bound to anti-V5-agarose beads after elution with glycine buffer. Samples from anti-V5-agarose purification fractions (Supernatant, Washes and Elutions) were analyzed by immunoblotting with anti-V5-HRP and anti-SUMO-1 antibodies (Figure S2.4 B).

Inclusion of EDTA in the resuspension buffer was crucial to prevention of proteolytic degradation of Cav-3-V5, presumably by metalloproteases, during the purification procedure. Therefore the lysis, wash and elution buffers were supplemented with 0.1 mM EDTA to inhibit degradation, but minimize interference with the Ni-NTA resin.

S2.5.2 Immunoflourescence

HEK AD-293 cells were transfected with WT Cav-3-V5 or the K38R sumoylation site mutant. WT transfected cells were co-transfected with and with out the sumoylation machinery (SUMO-3, Ubc9 and PIASy). 48 h post-transfection cells were washed twice with PBS, fixed with 3% paraformaldehyde buffer (Wako Chemicals) at 4°C for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were then washed three times with PBS, blocked (PBS + 10% FBS) 20 min at RT and incubated with anti-V5-FITC antibodies (1:500 in PBS) for 1 h at RT, protected from light. After washing twice with PBS, coverslips were fixed to microscope slides with Prolong Gold (Invitrogen). Images were collected using an Olympus IX70, multi-channel fluorescence microscope and deconvoluted with Softworx Suite software.

2.6 Acknowledgments

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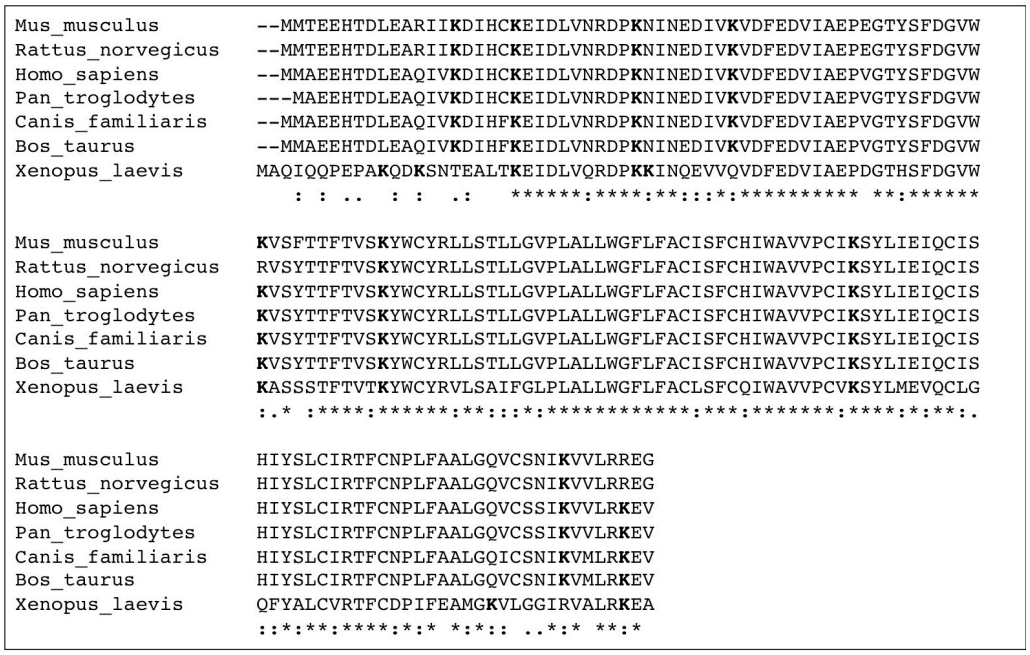


Figure S2.1 Evolutionary Conservation of Lysine Residues in Caveolin-3
ClustalX2.0.12 was used to align amino acid sequences of: mouse, rat, chimpanzee, dog, cow and frog caveolin-3 to determine the extent of conservation of lysine residues. The SUMO consensus site lysine at K38 is conserved except in frog. Human caveolin-3 has nine lysines while the rat caveolin-3, which was used in these studies, has only seven. One of the additional lysines in human caveolin-3 lies in the CSD at K59 and is present in all other species shown. In rat, an arginine is present instead, indicating that a positive charge may be favored at this position rather it being a site of regulation. This raises an interesting possibility that sumoylation at this site could regulate binding of proteins to the CSD, however the surrounding residues do not resemble a SUMO motif.

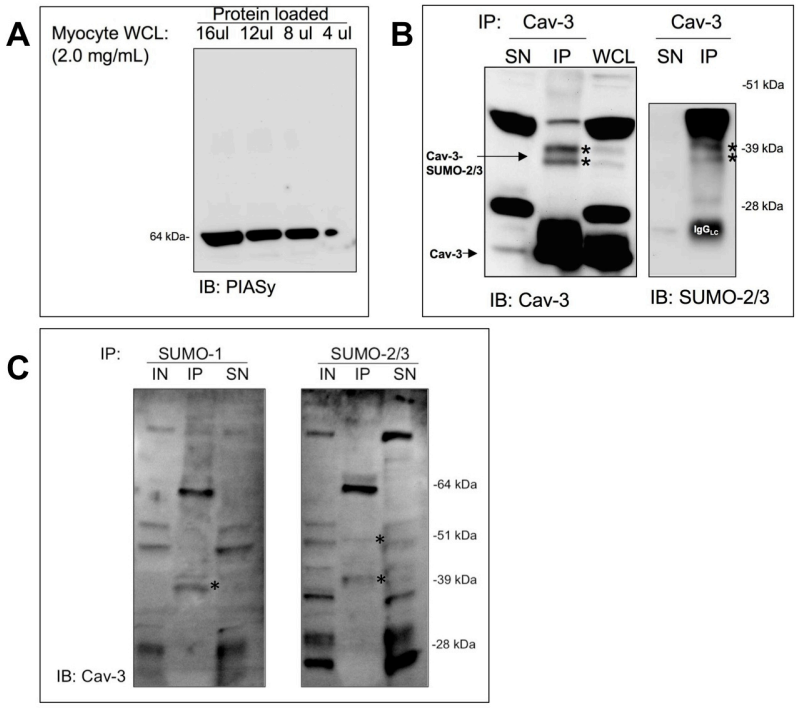


Figure S2.2 (A-C). *In Vivo* Sumoylation of Caveolin-3 in Adult Rat Cardiac Myocytes

(A) *Endogenous Expression of PIASy in Adult Rat Cardiac Myocytes.* Freshly isolated rat myocytes were lysed and analyzed by immunoblotting with anti-PIASy antibodies.

(B) and (C) *Detection of Endogenously Sumoylated Caveolin-3*. **(B)** Rat myocyte lysates were immunoprecipitated with anti-Cav-3 antibodies and immunoprecipitates (IP) and supernatants (SN) and whole cell lysates (WCL) were analyzed by immunoblotting with anti-Cav-3 and anti-SUMO-2/3 antibodies.

(C) Rat myocyte lysates were immunoprecipitated with anti-SUMO-1 and anti-SUMO2/3 antibodies and inputs (IN), immunoprecipitates (IP) and supernatants (SN) were analyzed by immunoblotting with anti-Cav-3 antibodies.

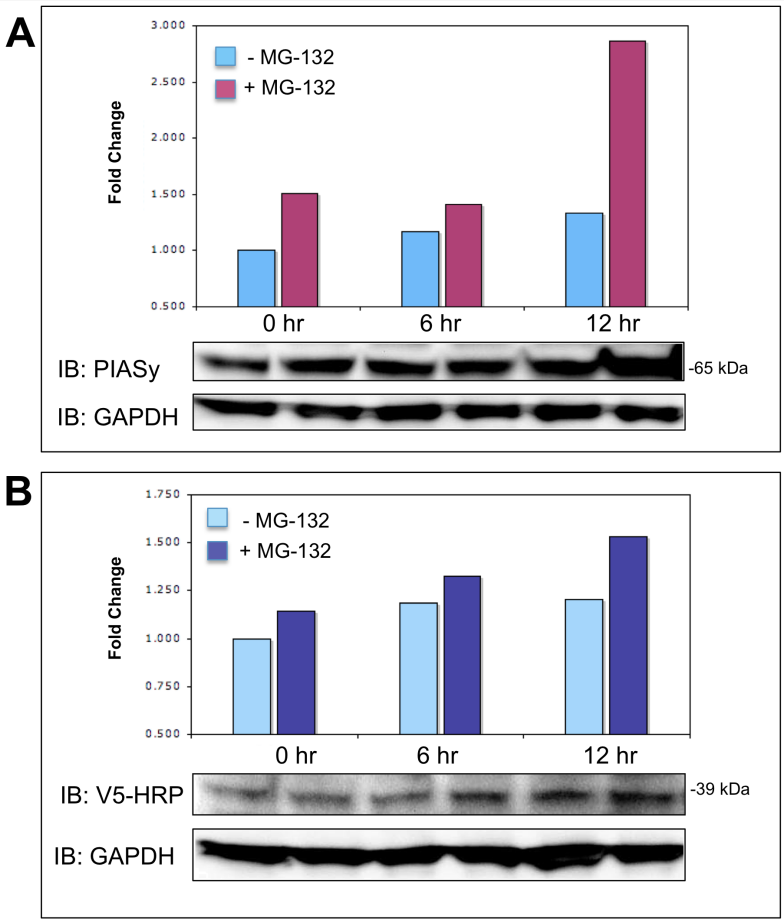


Figure S2.5 (A-B) TGFβ Stimulates PIASy Protein Expression and Sumoylation of Caveolin-3 in HEK 293 Cells

HEK 293 cells were co-transfected with Cav-3-V5, SUMO-3 and Ubc9. 24 h post-transfection, cells were serum starved for 12 h and stimulated with TGFβ for 6 or 12 h. Cells were treated with either 10 uM MG-132 or vehicle. Cell lysates were analyzed by immunoblotting with anti-V5-HRP **(A)** or anti-PIASy **(B)** and anti-GAPDH **(A-B)** antibodies. Protein expression was quantified by densitometry and values were normalized to GAPDH loading controls. Relative protein expression is expressed as fold change over untreated controls (i.e. 0 h time points).

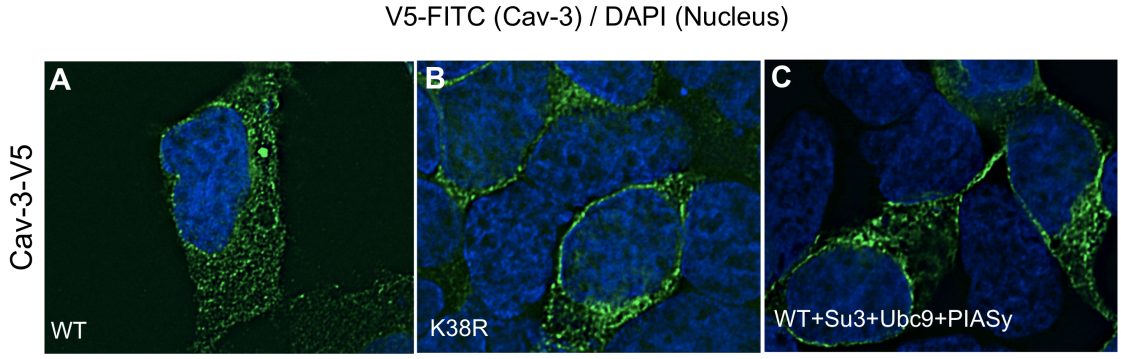


Figure S2.6 (A-C) Cav-3-V5 and the Sumoylation Site Mutant K38R Localize to the Cytoplasm and Plasma Membrane in HEK 293 Cells

(A-C) HEK 293 cells were plated and grown on glass coverslips coated with poly-D-lysine. Cells were transfected with WT Cav-3-V5 **(A)** or the sumoylation site mutant K38R **(B)**. Cells were also co-transfected with WT Cav-3-V5 and the sumoylation machinery (SUMO-3, Ubc9 and PIASy, **(C)**). 48 h post-transfection cells were fixed with paraformaldehyde, stained with DAPI and FITC-conjugated anti-V5 antibodies.

Figure S2.7 Alignment of SIMs in Caveolin-1 and -3 with SIMs in SUMO-Binding Proteins

(A) *Caveolin-3 has tandem SIMs that resemble SIMs present in RNF4 protreins*. Proteins bind SUMO non-covalently via SUMO-Interacting Motifs (SIMs). The poly-SUMO specific E3 ubiquitin ligase RNF4 has four tandem SIMs that are evolutionarily conserved (Tatham et al., 2008). SIMs from human, mouse, frog and zebrafish RNF4 homologs are aligned with putative, tandem SIMs (SIM1 and SIM2) in caveolin-1 and caveolin-3. The hydrophibic (blue) SIM binds in either orientation (`V/I-X-V/I/L-V/I/L or V/I/L-V/I/L-X-V/I/L`) and is enhanced by flanking acidic residues (underlined and bold).

(B) *SIM2 of Caveolin-3 resembles SIMs present in proteins known to interact non-covalently with SUMO.* A search for the motif [VI]-[VI]-X-[VIL]-X-X-[D/E] ((http://expasy.org/tools/scanprosite/)) turned up multiple SUMO-interacting proteins; SAE1 and SAE1 are the SUMO E1 heterodimer, Siz2 is a yeast E3 SUMO ligase, PIAS proteins, PC2 and TORPORS are mammalian E3s and Slx5 is the yeast homolog of RNF4. USP25 is a SUMO substrate (ubiquitin specific protease 25). RN111 (E3 ubiquitin ligase Arkadia). MCAF (Activating transcription factor 7-interacting protein).

Table 2.1 Cav-3-V5 Single 'K to R' Mutants Used in this Study

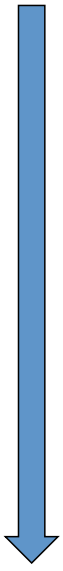
The Cav-3-V5 construct has seven lysine residues (K15, K20, K30, K38, K69, K108 amd K144) that are potential sites of sumoylation. Each lysine was site-directed mutagenesis was used to create these 'K to R' mutants for identification of sumoylation sites on caveolin-3. K38 lies in the tetrapeptide SUMO consensus motif. Each mutant was sequenced to validate presence of desired mutations.

Lysine	15	20	30	38	69	108	144
K to R Mutants							
K15R	X						
K20R		X					
K30R			X				
K38R				X			
K69R					X		
K108R						X	
K144R							X

Table 2.2 Cav-3-V5 Multiple 'K to R' Mutants Used in this Study

Mutation of the consensus site lysine, K38R, reduced sumoylation of Cav-3-V5, but none of the single 'K to R' mutants (Table 2.1) abolished SUMO modification. Multi-site mutagenesis was thus used to create a series of multiple 'K to R' mutants. A K15R_K38R double mutant was used as a template for simultaneous mutagenesis with up to four mutagenic primers. After two rounds of mutagenesis, a lysine-less mutant, Cav-3-V5-K7R was obtained. Each mutant was sequenced to validate presence of desired mutations.

Wild-type



K7R

Multi-site Mutagenesis Round 1	K15	K20	K30	K38	K69	K108	K144
K15,38R	X			X			
K15,38,144R	X			X			X
K15,30,38,108R	X		X	X		X	
K15,30,38,69R	X		X	X	X		
K15,38,108,144R	X			X		X	X
K15,20,38,108R	X	X		X		X	
K15,20,38,108,144	X	X		X		X	X
K15,30,38,69,108R	X		X	X	X	X	
Multi-site Mutagenesis Round 2							
K15,20,30,38,69,108R	X	X	X	X	X	X	
K15,20,30,38,108,144R	X	X	X	X		X	X
K15,20,38,108,144R	X	X		X		X	X
K15,20,30,38,69,108R	X	X	X	X	X	X	
K15,30,38,69,108R	X		X	X	X	X	
K15,20,30,38,69,108R	X	X	X	X	X	X	
K15,20,38,69,108,144R	X	X		X	X	X	X
K15,20,30,38,69,108,144R	X	X	X	X	X	X	X

Table 2.3 Cav-3-V5 'R to K' Mutants Used in this Study

The Cav-3-V5-K7R mutant was used as a template to systematically reverse each mutated residue back to lysine one at a time. The resulting 'R to K' mutants each have a single available lysine residue at each position present in wild-type Cav-3-V5. Presence of desired mutations was validated by sequencing each mutant.

Lysine	15	20	30	38	69	108	144
R to K Mutants							
R15K		X	X	X	X	X	X
R20K	X		X	X	X	X	X
R30K	X	X		X	X	X	X
R38K	X	X	X		X	X	X
R69K	X	X	X	X		X	X
R108K	X	X	X	X	X		X
R144K	X	X	X	X	X	X	

CHAPTER 3: Deconjugation of SUMO-3 from Caveolin-3 by the SUMO-Specific Proteases (SENPs)

3.1 Summary

Results shown in Chapter 2 indicate that sumoylation of caveolin-3 by SUMO-3 and poly-SUMO-3 chains is stimulated by the expression of the SUMO E3 ligase PIASy. SUMO-specific proteases (SENPs) deconjugate SUMO from targets and de-polymerize or 'edit' poly-SUMO chains. There are seven SENPs in humans, each of which has distinct subcellular localizations as well as substrate and SUMO paralog specificities. Having identified, a specific SUMO E3 ligase that promoted sumoylation, I sought to determine if a specific SENP reversed sumoylation of caveolin-3. SENPs were screened for their ability to de-sumoylate caveolin-3 by using "*in vivo* sumoylation" assays performed in HEK 293 cells. The results indicated that expression of either SENP1 or SENP2 dramatically reduced the accumulation of caveolin-3-SUMO-3 conjugates when PIASy was co-expressed. Thus, I conclude that sumoylation of caveolin-3 is regulated by the reciprocal activities of PIASy and SENP1/SENP2.

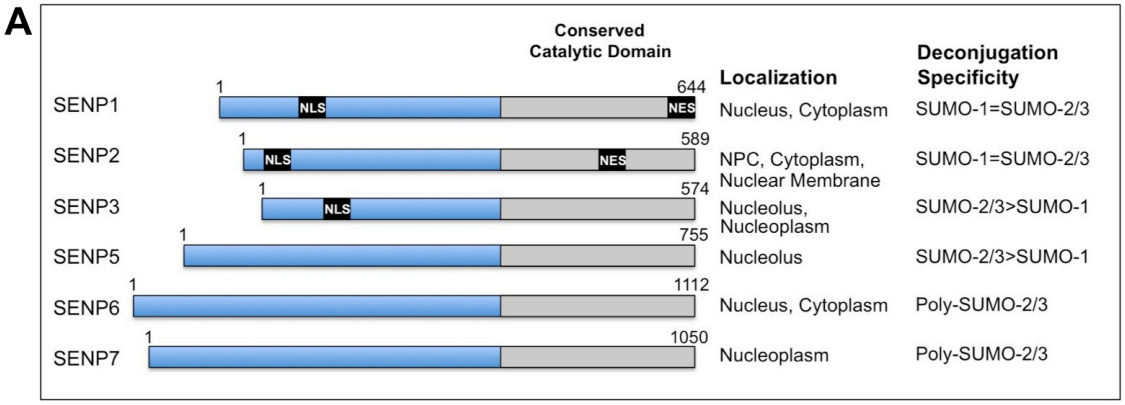
3.2 Introduction

Sumoylation is the reversible posttranslational modification of proteins by small ubiquitin-like modifier (SUMO) proteins. A growing number of proteins have been identified as SUMO modified substrates and sumoylation plays a key role in numerous

biological processes that include signal transduction, cell cycle regulation, gene transcription and cellular localization (Wilkinson and Henley, 2010). In humans, there are three conjugatable SUMO paralogs; SUMO-1, -2 and -3 that can be grouped into two subfamilies, SUMO-1 and SUMO-2/3 which are 97% similar. SENPs belong to the family of cysteine proteases and possess conserved, C-terminal catalytic domains with unique N-terminal regulatory domains (Yeh, 2009, Figure 3.1 A). SENPs carry out essential functions in SUMO metabolism. They have endopeptidase (C-terminal hydrolase) activity and process inactive SUMO precursors (Hay, 2007). SENPs also have isopeptidase activity that allows them to remove SUMO from targets and de-polymerize or 'edit' poly-SUMO chains (Figure 1.4).

Yeast possesses two Smt3-specific (the yeast SUMO homolog) proteases (Ulp1 & Ulp2) that both can remove Smt3 from proteins and process precursors via a homologous catalytic domain of ~200 amino acids (Figure 3.1 A). Seven human genes have been identified by sequence homology that may have SUMO protease activity (SENP1, 2, 3, 5, 6, 7 & 8); however, SENP8 is specific for NEDD8 and cannot deconjugate SUMO. Based on evolutionary relationships, human SENPs can be divided into two groups; SENP1, 2, 3 and 5 are related to Ulp1, while SENP6 and 7 are related to Ulp2 (Hay, 2007). The expansion of SENPs from yeast to humans likely reflects their evolution to perform specific functions. SENPs are among the most specific of all cellular proteases and each SENP has distinct subcellular localization as well as substrate and SUMO paralog preferences (Drag and Salvesen, 2008).

The mechanism of sumoylation is distinct from ubiquitination, but the basic biochemical pathway, utilizing a unique set of E1, E2 and E3 ligases, is similar. Inactive



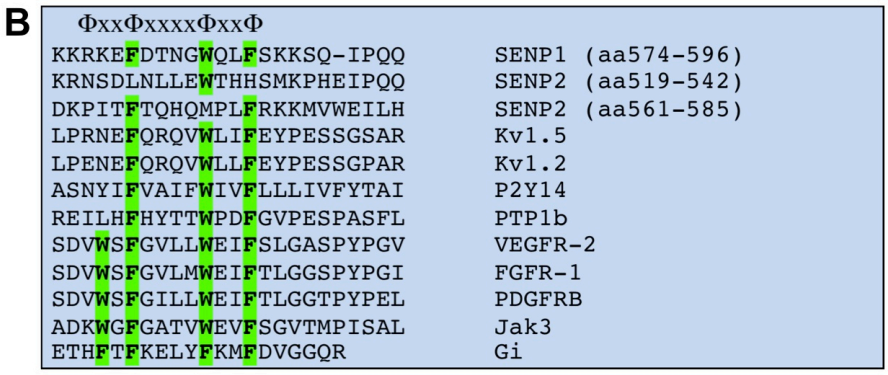


Figure 3.1 (A-B). SUMO-Specific Protease Family: Catalytic and Regulatory Domains

(A) *SENPs share a conserved C-terminal catalytic domain and each has a unique N-terminal regulatory domain*. Both SENP1 and SENP2 shuttle between nucleus and cytoplasm via nuclear localization signals (NLS) and nuclear export signals (NES). SENP1 (aa171-177 NLS, aa635-644 NES). SENP2 (aa228-31 NLS, aa46-51 NLS, aa317-332 NES (Itahana et al., 2006)). SENP2 localizes to the nuclear pore complex (NPC) and nuclear membrane. SENP2's cytoplasmic localization is regulated by ubiquitin proteasome system (Itahana et al., 2006). SENP3 (aa125-128, aa153-159 NLS) localizes primarily to the nucleolus and redistributes between nucleolus and nucleoplasm in response to oxidative stress (Huang et al., 2009). SENP5 also localizes to the nucleolus and SENP3 and SENP5 deconjugate SUMO-2/3 more efficiently than SUMO-1. SENP6 and SENP7 deconjugate SUMO-2/3 and poly-SUMO2/3 chains, but not SUMO1. Both have low efficiency in processing SUMO precursors to their mature forms (Lima and Reverter, 2008). SENP6 localizes to the nucleus and cytoplasm while SENP7 is primarily in the nucleoplasm (Shen et al., 2009).

(B) *SENP1, but not SENP2 has a caveolin-binding motif*. Bioinformatic analysis of SENP primary amino acid sequences revealed that SENP1, has a caveolin-binding motif (ΦxΦxxxxΦ or ΦxxxxΦxxΦ: Φ = aromatic residue; W, F or Y) in its C-terminus (aa579-587, F-X-X-X-X-W-X-X-F). A UniProt database search for this motif (http://expasy.org/tools/scanprosite/) showed that many caveolin binding partners also have similar motifs (e.g. Kv1.5, Kv1.2, P2Y14, PTP1B, VEGFR-2, FGFR-1 PDGFRB, JAK3 and GNAI2). A homologous region of SENP2 (aa519-542) lacked two of the aromatic residues that define the motif, however a nearby region (aa561-585) contained two phenylalanine residues in the correct positions.

SUMO precursors are processed by SENPs to expose the conserved C-terminal di-glycine motif that eventually forms isopeptide bonds with the ε-amino group of target lysines. The catalytic cysteine of the SUMO E1 activating enzyme (a heterodimer of SAE1 (Aos1) and SAE2 (Uba2)) forms a thioester bond with SUMO in a Mg^{2+}-ATP-dependent manner (Johnson et al., 1997). The SUMO~E1 thioester is then transferred to the catalytic cysteine of the single SUMO E2 conjugating enzyme (Ubc9), which can directly modify substrates (Figure 1.4). Many SUMO targets have a consensus motif (Ψ-K-X-[D/E]) that is recognized by Ubc9 (Chapter 1.6; Sternsdorf et al., 1999; Sampson et al., 2001); however, not all consensus sites are sumoylated. Likewise, not all sumoylation sites occur within SUMO consensus motifs indicating that other factors are important for providing specificity, including SUMO E3 ligases. The SUMO E1 and E2 are sufficient for sumoylation of many substrates *in vitro*, however, *in vivo* an E3 ligase (e.g. PIAS family of SUMO E3s) is often required (Bohren et al., 2007).

Caveolins are the principal protein components of caveolae, a type of membrane microdomain enriched in cholesterol, sphingolipids and signaling proteins (Insel et al., 2005). Caveolins are 22-24 kDa integral membrane proteins that form a hairpin loop in the plasma membrane such that both the N- and C-termini face the cytoplasm. Caveolae are 50-100 nm, flask-shaped invaginations of the plasma membrane that are found in most cell types and are particularly abundant in adipose, endothelial and muscle tissues. The caveolin family has three members: caveolin-1 and -2 are co-expressed in most cell types, while caveolin-3 is expressed primarily in cardiac, skeletal and smooth muscle cells (Song et al., 1996). Caveolin-1 and -3 have conserved SUMO consensus motifs in their N-termini and data presented in Chapter 2 identified the consensus site lysine (K38)

as the preferred sumoylation site on caveolin-3. Modification of caveolin-3 by SUMO-3 and poly-SUMO-3 chains on K38 was dramatically increased by co-expression of the SUMO E3 ligase PIASy. Mutation of the consensus site lysine to arginine reduced, but did not abolish sumoylation of caveolin-3 (Chapter 2). Mutation of all the lysine residues to arginine (K7R) of caveolin-3 created a sumoylation-deficient mutant. The K7R mutant provides a useful tool to assess SENP activity because it is a negative control for caveolin-3-SUMO-3 conjugates in immunoblots for accurate screening of SENP activity.

Sumoylation is dynamically regulated and SENPs are highly active suggesting that regulation of *de-conjugation* by SENPs is as important as the regulation of SUMO *conjugation* by E3 SUMO ligases. Identification of SENPs that target caveolin-3 is thus important for understanding how the rate and extent of its sumoylation are regulated as well as identifying specific functions of caveolin-3 regulated by sumoylation.

3.3 RESULTS

3.3.1 SENP Screening in an *In Vivo* Sumoylation Assay Reveals that SENP1 and SENP2 Deconjugate SUMO-3 from Caveolin-3

Endogenous levels of sumoylation are typically quite low and notoriously difficult to detect because of the potent activity of SENPs in cell lysates (Wilkinson and Henley, 2010). *In vivo* sumoylation assays are performed by transfecting cells with SUMO and components of the sumoylation machinery (e.g. Ubc9 and E3 ligases) to enhance endogenous sumoylation. PIASy stimulates the modification of caveolin-3 by SUMO-3 and poly-SUMO-3 chains in HEK 293 cells (Chapter 2). Therefore, HEK 293 cells were

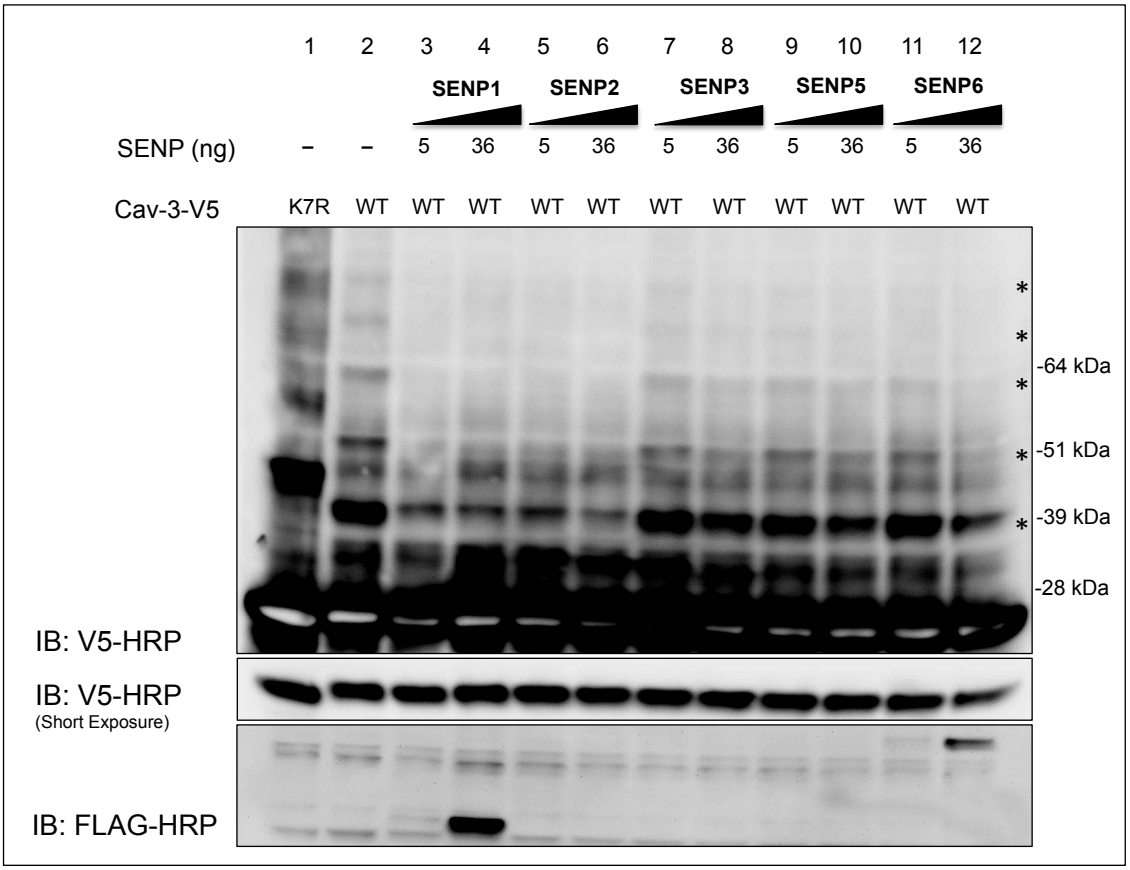


Figure 3.2 SENP Screening: SENP1 and SENP2, but Not SENP3, SENP5 or SENP6, Efficiently Deconjugate SUMO-3 From Cav-3-V5

In vivo sumoylation assays were performed in HEK 293 cells to assess the relative ability of SENPs (FLAG-SENP1, FLAG-SENP2, RGS-SENP3, RGS-SENP5 and FLAG-SENP6) to deconjugate SUMO-3 from caveolin-3. HEK 293 cells were co-transfected with a low and high dose of each SENP (50 or 360 ng) with the sumoylation machinery (SUMO-3, Ubc9 and PIASy, lanes 1-12) and WT Cav-3-V5 (lanes 2-12) or the sumoylation-deficient K7R mutant (lane 1). 48 h post-transfection, cells were lysed in hot 2% SDS lysis buffer to preserve SUMO conjugates and prevent post-lysis deconjugation by the overexpressed SENPs. Lysates were analyzed by immunoblotting with anti-V5-HRP and anti-FLAG-HRP antibodies.

co-transfected with WT Cav-3-V5 or the K7R mutant with the sumoylation machinery (SUMO-3, Ubc9 and PIASy) to stimulate sumoylation of Cav-3-V5. Cells were co-transfected or not with 50 or 360 ng of plasmid DNA expressing either FLAG-SENP1, FLAG-SENP2, RGS-SENP3, RGS-SENP5 or FLAG-SENP6 to determine their relative activity towards sumoylated caveolin-3. 48 h post-transfection, 2% SDS buffer was used to lyse cells and preserve SUMO conjugates (Itahana et al., 2006). Lysates were analyzed by immunoblotting with anti-V5-HRP, anti-FLAG-HRP antibodies.

Consistent with data presented in Chapter 2, co-transfection of PIASy stimulated modification of WT Cav-3-V5 by poly-SUMO-3 chains. The hot 2% SDS lysis buffer resulted in better preservation of Cav-3-V5-SUMO-3 conjugates relative to our previous studies using lysis buffer supplemented with NEM to inhibit SENPs (Chapter 2). Cav-3-V5-SUMO-3 conjugates are visible as a ladder of bands with electrophoretic mobility shifts at ~40, 50, 60, 70 and 80 kDa in cells expressing WT Cav-3-V5 and the sumoylation machinery (*, Figure 3.2, lane 2). Cells expressing the sumoylation-deficient K7R mutant lack these mobility shifts, however SDS-resistant dimers and oligomers are visible (Figure 3.2, lane 1). Co-transfection of as little as 50 ng (1.25% of the 4 μg total plasmid DNA transfected) FLAG-SENP1 or FLAG-SENP2 dramatically reduced detection of sumoylated Cav-3-V5. RGS-SENP3, RGS-SENP5 and FLAG-SENP6 had relatively negligible effect on Cav-3-V5 sumoylation at 50 ng, but showed a slight reduction of sumoylated Cav-3-V5 at 360 ng. It is unclear why the anti-FLAG-HRP antibody does not equally detect FLAG-SENP1 and FLAG-SENP2 protein expression (Figure 3.2, bottom panel) even though equivalent amounts of plasmid DNA were transfected and they appear to have equivalent activity in this assay. It is possible that the

N-terminal FLAG epitope is not as accessible to antibody detection due to conformational differences between SENP1 and SENP2. Assuming that the FLAG-tagged SENPs localize correctly when transfected, these results indicate that SENP1 and SENP2 are the major regulators of de-conjugation of SUMO-3 from caveolin-3.

3.3.2 SENP1 and SENP2 Dose-Dependently Deconjugate SUMO-3 from Caveolin-3 When PIASy is Co-expressed

To further investigate their relative ability to deconjugate SUMO-3 from caveolin-3, HEK 293 cells were transfected with increasing amounts of FLAG-SENP1 or FLAG-SENP2 (Figure 3.3). In the previous experiment, 50 ng of FLAG-SENP1 or FLAG-SENP2 was just as efficient as 360 ng in reducing sumoylation of Cav-3-V5. In order to observe a dose-dependent effect, cells were transfected with a range of concentrations starting at a 5-fold lower dose (10 to 160 ng) of FLAG-SENP1 or FLAG-SENP2. Cells were co-transfected with either WT Cav-3-V5 or the K7R mutant with the sumoylation machinery (SUMO-3, Ubc9 and PIASy) to stimulate sumoylation of Cav-3-V5. Both FLAG-SENP1 and FLAG-SENP2 prevented the accumulation of Cav-3-V5-SUMO-3 conjugates in a dose-dependent manner. FLAG-SENP2 appeared to have a slightly greater effect than FLAG-SENP1 at the lowest doses (10 - 20 ng lanes 3-4 vs. 8-9), but in general their activities towards caveolin-3 appear to be equivalent.

3.3.3 Proteasome Inhibition did not Significantly Alter SENP2 De-Conjugation of SUMO-3 from Cav-3-V5

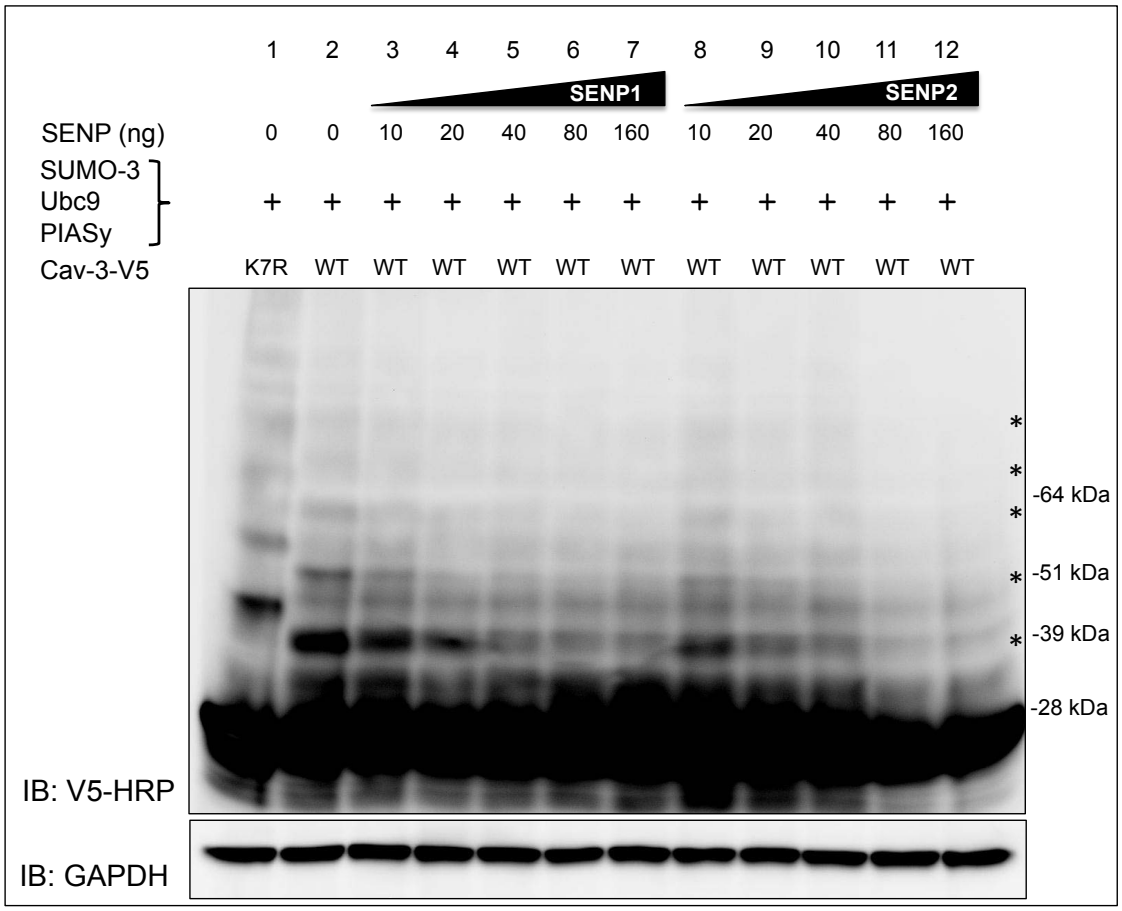


Figure 3.3 SENP1 and SENP2 Dose-Dependently Remove SUMO-3 from Caveolin-3

In vivo sumoylation assays were performed in HEK 293 cells to further assess the relative ability of FLAG-SENP1 vs. FLAG-SENP2 to de-conjugate SUMO-3 from caveolin-3. HEK 293 cells were co-transfected with the sumoylation machinery (SUMO-3, Ubc9 and PIASy, lanes 1-12) and WT Cav-3-V5 (lanes 2-12) or the sumoylation-deficient K7R mutant (lane 1). Increasing doses (10 – 160 ng) of FLAG-SENP1 (lanes 3-7) or FLAG-SENP2 (lanes 8-12) were co-transfected. 48 h post-transfection, cells were lysed in hot 2% SDS lysis buffer and analyzed by SDS-PAGE and immunoblotting with anti-V5-HRP and anti-GAPDH antibodies.

SENP2 has both a nuclear localization signal (NLS) and a nuclear export signal (NES) in the variable N-terminal SENP regulatory domain and these motifs are not found in other SENP family members. It has been reported that SENP2 shuttles between the nucleus and the cytoplasm and that SENP2 undergoes ubiquitination-dependent degradation through the 26S proteasome (Itahana et al., 2006). Cytoplasmic localization of SENP2 facilitated its poly-ubiquitination whereas nuclear localization impeded it. Co-localization of SENPs and their substrates is necessary for SUMO deconjugation and trafficking of SENPs between compartments could be a mechanism to regulate the modification state of substrates. Since caveolin-3 is primarily localized in the cytosol and caveolae membrane microdomains, I sought to determine if this trafficking behavior of SENP2 might regulate its co-localization with caveolin-3. I also wondered if proteasomal degradation of FLAG-SENP2 might explain the difference in detected protein expression levels between FLAG-SENP1 and FLAG-SENP2 (Figure 3.2, bottom panel). Therefore, I co-transfected HEK 293 cells with either WT Cav-3-V5 or the K7R mutant with the sumoylation machinery (SUMO-3, Ubc9 and PIASy) and FLAG-SENP2 (40 ng). Cells were lysed in hot 2% SDS buffer with or without pre-treatment with the proteasomal inhibitor MG-132 and analyzed by immunoblotting with anti-V5-HRP and anti-GAPDH antibodies. When WT Cav-3-V5 was transfected without SENP2, multiple bands were visible in V5-HRP immunoblots consistent with formation Cav-3-V5-SUMO-3 conjugates (*, Figure 3.4 lanes 2 and 8). No such bands were visible when the K7R mutant was expressed (lanes 1 and 7). MG-132 treatment was predicted to stabilize FLAG-SENP2 expression levels by preventing its cytosolic degradation, thereby causing a further decrease in sumoylated Cav-3-V5. No significant difference was observed on

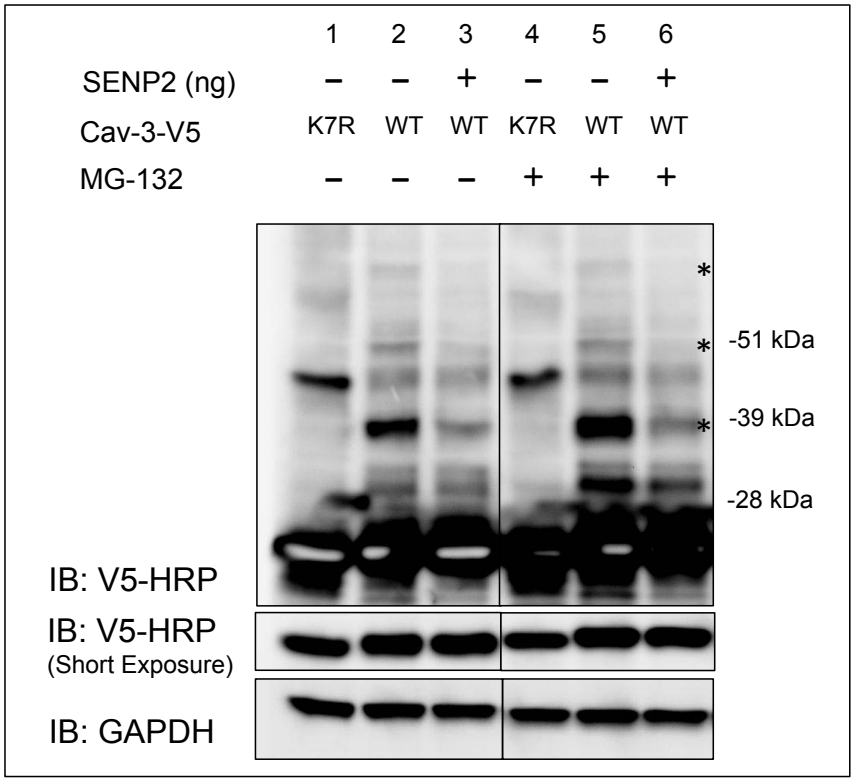


Figure 3.4 Proteasome Inhibition did not Significantly Alter SENP2 De-Conjugation of SUMO-3 from Cav-3-V5

HEK 293 Cells were co-transfected with WT or Cav-3-V5 or the K7R mutant and the sumoylation machinery (SUMO-3, Ubc9 and PISAy). Cells were co-transfected with and without FLAG-SENP2 (40 ng). Cells were incubated with or without 10 uM MG-132 for 3 h prior to lysis. 48 h post-transfection cells were lysed in hot 2% SDS lysis buffer and analyzed by immunoblotting with anti-V5-HRP and anti-GAPDH antibodies (immunoblots are cropped from single images for side-by-side comparison).

deconjugation in cells transfected with FLAG-SENP2 with or without MG-132 treatment (Figure 3.4, lane 3 vs. 6). However, there did appear to be a significant *increase* in Cav-3-V5-SUMO-3 conjugates (*, Figure 3.4, lane 2 vs. 5) with MG-132 treatment when FLAG-SENP2 was not co-expressed. This is consistent with data presented in Chapter 2 and may be due to stabilization of PIASy in the cytosol (Figure 2.3 D, lane 1 vs. 5).

3.4 DISCUSSION

Data in Chapter 2 show that caveolin-3 is a target of sumoylation and investigates how SUMO *conjugation* to caveolin-3 is regulated. Sumoylation of caveolin-3 was strongly enhanced by increased expression of the SUMO E3 ligase PIASy, however continued increase in PIASy expression was toxic to the cells (Figure 2.2). Even with overexpression of PIASy, only a small fraction of caveolin-3 is sumoylated at any given time. This is true for most SUMO targets (with the exception of RanGAP1), suggesting that the regulation of SUMO *de-conjugation* by SENPs may be the dominant force in regulating the level of sumoylated proteins in cells. I thus sought to identify the specific SENP(s) that regulate de-conjugation of SUMO from caveolin-3. PIASy has effects on many substrates besides caveolin-3 and its overexpression in cells can be toxic, therefore targeted knockdown of a specific SENP (as opposed to overexpression of PIASy) could be a better tool to investigate the functional consequences of caveolin-3 sumoylation *in vivo*.

SENPs are not inhibited by the protease inhibitors typically used in lysis buffers. In the studies here, I titrated expression of SENPs to determine the relative activity of each SENP towards caveolin-3. Efforts were made to preserve sumoylation in cell lysates

in order to distinguish real activity from post-lysis activity of over-expressed SENPs. I thus used hot 2% SDS lysis buffer since it has been demonstrated to better preserve SUMO conjugates than use of NEM to inhibit SENPs (Itahana et al., 2006).

Subcellular co-localization of SENPs with their substrates is clearly necessary for SUMO de-conjugation and only a handful of known SENPs exist, so one might predict candidates based on the localization of caveolin-3; however, the regulation and localization of SENP family members is yet to be fully understood. SENP7 was excluded based on evidence that it localizes to the nucleoplasm (Shen et al., 2009). Therefore I screened SENP1, 2, 3, 5 and 6 for activity using an *in vivo* sumoylation assay. Both SENP1 and SENP2 dramatically decreased the level of sumoylated caveolin-3, even when expressed at very low levels relative to the other components in the assay (i.e. 1.25% of total DNA transfected).. These data, combined with results presented in Chapter 2 strongly suggest that sumoylation of caveolin-3 is regulated by the reciprocal activities of PIASy and SENP1/SENP2.

SENPs are likely to interact transiently with their substrates, but are targeted through unique domains to specific sub-cellular compartments (Figure 3.1 A). Proteins targeted to caveolae often interact with caveolin via a caveolin-binding motif (ΦxΦxxxxΦ or ΦxxxxΦxxΦ: Φ = aromatic residue; W, F or Y), so I searched each SENP's amino acid sequence for such motifs. SENP1 has a caveolin binding motif in its C-terminal catalytic domain (aa579-587, Figure 3.1 B), however none of the other SENPs have complete motifs. The homolgous domain in SENP2 to the motif identified in SENP1 had just two out of three aromatic residues present. To test the ability of SENP1 and SENP2 to interact with caveolin-3, I transfected HEK 293 cells with Cav-3-V5 and

either FLAG-SENP1, FLAG-SENP2 or empty vector and immunoprecipitated cell lysates with anti-V5 and anti-FLAG antibodies. FLAG-SENP1 and FLAG-SENP2 were both detected in anti-V5 immunoprecipitates (Figure S3.1 A) and Cav-3-V5 was detected in anti-FLAG immunoprecipitates only when either FLAG-SENP1 or FLAG-SENP2 was co-expressed (Figure S3.1 B). Despite the potential caveolin-binding motif identified in SENP1, SENP2 seemed to pull down more Cav-3-V5 than SENP1 (even though less total FLAG-SENP2 protein was detected [Figure S3.1 B, upper panel]).

It has recently been demonstrated that SENP2 expression is induced in a cAMP-dependent manner in adipocytes and plays an essential role in the control of adipogenesis (Chung et al., 2010). Chung et al. demonstrated that elevated cAMP induces SENP2 expression through phospho-CREB binding to a functional CRE (*cis*-acting cAMP response element) in SENP2's promoter region. Such findings complement other recent data regarding the PIASy dependent-sumoylation of the β_2AR-specific phosphodiesterase subtype, PDE4D5 (Li et al., 2010) and results shown in this dissertation with regards to PIASy-dependent sumoylation of caveolin and regulation of the β_2AR desensitization. Together, the findings suggest the existence of a feedback mechanism in caveolae that regulates (in addition to other mechanisms) the compartmentation and activity of cAMP signaling pathway components by reversible sumoylation.

3.5 METHODS

3.5.1 Materials

FLAG-SENP1, FLAG-SENP2, RGS-SENP3, RGS-SENP5 and FLAG-SENP6 mammalian expression plasmids were obtained from Addgene. All other antibodies, plasmids and chemicals were described in Chapter 2.

3.5.2 *In Vivo* Sumoylation Assay

Confluent HEK 293 cells were transfected in 6-well plates as described previously (Chapter 2). After 48 h, cells were washed with PBS and lysed in 600 µl 2% sodium dodecyl sulfate (SDS) lysis buffer (2% [wt/vol] SDS, 50 mM Tris-HCl pH 6.8, 5 mM EDTA, 10% glycerol). The SDS lysis buffer was preheated to 95°C before being added to the cells to immediately denature SENPs and protect SUMO-conjugates during lysate preparation. Cells were scraped and pipetted into pre-chilled 1.5 ml Eppendorf tubes and incubated on ice for 30 min. Lysates were sonicated (3 x 10 sec) and clarified by centrifugation (5,000 x g for 15 min at 4°C). Protein concentrations were normalized by BCA protein assay and lysates were analyzed by SDS-PAGE and immunoblotting.

3.6 AKNOWLEGEMENTS

I thank my thesis advisor and mentor Dr. Paul A. Insel for his support, guidance and expertise, without which this project would not have been possible. I also thank Anna Busija for contributing to the planning and execution of experiments presented in this chapter. This chapter, in part, is being prepared for publication pending completion of further experiments to be performed by the dissertation author and Anna Busija. The dissertation author will be the primary author, Anna Busija will be a secondary author and Paul Insel the principal investigator.

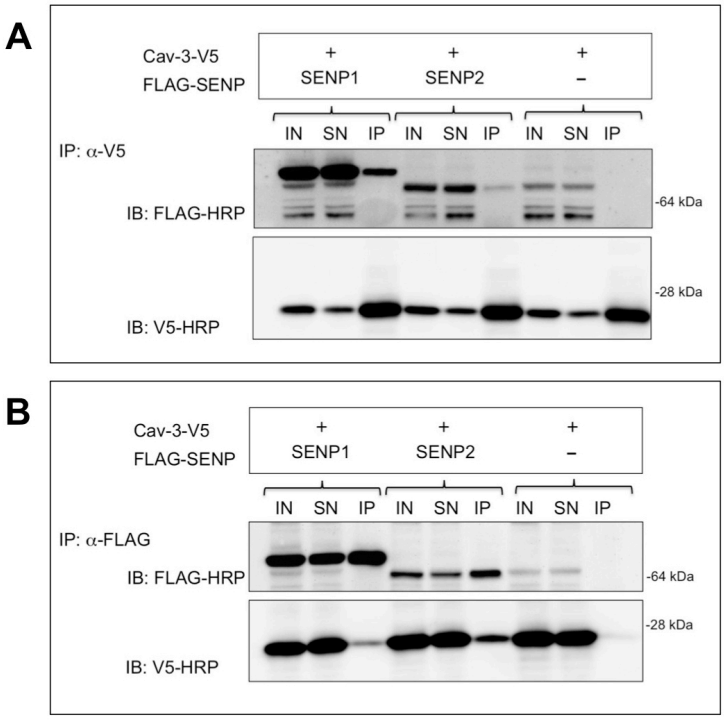


Figure S3.1 (A-B). FLAG-SENP1 and FLAG-SENP2 Co-Immunoprecipitate with Caveolin-3 when Co-Expressed in HEK 293 Cells
HEK 293 cells were transfected with Cav-3-V5 (1.5 μg) and either FLAG-SENP1, FLAG-SENP2 (1.5 μg) or empty vector. 48 hr post-transfection, cell lysates were immunoprecipitated with anti-V5 **(A)** or anti-FLAG antibodies **(B)**. The whole cell lysates (IN=Input), supernatant (SN) and immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted with anti-V5-HRP and anti-FLAG-HRP antibodies.

CHAPTER 4: Conclusions and Future Directions

4.1 Conclusions

Caveolins function as scaffolds that compartmentalize, organize and regulate numerous proteins in caveolar membrane microdomains. How this occurs in a dynamic manner in response to specific stimuli is poorly understood but PTMs of caveolins and their binding partners are likely important contributors to such protein-protein interactions. Sumoylation is a highly dynamic PTM that has recently emerged as a key regulator of numerous extranuclear processes, including signal transduction by plasma membrane proteins. Several of these proteins localize to caveolae and interact directly with caveolins. The identification of caveolin as a SUMO-modified substrate and the observation that caveolins contain tandem SIMs strongly suggest that both covalent and non-covalent interactions with SUMO are important for the regulation of signaling proteins in caveolae.

4.2 Future Directions

Data presented in this dissertation lay the groundwork for future studies that will address important unanswered questions regarding both the biochemical interactions and the biological significance of covalent and non-covalent interactions of caveolins with SUMO proteins.

4.2.1 Effect of Caveolin-3 K7R Mutant on Agonist-induced Desensitization of β2AR

Further studies are needed to validate the sumoylation-dependent effects of caveolin-3 on the desensitization of β_2AR and probe the mechanism by which the Cav-3-V5-K7R mutant affects it. HEK 293 cells are a well-validated system to assess this phenomenon; however, it may be advantageous to make stably transfected HEK 293 cells that express FLAG-β_2AR, as used by numerous previous investigators in studies on β_2AR desensitization (Baillie et al., 2003). An alternative approach would be to use cells stably transfected with WT or Cav-3-V5 mutants and selected for equivalent expression levels. In fact, I am in the process of making such stable cell lines in a caveolin-null cancer cell line (HT29).

The experiments presented here revealed an unexpected consequence of catecholamines present in FBS, which led to the stimulation of transfected β_2ARs. While the reversal of β_2AR destabilization by reducing serum concentration (i.e., reduced agonist exposure) or by treatment with the βAR antagonist (-)-propranolol strongly suggests that the effect is the result of agonist-induced destabilization, improvements to the experimental design will help solidify these results. The media could be supplemented with dialyzed FBS to remove catecholamines from the serum. The β-AR agonist isoproterenol could then be used to stimulate cells for specific time-points (e.g. 1-360 min…to 24 h and 48 h) to differentiate between short-term desensitization and long-term downregulation, the latter of which occurs by lysosomal degradation. In such studies, cAMP assays and immunoprecipitation and immunoblotting with anti-FLAG antibodies could be used to monitor changes in β_2AR protein and responses to agonist at

the different time points and determine the effect of co-expression of WT Cav-3-V5, K38R, R38K or K7R.

This system could also be used to determine the mechanism of caveolin-3's effects since much is known regarding the desensitization of β_2AR in HEK 293 cells (Chapter 1.10). For example, one might manipulate molecules shown to be important for β_2AR internalization, desensitization and resensitization (e.g. GRK2, β-arrestin-2, Mdm2, Nedd4, USP20 USP33 and dynamin) using specific siRNAs or transfection of mutants (e.g. K44A dynamin, GRK2 phospho-site β_2AR mutant or a 'lysine-less' 0K-β_2AR (Shenoy et al., 2001)) that disrupt β_2AR trafficking.

4.2.2 In Vivo Studies: Construction and Injection of AAV9 Virus Encoding WT, K38R, R38K and KR2 Cav-3-V5 Mutants in Cav-3 -/- Mice

Studies presented here have begun to probe the effect of sumoylation on protein-protein interactions of caveolin and demonstrate an unexpected effect of the Cav-3-V5-K7R mutant on the desensitization of β_2AR in HEK 293 cells. However, *in vivo* studies are necessary to determine if this effect occurs in animals. To this end, studies are underway in a collaborator's laboratory to infect Cav-3 KO mice to assess a role in myocytes by using a cardiac myocyte-specific AAV9 virus that encodes WT, K38R, R38K or K7R Cav-3-V5 constructs. In addition to confirming the *in vivo* significance of sumoylation on the regulation of β_2AR by caveolin-3, this approach should allow for identification of other effects by comparison of phenotypes and biochemical analyses of mice injected with viruses that contains WT or sumoylation-site mutants.

4.2.3 Subcellular Localization, Co-localization and Caveolin-Dependent Caveolae Formation and Morphology: Fluorescent and Electron Microscopy

Other remaining questions include whether or not sumoylation affects the subcellular localization of caveolin-3, its co-localization with signaling partners and sumoylation machinery, the ability of caveolin-3 to oligomerize and drive the formation of caveolae microdomains and the identification of the compartment(s) in which sumoylation and de-sumoylation of caveolin-3 occur.

Preliminary immunoflourescence studies performed in HEK 293 cells using the Cav-3-V5 construct did not show an obvious difference in localization between cells expressing WT vs. K38R Cav-3-V5 (Figure S2.6). These cells were not ideal for microscopy; however, due to their poor adherence to slides, even ones coated with poly-D-lysine. Further immunofluorescence studies need to be performed in a different cell type, such as Cos-7 cells, since these have been used successfully in the expression and visualization of caveolin-3 (Ohsawa et al., 2006). Myocytes isolated from mice infected with the different versions of the cardiac-specific AAV9 viruses, as described above, should prove very useful for immunofluorescence and electron microscopy studies. Indeed, work is underway with collaborators to perform electron microscopy on transfected cells as well as Cav-3 KO mouse myocytes infected with the viruses that contain WT or the sumoylation site mutants to determine if sumoylation effects formation or morphology of caveolae.

Ultracentrifugation and sucrose density fractionation are used to isolate caveolar membranes (Cav-fractions) and identify protein components of caveolae. Therefore it would be informative to probe caveolin-enriched fractions obtained from myocytes

(taking care to preserve SUMO modifications by using NEM-denaturing lysis conditions) for Cav-3-SUMO conjugates as well as the relevant sumoylation machinery identified here including: PIASy, Ubc9, SUMO-1, SUMO-2/3, SENP1 and SENP2. A preliminary experiment was performed on adult rat myocyte lysates using this technique; however, a satisfactory fractionation of caveolar membranes was not achieved and the results were inconclusive (unpublished data).

4.2.4 Investigation of Caveolin-1 and Cavin Proteins as Targets of Sumoylation

The SUMO motifs present in caveolin-3 that are important for covalent and non-covalent interactions with SUMO are also present in caveolin-1. Therefore future studies should test whether caveolin-1 undergoes sumoylation. A construct similar to the affinity tagged caveolin-3 (Cav-3-V5/His) used in these studies was obtained (from Dr. Timothy Thompson [MD Anderson, U Texas-Houston]; Tahir et al., 2003) for use in studying sumoylation of caveolin-1 (Cav-1-V5/His); however, I chose to focus on caveolin-3 based on initial data I obtained related to caveolin-3. The much wider tissue distribution of caveolin-1 vs. caveolin-3 implies that regulation of caveolins by sumoylation may have physiological and pathopysiological implications beyond muscle tissues.

Cavins are a recently recognized family of proteins (cavin-1, -2, -3 and -4) that are also critical for the formation, regulation and morphology of caveolae (Hill et al., 2008; Bastiani et al., 2009; Chidlow et al., 2010). MURC (cavin-4) also has a consensus SUMO motif (Figure 1.7) and like caveolin-3, MURC displays muscle-specific expression. The sumoylation-dependent interaction of MURC and caveolin-3 as well as the other cavin proteins should therefore be investigated.

4.2.5 Investigation of De-Sumoylation by SENP1 and SENP2

Screening of SENPs in HEK 293 cells revealed that SENP1 or SENP2 potently reduces SUMO-3 modification of caveolin-3 (Chapter 3). siRNA knockdown and co-localization studies of SENP1 and SENP2 performed in isolated myocytes or a muscle cell line (e.g. C2C12 or Human Skeletal Muscle Cells (HSkMC, ScienCell Research Laboratories) may help determine which of these homologs is the primary regulator of caveolin-3 de-sumoylation. I prepared a catalytically inactive SENP2 (C548S) by site-directed mutagenesis of the catalytic cysteine (part of the 'catalytic triad') for use as a negative control in de-sumoylation assays; however, it has not yet been sequenced and validated for loss of catalytic activity.

4.2.6 Validation and Investigation of the Effects of Caveolin's Tandem SIMs on Covalent and Non-Covalent Interactions with SUMO

The highly conserved nature of residues in caveolin-3's SIMs and their similarity to known, functional SIMs (Figure S2.7) indicate that these may regions may be important for the regulation of the interaction of caveolin-3 with sumoylated proteins. Alternatively, they may help direct the nature of covalent modification of caveolin-3 by different SUMO paralogs and/or specific poly-SUMO chains. A seven amino acid SIM was sufficient for SUMO-2/3-specific binding and conjugation of USP25 (Meulmeester et al., 2008). Therefore, a mechanism for paralog-specific sumoylation may involve SIM-dependent recruitment and conjugation by either SUMO-1 or SUMO-2/3

Ubc9~thioesters. Thus, future studies should also focus on validating and investigating tandem SIMs of caveolins.

I used site-directed mutagenesis to make three Cav-3-V5-ΔSIM mutants in which two out of the three hydrophobic residues that make up the core of the SIM were changed to alanine in one of each or both SIMs; ΔSIM1, ΔSIM2 and ΔSIM12 (Figure 1.6). SIM mutants were co-expressed in HEK 293 cells with the sumoylation machinery; however, differences in their expression levels and electrophoretic mobility yielded inconclusive results with respect to their effects on the covalent modification of Cav-3-V5 (unpublished data). Optimization of expression levels or creation of stably transfected HEK 293 cell lines would help clarify these studies and help determine whether caveolin-3 SIMs regulate the covalent modification by SUMO, or whether they are important for binding non-covalently to other sumoylated proteins. Affinity chromatography with SUMO-conjugated agarose (unpublished data) could be used to assess differences in non-covalent interactions between WT and Cav-3-V5 ΔSIM mutants. Also, GST pulldown assays with WT or Cav-3-V5 ΔSIM mutants and cell lysates (e.g. of myocytes) could reveal differences in their ability to interact with specific proteins.

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