The life cycle of caveolae - from assembly to disassembly

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Summary

Caveolae are small, omega-shaped membrane invaginations present on the surface of many mammalian cell types. They are enriched in cholesterol and sphingolipids and therefore represent lipid-raft microdomains. Caveolae function in a variety of cellular and physiological processes including endocytosis, transcytosis, lipid regulation, signaling, pathogen entry and cancer. Caveolin-1, an integral membrane protein, is the major coat protein of caveolae. There is evidence that caveolar transport follows principles distinct from known vesicular transport mechanisms. Unlike clathrin-, COPI-, and COPII- mediated transport, caveolar transport does not involve cycles of coat assembly and disassembly. Instead, once assembled, the caveolar coat remains stable throughout transport.

The main questions addressed in this thesis were; how are caveolae assembled, where are they assembled, and how are they disassembled and degraded.

In the first part of this thesis, the cellular location where caveolae initially form was investigated. By using various light microscopy techniques in cells expressing GFP-tagged caveolin-1 (CAV1-GFP) we could consistently visualize newly assembled caveolar domains departing from the Golgi complex as distinct spots, and proceeding to the plasma membrane following curvilinear tracks. Unlike VSVG-protein-carrying vesicles, these were homogenous and their fluorescence intensity was indistinguishable from surface caveolae. Upon arrival at the surface, the CAV1-GFP did not diffuse away, in contrast to VSVG-protein. Taken together with previous observations, the results indicated that caveolar domains assemble in the Golgi complex, that they are individually transported as caveolar vesicles to the plasma membrane, and that upon arrival and thereafter they remain stable.

In the second part, we studied the assembly of CAV1 in more detail by a combination of biochemical and live-cell microscopy approaches. Using sucrose gradient centrifugation we identified a novel oligomeric complex of CAV1 that forms during transit of CAV1 through the secretory pathway. Characterization of this complex indicated that it likely represents mature caveolae. FRAP analysis of newly synthesized CAV1 revealed that an essential oligomerization step for caveola
formation occurred in the Golgi. We furthermore present evidence for differential molecular requirements for Golgi exit of CAV1 and GFP-GPI versus VSVG and that newly assembled caveolar domains can incorporate cargo for surface delivery. Finally, the recently discovered caveolar coat component PTRF is shown to require newly synthesized CAV1 for association with caveolae.

The third part investigates degradation of caveolae. We first show that saturation of caveolae assembly in the Golgi caused delivery of non-assembled caveolin to the plasma membrane and subsequent degradation in the endo/lysosomal pathway. Moreover, we found that CAV1 also entered the endo/lysosomal pathway in unperturbed cells and that inhibitors of lysosomal degradation caused accumulation of CAV1 in endosomal organelles. Coexpression of GFP- and RFP-tagged CAV1 allowed monitoring of how CAV1 was targeted to the lumen of acidic endosomal organelles in living cells, as GFP-fluorescence is more sensitive to acidic pH than RFP. Taken together, our results are consistent with degradation of CAV1 in the endo/lysosomal pathway.

In the last part we studied assembly and biosynthetic trafficking of flotillins that were found to be related to caveolins in several aspects. Live-cell imaging showed that Golgi-derived flotillin-domains were stable when they arrived at the cell surface. Similar to caveolae, they formed microdomains in the plasma membrane that were stable over several hours. We propose that flotillin microdomains and caveolae may follow similar underlying principles.
Zusammenfassung


Die vorliegende Arbeit untersucht diese einzigartige Eigenschaft von Caveolae, indem sie der Frage nach der Biogenese dieser Strukturen nachgeht.


Der zweite Teil dieser Arbeit beschäftigt sich eingehender mit der Biogenese von Caveolae unter Verwendung von biochemischen und Lichtmikroskopie-Methoden. Mithilfe von Sukrosegradientenzentrifugation konnte ein neuer oligomerer Komplex von CAV1 identifiziert werden, der sich während des Transports von CAV1 durch

Im dritten Teil haben wir den Abbau von Caveolae untersucht. Zunächst zeigen wir, dass unter bestimmten Bedingungen freies, nicht mit Caveolae assoziiertes Caveolin vom Golgi an die Zelloberfläche transportiert werden kann, insbesondere dann, wenn der Caveolae-Bildungsprozess gesättigt ist. Dieses freie Caveolin wird schnell in die Zelle aufgenommen, wo es mit Markern der endosomalen Organellen kolokalisiert. Wir konnten nun zeigen, dass Caveolin auch unter physiologischen Bedingungen in den endosomalen/lysosomalen Weg eintritt. Eine neue experimentelle Strategie hat uns erlaubt, die Translokation von Caveolin ins saure Lumen von endosomalen Organellen mit Mikroskopie zu beobachten. Dabei werden GFP- und RFP-markiertes CAV1 zusammen exprimiert; wegen der höheren pH-Empfindlichkeit verliert GFP zunehmend an Fluoreszenz, was am Mikroskop verfolgt werden kann. Aus diesen Beobachtungen schließen wir, dass CAV1 in späten Endosomen oder Lysosomen abgebaut wird.

Chapter 1
Caveolae and their multiple roles in cell and animal physiology
1. Introduction

1.1 Caveolae and caveolins

1.1.1 Caveolae and discovery of caveolin

Caveolae are flask-shaped membrane invaginations present on the surface of most mammalian cell types. Their size ranges from 50-100 nm and they appear to have a striated coat on their cytoplasmic surface that can be visualized by rapid-freeze-deep etch electron microscopy (Rothberg et al., 1992, Figure 1.1 A-D, F). They are most abundantly found in endothelial cells and adipocytes, where they cover a surface of up to one third of the plasma membrane (Thorn et al., 2003). In Fibroblasts, they cover only 3-5% of plasma membrane surface, while they are completely absent in red blood cells, platelets, lymphocytes (Fra et al., 1995), neuroblastoma (Gorodinsky and Harris, 1995), and other neuronal cell types.

Caveolae were first described in the endothelium of the heart by Palade in 1953, who called them plasmalemmal vesicles (Palade, 1953). Yamada described similar structures in the gall bladder epithelium naming them caveolae intracellularae (Yamada, 1955), as they resembled “little caves”. The morphology of caveolae was suggestive of a potential role in transport and they were thought to allow for communication between intra- and extracellular milieu and transport of macromolecules across an endothelial cell layer, although the molecular mechanism was unknown. Also the components of the caveolar coat were not known at this time.

In 1989, a 22kDa protein was found to be a substrate of v-Src tyrosine kinase in Rous sarcoma virus-transformed chick embryo fibroblasts (Glenney, 1989). Later, antibodies raised against this phosphoprotein recognized the filamentous coat of caveolae in electron microscopy and punctate structures in immunofluorescence microscopy. The 22 kDa protein was named caveolin, as it likely was part of the caveolar coat (Rothberg et al., 1992). At around the same time, VIP21 (vesicular integral membrane protein, 21 kDa) was identified as component of trans-Golgi-network-derived transport vesicles (Kurzchalia et al., 1992). When VIP21 and
Figure 1.1 | **Caveolae and membrane topology of caveolin.** Panels A and B show electron micrographs of Ruthenium-red surface-labeled adipocytes. Caveolae appear as omega-shaped invaginations (A) or as circular cross-sections, sometimes in multi-caveolar assemblies that are surface-connected (B), schematic in (F). The regular morphology of plasma-membrane caveolae is apparent in the thin-section electron micrograph in (C). In deep-etch, rapid freeze electron microscopy caveolae appear with a striated coat that covers plasma membrane domains of variable curvature (D). (E) Caveolae membrane organization by caveolin-1. Caveolin-1 inserts into the membrane by a central hydrophobic segment as a hairpin with flanking N and C-termini projecting into the cytoplasm. The scaffolding domain is thought to be involved in cholesterol binding through conserved basic (+) and bulky hydrophobic residues (red circles). Palmitoylated cysteins in the C-terminal domain stabilize caveolin-1 homo-oligomers. Panels A, B, E, F are adapted by permission from Macmillan Publishers, Ltd: Nat Rev Mol Cell Biol (Parton and Simons, 2007) © 2007. C and D are adapted from (Shaul and Anderson, 1998). Scale bars, C 0.2 µm, D 0.1 µm.
caveolin were cloned and their sequence compared, they were found to be identical (Glenney, 1992).

1.1.2 The family of mammalian caveolin proteins

Whether caveolin is an essential component of the caveolar coat was not known until expression of caveolin was shown to induce de novo formation of caveolae in cells otherwise lacking caveolae (Fra et al., 1995). The requirement of caveolin for expression of morphologically identifiable caveolae was thus demonstrated. Caveolin was termed caveolin-1 (CAV1) when proteins with homologous primary sequence were identified (Scherer et al., 1996; Tang et al., 1996).

The caveolin protein family consists of CAV1, caveolin-2 (CAV2) and caveolin-3 (CAV3), 18-22 kDa proteins with relatively high degree of sequence identity between CAV1 and CAV3. CAV2 is the most divergent of the three proteins. CAV3 is exclusively expressed in muscle, while CAV1 expression is restricted to non-muscle cells (Way and Parton, 1996). In muscle, CAV3 localizes to the sarcolemma, where it is associated with the dystrophin-glycoprotein complex (Song et al., 1996). Expression of either CAV1 or CAV3 is sufficient to induce caveolae, whereas expression of CAV2 alone does not support caveola formation. CAV2, generally coexpressed with CAV1, is therefore thought to have a regulatory rather than a structural role in caveola biogenesis (Mora et al., 1999; Sowa et al., 2003). CAV2 is retained in the Golgi complex and rapidly degraded in absence of CAV1. When CAV1 is coexpressed with CAV2, they form hetero-oligomeric complexes and CAV2 is delivered to the cell surface where it localizes to caveolae (Mora et al., 1999).

The essential role of caveolins in caveolae formation was further demonstrated by loss of caveolae in CAV1 or CAV3 gene-disrupted mice (Drab et al., 2001; Galbiati et al., 2001).
1.1.3 Non-mammalian caveolins

Expression of caveolins is not restricted to mammals. While absent in plants and fungi, they are found in many branches of metazoans, including the nematode *C. elegans*, zebrafish *D. rerio*, the frog *X. laevis*, and honey bee *A. mellifera*. However, they are apparently absent from *D. melanogaster*. Expression of caveolins does not necessarily confer the ability to form caveolae in all species. *C. elegans* caveolin, for instance, localizes to the plasma membrane of cells within the early embryo, from where it is endocytosed in clathrin-dependent manner (Sato et al., 2006). When expressed in mammalian cells, *C. elegans* caveolin does not support caveola formation (Kirkham et al., 2008). Caveolae were identified in zebrafish *D. rerio* that expresses five different caveolin genes (Nixon et al., 2005). In zebrafish, knockdown of the caveolin ortholog to mammalian CAV1 caused embryonic lethality (Fang et al., 2006). Caveolin from honey bee *A. mellifera*, when heterologously expressed in mammalian cells, induced formation of caveolae (Kirkham et al., 2008). From these observations one may postulate cellular functions of caveolins outside caveolae, but clearly more research is needed to gain insight into the function of non-mammalian caveolins.

1.1.4 Structure and features of mammalian caveolin-1

CAV1 is a protein of 21 kDa encoded by 178 amino acids. Although CAV1 behaves as an integral membrane protein, cytosolic and secreted pools of CAV1 have also been described (Liu et al., 1999; Uittenbogaard et al., 1998). In membranes, CAV1 assumes an unusual hairpin-topology, in which both amino- and carboxy-termini are facing the cytoplasm (Dupree et al., 1993; Monier et al., 1995). The central hydrophobic stretch of 33 residues (CAV1102-134) is thought to be embedded in the cytosolic leaflet of membranes with no exposure to the extracellular space (Figure 1.1E).

CAV1 is expressed in two isoforms, CAV1α (CAV11-178) and CAV1β (CAV132-178). Both isoforms are likely translated from the same transcript, CAV1β from an internal start codon (Scherer et al., 1995). CAV1α and CAV1β are expressed together in the same cell types and it is not known how they differ functionally.
The oligomerization domain (CAV1_{61-101}) in the amino-terminal portion mediates homo-oligomerization, the scaffolding domain (CAV1_{82-101}) interacts with signaling proteins and can modify their activity (Okamoto et al., 1998). The caveolin signature domain (CAV1_{68-75}, FEDVIAEP) is located within the oligomerization domain and shared with all caveolins.

CAV1 oligomers are stabilized by palmitoylation at cysteins in the carboxy-terminal domain at positions 133, 143, 156. However, this modification is not required for localization of CAV1 to caveolae (Dietzen et al., 1995; Monier et al., 1996). CAV1 can directly bind cholesterol at a stoichiometric ratio of 1:1 (Murata et al., 1995) and interaction with the lipid is required to maintain the structure of caveolae (Rothberg et al., 1992). Phosphorylation of CAV1 at serine-80 may regulate interaction with cholesterol (Fielding et al., 2004; Schlegel et al., 2001). CAV1 is also phosphorylated at the amino-terminus at tyrosin-14. This modification was shown to modulate Src signaling and directional migration of cultured fibroblasts (Grande-Garcia et al., 2007).

1.1.5 Oligomerization of caveolin-1 and assembly of caveolae

CAV1 inserts cotranslationally into the membrane of the endoplasmic reticulum (ER) in an SRP-dependent manner (Monier et al., 1995). Soon after synthesis, CAV1 forms homo-oligomers of 7-10 monomers (Scheiffele et al., 1998). These 150-200 kDa primary oligomers are remarkably stable as they resist solubilization in ionic detergents (SDS). They can be visualized by SDS-PAGE when samples are not boiled in sample buffer (Monier et al., 1995; Sargiacomo et al., 1995). Primary oligomerization depends on the oligomerization domain (CAV1_{61-101}) and can also be observed when the amino-terminal domain of CAV1 (CAV1_{1-101}) is heterologously expressed in bacteria (Fernandez et al., 2002).

Following oligomerization in the ER, CAV1 enters the secretory pathway and reaches the plasma membrane via the Golgi complex (Figure 1.2). During this transit, CAV1 acquires detergent resistance, i.e. it becomes insoluble in 1% TX100 at 4°C (Pol et al., 2005). Mature caveolar membranes are enriched in
Caveolin-1 inserts cotranslationally into the ER membrane where it forms homo-oligomers of 7-10 caveolin-1 monomers. These are transported to the Golgi complex, where they may associate with cholesterol, glycosphingolipids and sphingomyelin to become part of detergent resistant membrane (DRM) complexes. Surface-delivered caveolin-1 is found in caveolae and caveolar vesicles. Once assembled, the caveolar coat represents a stable unit that does not undergo cycles assembly and disassembly during transport. Many mutants of CAV1 do not reach the plasma membrane but remain trapped in the Golgi complex from where they are degraded. Adapted by permission from Macmillan Publishers, Ltd: Nat Rev Mol Cell Biol (Parton and Simons, 2007) © 2007.

Dasho et al (1995) and Scheiffele et al (1998) studied the assembly of caveolin-1 by biosynthetic and gel permeation chromatography. They showed that caveolin-1 forms homo-oligomers in the ER membrane that are transported to the Golgi complex, where they associate with cholesterol, glycosphingolipids and sphingomyelin to become part of detergent resistant membrane (DRM) complexes. Surface-delivered caveolin-1 is found in caveolae and caveolar vesicles. Once assembled, the caveolar coat represents a stable unit that does not undergo cycles assembly and disassembly during transport. Many mutants of CAV1 do not reach the plasma membrane but remain trapped in the Golgi complex from where they are degraded. Adapted by permission from Macmillan Publishers, Ltd: Nat Rev Mol Cell Biol (Parton and Simons, 2007) © 2007.

glycosphingolipids, sphingomyelin and cholesterol and share the general characteristics of detergent resistant membranes (DRMs, Brown and Rose, 1992). Pulse chase experiments and subsequent analysis of CAV1 oligomers by sucrose gradient centrifugation indicated that the CAV1 complex increased in size during passage through the secretory pathway. That this increase in size was sensitive to cholesterol-depletion was consistent with a role of cholesterol in higher-order oligomerization of CAV1 (Scheiffele et al., 1998).
Export from the Golgi complex seems to be rate-limiting during surface delivery, as a Golgi pool of CAV1 is readily detectable in many cell types at steady state (Nichols, 2002). Passing through the Golgi may therefore represent a “quality control” step during caveolae assembly as several mutants of CAV1 incapable of forming caveolae accumulate in this compartment (Machleidt et al., 2000; Ren et al., 2004, Figure 1.2). While the formation of the primary 150-200 kDa CAV1 oligomer is relatively well understood, it is currently unknown how these primary oligomers assemble to form caveolae.

Extensive mutational analysis has aimed at dissecting the process of caveolae assembly (Machleidt et al., 2000; Ren et al., 2004). In these studies, the mutants were analyzed according to (i) their capacity to form oligomers, (ii) their association to DRMs, and (iii) their trafficking phenotype. The fact that a variety of mutations in different portions of the CAV1 molecule caused similar phenotypes did not allow assignment of domains or residues of CAV1 required for higher-order oligomerization. However, it became clear that CAV1 is a molecule with highly optimized conformation and that oligomerization, DRM-association, and correct targeting of the CAV1 were all required for caveolae formation. A clear hierarchy between those criteria, however, could not be established (Machleidt et al., 2000; Ren et al., 2004). A recent study took advantage of the divergent properties of CAV1 and C. elegans-caveolin (CeCAV). While efficiently delivered to the surface of cells, CeCAV is incapable of forming caveolae when heterologously expressed in mammalian cells (Kirkham et al., 2008). Expression of chimeric constructs based on CAV1 and CeCAV1 in CAV1-/- fibroblasts and electron microscopic analysis allowed definition of the minimum sequence requirement for caveolae assembly to CAV1\(^{49-156}\). This lead to a refined model of how CAV1 may associate with membranes, but did not allow prediction of how CAV1 oligomers form caveolae (Figure 1.3).

Together with previous studies this argues that the oligomerization domain CAV1\(^{61-101}\) is required for primary oligomerization into 150-200 kDa complexes, and the flanking sequences CAV1\(^{49-60}\) and CAV1\(^{102-156}\) for association of primary oligomers into higher-order assemblies.
Figure 1.3 | **Model of caveolin-1 membrane association.** Structural modeling suggests an N-terminal amphipathic α-helix at the cytoplasm-membrane interface (α1). The α-helix at the C-terminus (α4) may be of similar characteristics, but with lower probability. The central domain is flanked by Ser80 and Cys133, both of which likely are in the cytoplasm because these residues are phosphorylated and palmitoylated, respectively. The central domain (80-130) is likely associated with the membrane and organized into the α-helices α2a, α2b and α3. The helix α3 is predicted as transmembrane helix spanning from Pro110 to the highly conserved Pro132. Phosphorylation of Ser80 likely controls the extent of membrane association of α2a. Note that only residues 49-156 are needed for oligomerization and caveolae formation. Adapted from (Kirkham et al., 2008; Parton et al., 2006).

### 1.1.6 Container traffic in the cell - caveolae as stable vesicular carriers

Coated vesicle transport in eukaryotic cells, such as clathrin or COPI/II transport, generally occurs in the following sequence of events. (i) Assembly of cytoplasmic coat proteins at the source membrane, (ii) morphogenesis of a vesicle, (iii) scission and release of the vesicle from the source membrane and (iv) disassembly of the protein coat from the vesicle. Work from the lab has demonstrated that the caveolar mode of vesicular transport functions fundamentally differently. Caveolar coats, once assembled, remain attached to their own membrane and do not undergo cycles of assembly and disassembly during transport (Tagawa et al., 2005, Figures 1.2 and 1.5). Caveolae may therefore retain during transport their unique lipid-raft-type membrane composition including lipid-anchored signaling components and their adaptors.
**1.1.7 Novel caveolar coat components - PTRF, SDRP, SRBC**

Recent studies have identified PTRF, SDRP and SRBC, a novel family of caveolar proteins that localize to caveolae and regulate their assembly, stability or trafficking (Hill et al., 2008; Liu et al., 2008; Liu and Pilch, 2008; McMahon et al., 2009). From their primary sequence they are all predicted to be cytosolic proteins, however, they are efficiently targeted to plasma membrane caveolae. They are homologous to some extent and share a common overall domain structure. Their common structural motifs include leucine zipper(s), several PEST domains, and a putative phosphatidylserine (PS)-binding site (Figure 1.4).

**PTRF.** Polymerase I and transcript release factor (PTRF, also called cavin or cavin-1) is the best studied of the novel caveolar coat proteins. PTRF, a 390-amino acid protein, was first identified as a protein involved in transcriptional regulation, where it stimulated the release of pre-rRNA and Pol I from templates during Pol I-dependent transcription (Jansa et al., 1998). However, several proteomic studies have identified PTRF as a component of caveolin-enriched membranes or caveolae and surprisingly, in many cell types PTRF localizes to caveolae rather than to the nucleus (Aboulaich et al., 2004; Hill et al., 2008; Liu and Pilch, 2008; McMahon et al., 2006; Vinten et al., 2005). In tissues, PTRF is most abundantly expressed in lung, muscle and adipose tissue, correlating with the expression of CAV1. In cells, PTRF is associated with surface caveolae, but not with the Golgi pool of CAV1. While PTRF and CAV1 are in close proximity in surface caveolae as demonstrated by FRET, a direct interaction could not be demonstrated by co-immunoprecipitation experiments (Hill et al., 2008; Liu and Pilch, 2008). PTRF may therefore be recruited to the lipid-environment generated and stabilized by clustered CAV1. In fact, PTRF was shown to bind to the negatively charged lipid phosphatidylserine (PS) in vitro (Hill et al., 2008). However, the domain of PTRF required for its targeting to caveolae has not been identified.

Knockdown of PTRF in tissue culture cells causes loss of caveolae, dispersion of punctuate distribution of CAV1 in caveolae, and subsequent degradation of CAV1 in the endosomal/lysosomal pathway (Hill et al., 2008). Exogenous expression of PTRF in cells lacking PTRF, but not CAV1 restored caveolae at the plasma
Figure 1.4 | Structural motifs in PTRF, SDPR and SRBC. Domain organization of novel soluble proteins that associate with the caveolar coat. All PTRF-family proteins share the presence of leucine-zipper(s), PEST domains and a putative phosphatidylserine (PS) binding domain. In addition, PTRF has two nuclear localization sequences, one of which overlaps with the putative PS binding domain. SDPR and SRBC have protein kinase C (PKC) binding sites. The leucine zipper, but not the PS binding site was shown to be essential for association of SDPR and SRBC to the caveolar coat. SDPR and SRBC may recruit PKC to caveolae. Compiled from (Aboulaich et al., 2004; McMahon et al., 2009).

membrane (Hill et al., 2008). The phenotype of PTRF gene disrupted (knockout) mice resembles caveolin knockout mice in several aspects. PTRF knockout causes a global loss of morphologically identifiable caveolae, while animals are viable and fertile. Moreover, loss of either PTRF or CAV1 is associated with a lipodystrophic phenotype (see also section 1.2.5, Heimerl et al., 2008; Le Lay and Kurzchalia, 2005; Liu et al., 2008). The interpretation of the above findings regarding a role of PTRF in caveola formation is difficult, because loss of PTRF consistently caused concomitant loss of caveolin expression, which is known to be essential for caveola biogenesis.

PTRF might directly be involved in caveola formation by binding to CAV1 patches at the plasma membrane and inducing membrane curvature. Alternatively, PTRF could be involved in stabilizing existing caveolae and preventing their disassembly. Signal-induced dissociation of PTRF from caveolae could then trigger disassembly of caveolae and degradation of CAV1. Both
interpretations are consistent with the available experimental data, and further studies are needed to clarify how PTRF is involved in caveola formation.

**SDPR.** Serum deprivation protein response (SDPR, also called SDR, PS-p68 or cavin-2) is a phosphatidylyserine (PS-) binding protein expressed in several splice variants, up to 425 amino acids in length. SDPR was originally identified as a gene whose expression was specifically induced upon serum starvation (Gustincich and Schneider, 1993). Later it was found that SDPR recruits PKCα for targeting to caveolae (Mineo et al., 1998). Since PKCα is known to regulate caveolae budding/internalization (Smart et al., 1994), this may indicate a role of SDPR in inducing membrane curvature in caveolae. Consistent with localization to caveolae, SDPR was identified in several proteomic studies on the composition of caveolae (Aboulaich et al., 2004; Hill et al., 2008; McMahon et al., 2006). Although SDPR can bind to phosphatidylyserine (PS, Gustincich et al., 1999) similar to PTRF, the leucine-zipper motif was identified to be essential for binding of SDPR to caveolae (McMahon et al., 2009). Currently, there are no studies published addressing involvement of SDPR in caveola formation or stability.

**SRBC.** SDR-related gene product that binds to c-kinase (SRBC, also called PRKCDBP or cavin-3) is a protein expressed in up to five potential splice variants and up to 293 amino acids in length. SRBC binds to PKCδ and similar to SDPR, its expression is induced upon serum starvation (Izumi et al., 1997). The beta-splice variant of SRBC (SRBCβ, 261 amino acids) has been studied with regard to its relationship to caveolae. The leucine-zipper motif in the amino-terminal domain was shown to be responsible for targeting SRBC to caveolae and for its interaction with CAV1 in immunoprecipitation experiments (McMahon et al., 2009). Upon budding of caveolae into the cell, GFP-tagged SRBC remained associated with caveolae and together they were transported along microtubules through the cell. Absence of SRBC caused defects in caveolae trafficking, suggesting a potential role of SRBC in regulating association of caveolae with microtubules (McMahon et al., 2009). Whether SRBC has a structural role in caveolae is not known.
1.1.8 Cellular pools of caveolin-1

Multiple cellular pools of CAV1 with established or predicted functions in biosynthetic trafficking, endocytosis, signaling, and lipid storage have been described.

ER- and Golgi pools are considered as intermediate biosynthetic pools during caveolae assembly as these pools can be depleted by incubating cells with protein synthesis inhibitors (Nichols, 2002). Interestingly, ER- and Golgi pools are recognized by a subset of anti-CAV1 antibodies and this antigenicity is lost upon Golgi-exit, consistent with a conformational change of CAV1 during transit through the secretory pathway (Pol et al., 2005). While an ER pool is not observed at steady state, the Golgi pool represents 10-15% of total cellular CAV1 (Pelkmans et al., 2004). CAV1 can also be found in TGN-derived exocytic vesicles of epithelial cells (Kurzchalia et al., 1992; Scheiffele et al., 1998).

Treatment of cells with the fungal toxin brefeldin A (BFA) that causes a collapse of the Golgi into the ER, re-routes caveolin from the ER to lipid droplets (also called lipid bodies, Ostermeyer et al., 2001). A similar phenotype was observed when certain mutants of CAV1 were overexpressed (Ostermeyer et al., 2001; Pol et al., 2001). However, also under physiological conditions, CAV1 is observed on lipid droplets and CAV1 trafficking to and from lipid droplets was shown to be regulated by cholesterol, dynamin-2, and PKC (Le Lay et al., 2006). Lipid droplets represent cellular lipid storage organelles, most prominently observed in adipocytes. They consist of a core of triglycerides that is surrounded by a phospholipid monolayer. That CAV1 and PTRF knockout mice display lipodystrophies (Le Lay and Kurzchalia, 2005; Liu et al., 2008) suggests a regulatory role of CAV1 in lipid droplet dynamics important for animal physiology.

The majority of cellular CAV1 is located in plasma membrane caveolae. These are, at least in tissue culture cells, rather immobile structures (Thomsen et al., 2002). However, upon activation by phosphatase inhibitors (okadaic acid or NaVO₄) or Simian virus 40 (SV40), they become highly dynamic and undergo microtubule-dependent long-range movement across the cell (Tagawa et al.,
2005). This indicates that caveolae dynamics are signal-activated and can switch between quiescent and activated state. Several kinases might be involved in regulating dynamics and clustering of caveolae at the plasma membrane (Pelkmans and Zerial, 2005).

A significant pool of CAV1 is observed in early endosomes. Overexpression of constitutive active RAB5 dramatically increases the early endosomal pool of CAV1 and causes re-routing of SV40 from caveosomes to early endosomes, where the virus accumulates and remains non-infectious (Pelkmans et al., 2004). Stable caveolar domains were observed to dock onto early endosomes and release their cargo, followed by detachment and onward traffic, consistent with the notion of “container traffic” in the cell (Pelkmans et al., 2004).

**Caveosomes** are intracellular endosome-like organelles characterized by a neutral pH and the presence of CAV1. They serve as intermediate organelles during infectious entry of SV40 (Pelkmans et al., 2001), but also accumulate markers of fluid-phase endocytosis (Nichols, 2002). They are thought to be grape-like structures, built of caveolar vesicles, serving as intracellular distribution stations for caveolar membrane traffic (Pelkmans, 2005). Clearly, additional defining features are needed and physiological ligands passing through caveosomes have to be identified in order to understand the significance of this organelle.

Additional pools of CAV1 have been described. These include a cytosolic chaperone-CAV1 complex that was suggested to be involved in cholesterol trafficking (Uittenbogaard et al., 1998), CAV1 in lipoprotein particles in exocrine cells (Liu et al., 1999), and CAV1 in the lumen of multi-vesicular bodies (Botos et al., 2008).

### 1.1.9 Disassembly of caveolae and degradation of caveolin-1

While assembly and biosynthetic trafficking of CAV1 have been extensively studied, very little is known about disassembly of caveolae and degradation of CAV1. Estimations of the half-life of CAV1 range from 5-10 h (Conrad et al., 1995; Forbes et al., 2007). Mutants of CAV1 that fail to assemble into caveolae and
remain trapped in the Golgi, or CAV2 expressed in the absence of CAV1 are known to be degraded by the proteasomal pathway (Galbiati et al., 2000; Razani et al., 2001). However, it remains unknown whether mature caveolae are degraded as entire structures, whether they are disassembled into smaller units, or whether CAV1 is even extracted from membranes for degradation.

One possibility is that caveolae are disassembled at the plasma membrane, and the dissociated subunits endocytosed into the endosomal pathway for degradation. Consistent with this model, an increased rate of CAV1 degradation after knockdown of PTRF, which is thought to stabilize caveolae, has been described. Importantly, the increased rate of degradation could be reversed by addition of lysosomal inhibitors (Hill et al., 2008). Further support for lysosomal degradation of CAV1 is provided by studies on the control of CAV1 levels by the tetraspanin EMP2 (epithelial membrane protein 2). Cells overexpressing EMP2 displayed a decreased half-life of CAV1, but this decrease could be blocked by addition of lysosomal inhibitors. Additionally, the increased degradation was accompanied by increased colocalization of CAV1 with the endosomal markers EEA1 and LAMP1 (Forbes et al., 2007). Consistent with lysosomal degradation, CAV1 was found in the lumen of multi-vesicular bodies (MVBs) following caveolar uptake stimulated by albumin (Botos et al., 2008). Further studies are needed to identify putative signals causing disassembly of the remarkably stable caveolar coat and to address the intriguing question of how a membrane protein with hairpin-topology can be targeted to the lumen of multi-vesicular bodies (MVBs).

1.2 Caveolae in cell and animal physiology

1.2.1 Caveolar endocytosis and transcytosis

Caveolar endocytosis is characterized by budding of caveolae into the cytoplasm to form endocytic caveolar carriers (Figure 1.5). Well-studied ligands of caveolar endocytosis include the bacterial cholera toxin and the non-enveloped virus SV40, both of which bind to the ganglioside GM1 that is enriched in caveolae. Besides these exogenous ligands, albumin, transforming growth factor
(TGFβ)-receptors, and EGFR may also be endocytosed by a caveolar mechanism (Botos et al., 2008; Di Guglielmo et al., 2003; Khan et al., 2006). With only 2% of surface-caveolae endocytosed per minute at steady state caveolar endocytosis is likely to be a highly regulated process (Kirkham and Parton, 2005).

Budding of caveolae can be stimulated by addition of the lipid lactosylceramide, the phosphatase inhibitor okadaic acid, and the virus SV40 (Kirkham and Parton, 2005; Tagawa et al., 2005). The molecular requirements for caveolar endocytosis include dynamin-2, that localizes to the neck of caveolae (Henley et al., 1998; Oh et al., 1998; Yao et al., 2005), Src family kinases and protein kinase C (PKC), actin (Pelkmans et al., 2002; Anderson et al., 1996), and
cholesterol (Sharma et al., 2004, Figure 1.5). Many of these characteristics of caveolar endocytosis are shared with a related caveolin-independent, but lipid raft-dependent pathway that is used by ligands of caveolar endocytosis both in presence and absence of CAV1 (Damm et al., 2005; Kirkham et al., 2005). The observation that cellular uptake of SV40, a ligand of caveolar endocytosis, was increased in cells lacking both CAV1 and caveolae, raised the possibility that caveolin contributes a regulatory function to a more general raft-dependent endocytic pathway (Damm et al., 2005). The term caveolae/raft-dependent endocytosis is now generally used for this pathway to take account of the dispensability of the caveolar coat (Marsh and Helenius, 2006).

A specialized form of endocytosis that involves caveolae is observed in endothelial cells. There, caveolae were shown to mediate transcytosis of plasma albumin across the thin cell layer of the endothelium (Heltianu et al., 1989; Schubert et al., 2001). This suggested a general role for caveolae in vascular permeability and transendothelial transport. Other than in cultured epithelial cells and fibroblasts, caveolar transport in endothelial cells in vivo was shown to be a highly efficient process (Oh et al., 2007). Studies with CAV1-deficient mice, however, questioned the role of caveolae in mediating transendothelial transport as these mice displayed vascular hyperpermeability (Schubert et al., 2002). Although this phenotype might be explained by a dual role of CAV1 in transport and regulation of eNOS (Schubert et al., 2002), additional studies are needed to further establish the transport function of caveolae in endothelia.

1.2.2 Caveolin-1 in exocytic transport and sorting

The plasma membrane of polarized epithelial cells is organized into an apical and a basolateral domain, separated by tight junctions. Each of these domains exerts specific functions essential for many cellular processes including absorption and secretion, signaling, development and morphogenesis. The biogenesis and maintenance of this cellular asymmetry has been subject of intense research over the past two decades. While tight junctions between neighboring cells support maintenance of apical and basolateral domains, the Golgi complex is thought to be
Figure 1.6 | **Exocytosis and sorting in epithelial cells.** Apical exocytic routes (1 and 4): glycosylphosphatidylinositol (GPI) anchors, N-glycans, and O-glycans sort apical proteins at the trans-Golgi network (TGN), common recycling endosomes (CREs) and apical recycling endosomes (AREs). This sorting might involve clustering of small lipid rafts into larger functional lipid rafts, a process that might be promoted by a luminal lectin and caveolin oligomers. Basolateral exocytic routes (2 and 5): basolateral signals interact with adaptors of the clathrin or the non-clathrin type at the TGN and CREs. Newly synthesized lysosomal membrane proteins (LAMPS) seem to be transported to the lysosome via the basolateral membrane (route 2 followed by route 7) to basal sorting endosomes (BSEs), late endosomes (LEs) and lysosomes (LYS), although some believe they follow a direct intracellular route (3c). Biosynthetic route through endosomes (routes 3a-c): some newly synthesized basolateral proteins reach CREs directly from the TGN (routes 3a and 3b, via unknown adaptors) from where they are sorted to the basolateral membrane via AP1B (route 5). Mannose-6-phosphate receptors and their ligands (lysosomal hydrolases) move through clathrin-coated vesicles, possibly into LEs, (route 3c) from where they are transported back to the TGN. Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Mol Cell Biol (Rodriguez-Boulan et al., 2005) © 2005.

the central organelle responsible for the establishment of polarized organization (Keller et al., 2001; Rodriguez-Boulan et al., 2005). Newly synthesized proteins and membrane components are sorted into distinct classes of transport vesicles and delivered to either the apical or the basolateral domain in a vectorial manner, thus
establishing an asymmetric distribution of proteins. Several determinants for sorting of cargo proteins into either apically or basolaterally destined transport vesicles have been described (Figure 1.6).

Basolateral sorting signals are typically located in the cytoplasmic region of a transmembrane protein. They may consist of tyrosine, mono- or di-leucine motifs, often found in proximity to patches of acidic amino acids (Rodriguez-Boulan et al., 2005). Endocytic motifs, transplanted to otherwise apically targeted secretory proteins (p75, HA), were shown to cause their re-routing to the basolateral domain (Brewer and Roth, 1991; Le Bivic et al., 1991). This may relate to a recent study that identified clathrin as a major determinant of basolateral sorting at the Golgi complex (Deborde et al., 2008). Clathrin knockdown accordingly resulted in slowed Golgi-exit of basolateral cargo and its missorting into apical carriers.

Requirements for apical sorting seem more complex. In 1988, van Meer and Simons formulated the lipid-raft hypothesis. According to this model, apical proteins are sorted based on their affinity to microdomains enriched in cholesterol and sphingolipids. These clusters of proteins and lipids then serve as sorting platforms that allow segregation within the Golgi and packaging for delivery to the apical surface (van Meer and Simons, 1988). The lipid-raft hypothesis has gained substantial experimental support through work with GPI-anchored proteins and influenza HA (Brown and Rose, 1992; Keller and Simons, 1998; Skibbens et al., 1989). However, the lipid-raft hypothesis was not sufficient to fully explain sorting of all apical proteins, as some apical proteins were shown not to be associated with lipid rafts (Kreitzer et al., 2000). Thus, other signals were identified mediating sorting of apical cargo. Construction of a synthetic GPI-anchored protein demonstrated that addition of GPI-anchor alone to a soluble protein was insufficient, but together with N-glycosylation effective in targeting a protein to the apical domain (Benting et al., 1999). Additionally, a weak tendency of GPI-anchored proteins to oligomerize was demonstrated to support apical sorting (Paladino et al., 2008; Paladino et al., 2004).

Since CAV1 was found both in the TGN and in TGN-derived vesicles, and because of its property to associate with glycosphingolipid-enriched membrane
domains, CAV1 was suggested to be involved in sorting and transport of apical cargo to the plasma membrane (Dupree et al., 1993; Kurzchalia et al., 1992; Zurzolo et al., 1994). A later study addressed this possibility by making use of Fisher Rat Thyroid (FRT-) cells, an epithelial cell line deficient in CAV1 and apical sorting of GPI-anchored proteins. While CAV1 expression restored caveolae formation in FRT cells, it failed to restore apical sorting and thus questioned the role of CAV1 in apical sorting (Lipardi et al., 1998). Studies on CAV1 in the exocytic pathway of MDCK cells found that both apical and basolateral TGN-derived vesicles contained CAV1, however CAV1 was enriched in apical vesicles and in a higher oligomeric state. Moreover, microinjection of anti-CAV1 antibodies blocked post-Golgi trafficking of apically targeted influenza HA, but not basolaterally targeted VSVG (Scheiffele et al., 1998). However, studies on polarized exocytosis of GPI-anchored proteins in CAV1-depleted MDCK cells indicated no defects in transport kinetics or sorting upon CAV1 knockdown, demonstrating dispensability of CAV1 in apical sorting and transport (Manninen et al., 2005).

In contrast to GPI anchored proteins, there is evidence for a set of other exocytic cargo proteins that require CAV1 for efficient surface delivery. These include dysferlin (Hernandez-Deviez et al., 2005), the angiotensin receptor (Wyse et al., 2003), the insulin receptor (Cohen et al., 2003) and stretch-activated channel short transient receptor potential channel-1 (TRPC1, Brazer et al., 2003). Either downregulation of CAV1 or overexpression of Golgi-trapped mutants of CAV1 caused defects in surface targeting of these proteins.

1.2.3 Caveolae as signaling platforms?

The identification of CAV1 as a major component of the caveolar coat allowed for purification of caveolar membranes and establishment of an inventory of caveolar proteins. Initial studies identified a number of signaling components enriched in caveolae and this lead to the “signaling hypothesis”, in which caveolae were thought to serve as organizing platform of signaling components (Lisanti et al., 1994). The signaling hypothesis predicted that “compartmentalization of
certain signaling molecules within caveolae could allow efficient and rapid coupling of activated receptors to more than one effector system” (Lisanti et al., 1994).

Recent thought has questioned the universal role of caveolae in organizing signaling because the purification techniques originally used likely copurified other DRM-associated proteins that are not necessarily enriched in caveolae (Parton and Simons, 2007). The relatively mild phenotype observed in CAV1 knockout mice also suggested that caveolae may not have an essential role in signaling (Drab et al., 2001).

However, there are well established examples of how caveolae modulate signaling. One is endothelial nitric oxide synthase (eNOS). Tight control of eNOS activity is required for regulation of blood pressure, angiogenesis and platelet aggregation (Andrew and Mayer, 1999). Targeting of eNOS to caveolae is mediated by myristoylation and palmitoylation (Shaul et al., 1996). In caveolae, eNOS interacts with the scaffolding domain of CAV1 in endothelial cells and of CAV3 in cardiomyocytes. This interaction attenuates the activity of eNOS and only the release from CAV1 fully activates the enzyme (Garcia-Cardena et al., 1997). Ca$^{2+}$/Calmodulin and Hsp90 may support displacement of eNOS from caveolae to fully activate it (Gratton et al., 2000).

1.2.4 Regulation of cell polarity and migration by caveolin-1

In migrating endothelial cells there is an apparent asymmetric distribution of CAV1 suggestive of a potential role in establishment of cell polarity during migration or locomotion. Interestingly, the polarity seems to be dependent on whether cells are moving in the plane or in three dimensions (Parat et al., 2003). In cells migrating in the plane, CAV1 is concentrated in the rear of the cells, where it may prevent lamellipod protrusion (Beardsley et al., 2005). A portion of the amino-terminal domain of CAV1 (CAV1$_{46-55}$) was shown to be required for rear polarization of CAV1, although mutation of this region may also interfere with caveolae assembly (Kirkham et al., 2008; Sun et al., 2007). The observation that CAV1 -/- fibroblasts migrate randomly and that expression of CAV1 in those cells
restores directional movement underscores a function of CAV1 in this process (Sun et al., 2007).

Further studies employing CAV1 -/- cells suggested altered cell architecture due to modifications of RhoGTPase signaling with hyperactivated Rac and Cdc42, but low Rho activities (Grande-Garcia et al., 2007). Expression in CAV1-/- cells of wildtype CAV1, but not of CAV1 with non-phosphorylatable Tyr14 (CAV1-Y14F), restored cell morphology, polarization and directional persistence during migration (Grande-Garcia et al., 2007). This potentially puts CAV1 into a negative feedback-loop with Src kinase, with signaling output to Rho via p190RhoGAP (Figure 1.7), resulting in the observed phenotypic changes when CAV1 is absent or expressed in mutant form (Grande-Garcia et al., 2007).

![Figure 1.7](image.png)

**Figure 1.7 | Caveolin-1 in a Src-kinase feedback that controls directional migration.** Caveolin-1 (CAV1) phosphorylated by Src at Try14 recruits C-terminal Src kinase (Csk). Together they inhibit Src activity. Reduced Src activity affects Rho GTPase through p190RhoGAP, with consequences in cell architecture and polarization. Compiled from (Grande-Garcia et al., 2007).

1.2.5 Caveolae and lipid homeostasis

Both CAV1- and PTRF- deficient mice showed decreased adiposity and were resistant to diet-induced obesity (Liu et al., 2008; Razani et al., 2002a). Moreover, CAV1-deficiency altered plasma lipid composition in mice (Heimerl et al., 2008). Caveolae are also highly abundant in adipocytes, together this suggests an
involvement of caveolae and CAV1 in lipid regulation. CAV1 is associated with lipid droplets in cultured cells under special conditions (Ostermeyer et al., 2001; Pol et al., 2001) and in vivo (Fernandez et al., 2006). CAV1 -/- mice showed severe defects in liver regeneration after partial hepatectomy and this correlated with reduced lipid droplet formation in hepatocytes (Fernandez et al., 2006).

Figure 1.8 | Caveolin in the regulation of lipid homeostasis. Potential ways of how caveolin-1 may regulate lipid metabolism in adipocytes. (1) Delivery of exogenous lipids, such as cholesterol, to lipid droplets by caveolar endocytosis (see also Figure 5). (2) Involvement of surface caveolae in lipid synthesis from exogenous fatty acids with possible involvement of CD36/FAT (CD36/fatty acid translocase). Small lipid droplets may bud from the plasma membrane to fuse with the main lipid storage organelle. (3) Caveolin-1 can also positively regulate fatty acid mobilization from lipid droplets by facilitating cAMP-dependent protein kinase (PKA) phosphorylation of perilipin, a crucial step in adipocyte lipolysis. (4) Finally, because signaling from the insulin receptor occurs in caveolae, plasma membrane caveolin behaves as a positive regulator of insulin signal transduction in fat cells. Adapted from (Le Lay et al., 2008).
How is CAV1 related to lipid uptake and lipid droplet formation? Studies on cultured adipocytes showed that stimulation of cells with cholesterol caused redistribution of surface CAV1 to lipid droplets. This redistribution was dependent on dynamin, PKC, and Src, consistent with a contribution of caveolar endocytosis (Le Lay et al., 2006). The lipoprotein receptor CD36 (also called fatty acid translocase, CD36/FAT) may be a candidate contributing to CAV1-dependent fatty acid uptake. CD36 has been implicated in fatty acid transport, although the transport function is controversial (Ehehalt et al., 2006). CD36 localized to biochemically isolated caveolae (Aboulaich et al., 2004; Scherer et al., 1995) and CAV1 was required for surface expression of CD36 (Ring et al., 2006). Deficiencies in fatty acid uptake were observed in CD36 knockout mice (Coburn et al., 2000). CAV1 expression above a certain threshold, on the other hand, increased fatty acid flux across the membrane such that the flip from the outer to the inner membrane was increased 2-fold (Meshulam et al., 2006). In a possible scenario, fatty acids are captured by lipid synthesizing caveolae via caveolae-localized CD36. Newly synthesized lipids are then transported into the intramembrane space in caveolae. This leads to growth of a lipid-droplet-like organelle from within caveolae. These bud off in a dynamin-, PKC- and Src-dependent manner to fuse with the central lipid droplet where they release their lipid cargo (Le Lay et al., 2008, Figure 1.8).

1.2.6 Phenotypes of caveolin-deficient mice

Despite an essential role of caveolins in caveolae biogenesis, all caveolin knockout mice that have been generated, i.e. CAV1, CAV2 and CAV1+CAV3 double knockout, are viable and fertile. This suggests that compensatory mechanisms must exist that take over the function of caveolae in their absence. Interestingly, this is not true for zebrafish where knockout of the CAV1 ortholog causes embryonic lethality (Fang et al., 2006). In spite of their rather mild phenotype, studies on caveolin knockout mice have substantially contributed to the current view of how caveolae function in cell and animal physiology. CAV1 knockout causes loss of caveolae in all non-muscle tissues (Drab et al., 2001). Besides a lipodystrophic phenotype of CAV1 -/- mice (described above), CAV1
Caveolae and their multiple roles in cell and animal physiology

Deficiency has further been associated with vascular defects, pulmonary diseases and higher susceptibility to tumorigenesis (reviewed in Le Lay and Kurzchalia, 2005).

CAV2 gene-disrupted mice suffer from pulmonary dysfunction with alveolar septal thickening, endothelial hyperproliferation and exercise intolerance (Razani et al., 2002b). As CAV2 knockout did not affect abundance or localization of caveolae, these defects must arise from the lack of a regulatory function that CAV2 normally adds to caveolae. Further research is needed to identify such mechanisms.

CAV3 is expressed in heart, skeletal and smooth muscle and accordingly, CAV3-deficient mice are characterized by loss of sarcolemmal caveolae, without affecting caveolae in non-muscle tissues (Galbiati et al., 2001). Compared to wild-type mice, CAV3 -/- mice did not display major defects in growth or movement. However, knockout mice had myopathic changes in skeletal muscle similar to those observed in patients carrying dominant-negative mutations in the CAV3 gene (Galbiati et al., 2001; Hagiwara et al., 2000). Finally, CAV1 and CAV3 double knockout mice did not have any caveolae and showed an additive phenotype of CAV1 and CAV3 single knockouts (Park et al., 2002b). These animals developed severe cardiomyopathies characterized by cardiac hypertrophies (Park et al., 2002b). Defects in both vasculature through lack of CAV1 and muscle defects through lack of CAV3 may together contribute to this relatively strong phenotype.

1.2.7 Caveolin in human disease

There is strong evidence for involvement of caveolins in development of human diseases. CAV1-/- cells display increased proliferation and loss of CAV1 was shown to promote tumorigenesis (Williams and Lisanti, 2005). A mutation in the membrane spanning segment of CAV1, CAV1-P132L is found in up to 16% of human breast cancers (Lee et al., 2002). The mutation CAV1-P132L may be considered as a loss of CAV1 function since it acts as dominant negative. CAV1 P132L is retained intracellularly and also traps wild-type CAV1 so that no caveolae can be formed at the cell surface (Lee et al., 2002). Although CAV1 -/-
mice show precocious breast development and lactation during pregnancy concomitant with hyperactivated JAK/STAT signaling pathway, the precise mechanism of tumorigenesis supported by the CAV1-P132L mutation remains unknown (Park et al., 2002a).

Figure 1.9 | Caveolin-1 and caveolin-3 in human disease. Reported disease associated mutations of CAV1 and CAV3, shown relative to their domain organization. Numbers in blue denote mutations and associated diseases. Abbreviations: RMD, rippling muscle disease; DM, distal myopathy; FHCK, familial hyperCKaemia; SHCK, spontaneous hyperCKaemia; MYO, myopathy; LGMD1C, limb girdle muscular dystrophy 1C; BC, breast cancer. Adapted from (Parton et al., 2006)

Contrary to breast cancer, CAV1 is thought to function as tumor promoter in prostate cancer (Thompson et al., 1999). CAV1 may therefore act either as tumor suppressor or tumor promoter, depending on the tissue context. Mutations in the CAV3 gene are strongly linked to muscular dystrophies. These include limb girdle muscular dystrophy 1C (LGMD-1C), rippling muscle disease, distal myopathy and hyperCKemia (Woodman et al., 2004). They are caused by autosomal dominant mutations that often cause intracellular retention of CAV3 and mistargeting of other muscle plasma membrane proteins such as the muscle repair protein dysferlin (Hernandez-Deviez et al., 2005; Matsuda et al., 2001). Disease-associated mutations in both CAV1 and CAV3 are summarized in Figure 1.9.
1.3 Outline of this thesis

Although caveolae have been known as striking plasma membrane features for more than 55 years, their functional significance in cells and organisms remains poorly understood. The many cellular processes caveolae have been associated to, together with their apparent dispensability in mice, makes them enigmatic features. A detailed understanding of the basic cell biology of caveolae will certainly help to understand their physiological relevance. A recent finding indicated that the caveolar coat functions fundamentally differently compared to other coated vesicle transport mechanisms. Once assembled, the coat remains as a stable unit and does not undergo cycles of assembly and disassembly (Tagawa et al., 2005). The major questions addressed in this thesis are - where in the cell are caveolar domains originally assembled and how are they assembled? The stable nature of the domains furthermore raised the question how they are disassembled and by what mechanism CAV1 is degraded.

Chapter 2 shows that caveolar domains are assembled in the Golgi complex. Newly synthesized, CAV1-EGFP-positive domains budding from the Golgi remained stable upon arrival at the cell surface, indicating that they were stable when exiting the Golgi. The surface-delivery of newly assembled caveolar domains was characterized and it was found that the CAV1-post-Golgi transport vesicles were distinct from those used by VSVG, a cargo of constitutive exocytosis. This suggested that CAV1 may use an exocytic pathway distinct from the one used by VSVG.

Chapter 3 reports on the identification of a novel oligomeric complex of CAV1 that forms during transit of CAV1 through the secretory pathway. Characterization of this complex indicated that it likely represents mature caveolae. FRAP analysis of newly synthesized CAV1 revealed that an essential oligomerization step for caveola formation occurred in the Golgi. Chapter 3 furthermore demonstrates differential molecular requirements for Golgi exit of CAV1 and GFP-GPI versus VSVG and that newly assembled caveolar domains can incorporate cargo for surface delivery. Finally, the coat component PTRF is shown to require newly synthesized CAV1 for association to caveolae.
Chapter 4 addresses the degradation of CAV1. Saturation of caveola assembly caused delivery of non-assembled caveolin to the plasma membrane and subsequent degradation in the endo/lysosomal pathway. Coexpression of mEGFP-tagged and mCherry-tagged CAV1 allowed to follow targeting of CAV1 to the lumen of acidic endosomal organelles in living cells. The implications on disassembly of caveolae, on degradation of CAV1 and on caveosomes are discussed.

Chapter 5 reports on flotillins and their assembly into a related lipid-raft organizing scaffold. Consistent with the literature, flotillin assembly is shown to be independent of a functioning Golgi complex. However, flotillin uses the Golgi complex during biosynthetic trafficking and strikingly can, other than CAV1, directly access the Golgi without using COPII machinery.

Chapter 6 presents models for caveolae assembly and degradation of CAV1. Future challenges in caveolae research are discussed.
Chapter 2

Assembly of caveolae in the Golgi complex

This chapter is part of the publication

Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters

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2.1 Introduction

Caveolae are remarkably stable structures. They can be visualized by fluorescence microscopy of tissue culture cells expressing GFP-tagged versions of caveolin-1 (CAV1), the major coat protein of the caveolae. In fluorescence microscopy, they appear as subresolution punctae at the cell surface (Pelkmans et al., 2001). The coat stability was demonstrated by a cell fusion experiment in which separate populations of cells expressing either CAV1-GFP or CAV1-mRFP were plated together and fused using polyethylene glycol (PEG). The resulting heterokaryons contained red and green caveolae at their cell surface. In order to prevent \textit{de novo} synthesis of caveolae fused cells were incubated in presence of cycloheximide (CHX). In case of exchange of CAV1 protein between existing caveolae, red and green caveolae would become yellow over time. Strikingly, the caveolar spots did not become yellow but remained either red or green for at least 3 h. Importantly, activation of caveolar dynamics by SV40 or addition of vanadate did not affect stability of the coat (Tagawa et al., 2005). These observations raised the question as to the site of initial assembly of these domains.

2.2 Results

Regarding assembly of caveolae it is known that CAV1 monomers are synthesized and become membrane associated in the ER, and undergo oligomerization processes during transport via the secretory pathway (Monier et al., 1995; Sargiacomo et al., 1995; Scheiffele et al., 1998; Song et al., 1997). To determine where along the pathway caveolar domains arise, we fused HeLa cells stably expressing CAV1-GFP and -RFP using UV-inactivated Semliki Forest virus (SFV) to produce heterokaryons. Caveolar domains assembled from newly synthesized CAV1 were expected to be yellow and thus distinguishable from the preformed caveolae.

When CHX was omitted, heterokaryons of HeLa cells expressing CAV1-GFP and -RFP did display discrete yellow spots on the cell surface visible by TIR-FM in addition to the preexisting red and the green spots (Figure 2.1A, surface). The
yellow spots appeared within the first 2–3 h after fusion (16 ± 4% yellow spots of total at 3 h).

Figure 2.1 | **Newly assembled caveolar domains in the Golgi complex and in transit to the PM.**

(A) Appearance of yellow CAV1 in heterokaryons of HeLa cells expressing CAV1-GFP and -RFP, respectively, was detected on the PM (surface) by TIR-FM as well as in the Golgi complex by confocal microscopy in the absence of CHX, 3 h after fusion. CAV1-GFP, -RFP, merged image of CAV1-GFP and -RFP (merge), and anti-Giantin are shown. Bars, 5 µm. (B) Kinetics of CAV1 appearance on the PM in CV1 cells expressing CAV1-GFP observed by TIR-FM. The number of spots in three homogenously illuminated areas per cell was counted, normalized to the average total visible cell surface (671 ± 228 µm², disregarding indentations and non-flat areas of the PM), and plotted against time. Error bars are SEM of n=6-15 cells per time-point from three independent experiments. (C) Spinning disc confocal images of CAV1 structures leaving from the Golgi complex in CV1 cells expressing CAV1-GFP (arrowheads). Images taken at 2 Hz (0–4 s) (Movie 2.1, available at http://www.jcb.org/cgi/content/full/jcb.200506103/DC11) and a projected image over the 4 s (projection) are shown. Bar, 5 µm.
A pool of yellow CAV1 colocalizing with the Golgi complex marker Giantin was also visible by confocal microscopy 3 h after fusion (Figure 2.1A, CAV1-GFP, -RFP, merge, and anti-Giantin). Consistent with previous reports showing that the CAV1 in the Golgi complex corresponds to newly synthesized molecules, this pool gradually disappeared when protein synthesis was inhibited (unpublished data; Nichols, 2002; Pol et al., 2005). Although most of the CAV1-GFP and -RFP in the Golgi complex had a diffuse distribution, closer inspection revealed a few, yellow spots with intensities similar to surface caveolae (Figure 2.1A, merge). The results suggested that assembly of some CAV1 domains might occur in the Golgi complex followed by rapid departure, but assembly in the PM and elsewhere could not be excluded.

To obtain a more detailed sense for time and place during the assembly process, we transfected CV-1 cells with the CAV1-GFP construct but blocked the translation of CAV1 with CHX until the cells had attached to coverslips. CV-1 cells were used because they attached to coverslips faster than HeLa cells. The CHX was then washed out to allow protein synthesis, and the transport of newly synthesized CAV1-GFP was visualized. Although CAV1-GFP was not detectable in the ER at any time, it became visible in the region of the Golgi complex as early as 15 min after CHX washout. Later, it started to appear as discrete spots in the PM. When the kinetics on appearance of CAV1-GFP spots were quantified in uniformly illuminated areas of the bottom surface of cells using TIR-FM, the results showed that newly assembled caveolae began to appear 45 min after CHX washout (Figure 2.1B). Their number reached a steady-state after 4–5 h (Figure 2.1B). By analyzing three surface areas in each of 6–15 cells per data point, we estimated that the bottom surface visible by TIR-FM (671 ± 228 µm², disregarding indentations and nonflat areas of the PM) acquired 46 ± 20 newly assembled caveolae per min. This was comparable to the rates of surface arrival reported for vesicles containing marker proteins for apical and basolateral membrane (27 per min for the p75 neurotrophin receptor and 20 for an internalization-defective low-density lipoprotein receptor mutant, Kreitzer et al., 2003). The quantal nature of the CAV1 spots suggested that the caveolar domains were assembled before insertion into the PM.
Next, we visualized the Golgi complex in live cells after CHX washout. Using spinning-disc confocal microscopy, we were consistently able to visualize the Golgi exit of CAV1-GFP vesicles. They were fluorescent structures that budded from the rim of the Golgi complex and proceeded to move along straight or curvilinear trajectories until they exited the focal plane (Figure 2.1C; and Movie 2.1, available at http://www.jcb.org/cgi/content/full/jcb.200506103/DC11). That the structures did not change their appearances during the transport is apparent in the projected image shown in Figure 2.2A (arrowheads).

We could track the CAV1 structures from the Golgi complex to the PM using TIR-FM by adjusting the penetration depth of the evanescent field such that both the cell surface and part of the Golgi complex were illuminated. The video recordings showed numerous small CAV1-GFP–labeled spots that moved from the Golgi complex radially to the surface (Figure 2.2A; Movie 2.2, available at http://www.jcb.org/cgi/content/full/jcb.200506103/DC12). Figure 2.2A shows the frames of the movie superimposed. It is clearly visible that CAV1 spots display curvilinear trajectories. When arriving at the surface, the spots either remained stationary without loss of fluorescence intensity (Figure 2.2B, E), or they traveled back into the cytoplasm.

The surface arrival of CAV1-GFP was strikingly different from the well-characterized deposition of tsO45- vesicular stomatitis virus G-protein (VSVG)-GFP, a membrane-bound protein delivered to the PM by constitutive, TGN-derived secretory vesicles (Presley et al., 1997; Toomre et al., 1999). Although CAV1-GFP remained at the cell surface as distinct spots (Figure 2.2B, E), the VSVG-GFP rapidly diffused away from the site of vesicle discharge (Figure 2.2D, E, Movie 2.3, Toomre et al., 2000). Together, our results indicated that caveolae are assembled in the Golgi complex, are individually transported as vesicles to the PM, and remain together as stable units without dispersion.
Figure 2.2 | Stable, Golgi-derived caveolar domains arriving at the cell surface. Post-Golgi trafficking of CAV1-EGFP and VSVG-EGFP. CV-1 cells expressing either CAV1-GFP (A) or VSVG-GFP (C) were imaged at 1 Hz for 300 frames by TIR-FM, but with illumination such that cell surface and part of the Golgi complex were visible simultaneously (Movies 2.2 and 2.3). Consecutive frames were subtracted from each other to yield a stack of images with only moving objects. The frames were projected, and trajectories generated this way are shown. Note that the CAV1 spots in the trajectories do not change their appearance. Bars, 10 µm. (B) CAV1 structures leaving from the Golgi complex area (cloudy staining in the background) and arriving on the cell surface (arrowheads) in CV-1 cells expressing CAV1-GFP. Images were taken as in A (Movie 2.2), and selected frames (0, 3, 7, 9, 19, and 46 s) are shown. Bar, 2 µm. (D) A VSVG-loaded post-Golgi carrier arriving at the cell surface and depositing its cargo visible by diffusion of VSVG-GFP into
2.3 Discussion

The assembly of caveolar domains is a multistep process that begins with the synthesis and membrane insertion of caveolins in the ER. After rapid homooligomerization of CAV1 into 200-400 kD complexes (Monier et al., 1995), caveolins move to the Golgi complex where they occur diffusely, and where they are in mobile, detergent-soluble form (Monier et al., 1995; Pol et al., 2005). They undergo further assembly, become detergent resistant, and associate with cholesterol and sphingolipids (Fra et al., 1995; Lisanti et al., 1993; Murata et al., 1995; Scheiffele et al., 1998). Transport to the cell surface correlates with these maturation steps, and is regulated by cholesterol (Pol et al., 2005; Ren et al., 2004).

Our observations indicated that assembly of the stable caveolar domain as a unit occurs in the Golgi complex, and that the assembled domain moves rapidly after formation of a vesicle to the PM where it inserts itself as a mature, stable caveolar domain without further expansion. It was quite clear from TIR-FM that the CAV1-GFP did not disperse like VSVG protein upon arrival at the PM. That the CAV1-positive spots that arrived at the PM from the Golgi complex were identical in morphology to surface caveolae implied that caveolae are preassembled already in the Golgi complex.

Thus, according to our working model, assembly of a caveolar domain occurs in the Golgi complex from mobile, multimeric complexes of CAV1 (and CAV2) that associate tightly with each other and with cholesterol and sphingolipids. Immediately after a domain has formed, it detaches as a vesicle distinct from the TGN-derived carriers that transport VSVG protein to the cell surface. In CV-1 cells, we estimate that up to 100–200 caveolae can move to the PM every minute. After arrival, they remain as distinct caveolar domains that do not exchange CAV1 molecules with each other or with any other pool. Some may cluster into dense grape-like structures, others are tightly associated with the actin cytoskeleton that

(Figure 2.2 continued) the surrounding plasma membrane. Images were taken as in C (Movie 2.3) and selected frames (0, 1, 2, 3, 4, and 5 s) are shown. Bar, 2µm. (E) Fluorescence intensity profiles of individual CAV1-GFP and VSVG-GFP-loaded vesicles after arrival at the cell surface (t=0 s). The VSVG loaded vesicle released its cargo visible as a burst in fluorescence followed by exponential decay. The fluorescence intensity of the CAV1-GFP domain remained stable.
prevents their lateral movement, and a fraction cycles between free and fused forms remaining close to the PM in a volume limited by microfilaments. Stimulation by SV40 results in activation of previously immobile caveolar domains, loosening of the cortical actin cytoskeleton, release of mobile caveolar vesicles, and association with MTs. Long-range traffic is enhanced, and turnover of caveolar domains in caveosomes and in the PM are stimulated.

There are two main differences in modus operandi between caveolar and clathrin- or coatamer-mediated vesicle traffic. Internalization of caveolae is a triggered event involving cargo-mediated signals and a complex network of kinases. Whereas the clathrin, COPI, and COPII coats are assembled each time from soluble component for vesicle formation and cargo loading, CAV1 follows the vesicle from donor to acceptor membrane and helps to define the membrane as a permanent (or semipermanent) domain. Caveolar domains are therefore unique vesicular transporters that shuttle between membranes without exchange of CAV1 and without loss of domain identity.

2.4 Materials and methods

Cells and transfection

HeLa cells were grown in MEM-complete medium supplemented with 10% FCS and essential amino acids and CV-1 cells in DME complete medium with 10% FCS. To generate HeLa cells stably expressing CAV1-GFP and -RFP, HeLa cells were transfected with 2.5 µg of CAV1-GFP and -RFP, respectively, using AMAXA Nucleofector (Amaxa Biosystem). The cells were incubated for 24 h in MEM-complete medium before addition of G418 (0.5 mg/ml) and incubated for 10 d to select cells stably expressing the proteins of interest. The stable cell lines were maintained in MEM-complete medium containing G418 (0.5 mg/ml).

Cell fusion and DNA constructs

For cell fusion, 10⁶ HeLa cells were transfected with 1 µg of CAV1-GFP, CAV1-RFP, using AMAXA Nucleofector (Amaxa Biosystem). CAV1-GFP was
constructed as described previously (Pelkmans et al., 2001) and CAV1-RFP was constructed by inserting CAV1 part of CAV1-GFP construct into mRFP vector provided by R.Y. Tsien (University of California, San Diego, La Jolla, CA) and described in (Campbell et al., 2002). In cholesterol depletion/repletion and caveolar assembly experiments, HeLa cells stably expressing CAV1-GFP and -RFP were used. The HeLa cell lines were fused using UV-inactivated SFV as previously described (White et al., 1981). In brief, SFV was UV inactivated for 3 min in laminar flow hood, and 7 x 10^7 of particles in 20 µl were bound to 2 x 10^5 cells on 18-mm coverslips on ice for 1 h. Cell fusion was induced at 37°C by a transient (1 min) change of pH from 6.8 to 5.0 in MEM- medium containing 10 mM MES, 10 mM HEPES, and 0.2% BSA. SFV method was superior to PEG fusion in efficiency of fusion and lack of toxicity. SFV itself did not induce any detectable change in caveolar behavior.

**Confocal microscopy and analysis**

Cells were either visualized live or formaldehyde (4%) fixed. For live microscopy, the coverslips were mounted in custom-built stainless-steel chambers (Workshop Biochemistry) in CO_2-independent medium, and viewed on a stage at 37°C using an inverted confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.) equipped with a 100x/1.4 NA plan-Apochromat objective. Images were acquired using LSM510 software package (Carl Zeiss MicroImaging, Inc.).

**TIR-FM, spinning disc confocal microscopy, and analysis**

For examining surface dynamics of CAV1, TIR-FM was performed at 37°C in CO_2-independent medium. The images were acquired on Olympus IX71 microscope equipped with TILL IMAGO QE (TILL Photonics), TILL TIR condenser (TILL Photonics), an Argon-Krypton laser (Spectra Physics) at 488 and 568 nm, Acousto-Optic Tunable Filters (model AA.AOTF.nC.TN; Opto-electronic), and a 60x/1.45 NA oil immersion objective, and using TILLvision 4.0 software (TILL Photonics).

For visualizing surface arrival of newly synthesized CAV1, and transport of CAV1 from the Golgi complex to the cell surface, 10^6 CV-1 cells were transfected with 2.5 µg of CAV1-GFP DNA, using AMAXA Nucleofector (Amaha Biosystem).
After transfection, the cells were incubated for 2 h to allow attachment to the coverslip in the presence of 1 mM CHX (Sigma-Aldrich) to inhibit protein synthesis. The CHX was then washed out, and the cells further incubated before live-cell imaging in CO₂-independent medium or formaldehyde (4%) fixation at indicated time points. The exit of CAV1-GFP vesicles was visualized 40–90 min after CHX washout either by spinning disc confocal microscopy (Axiovert 200M [Carl Zeiss MicroImaging, Inc.]; ORCA ER [Hamamatsu]; Plan-Apochromat 100x [Carl Zeiss MicroImaging, Inc.]; QLC100 spinning disc confocal scanning system [VisiTech international]; Orbit AOTF and controllers [Improvision]) or by TIR-FM. The penetration depth was adjusted such that the PM and part of the Golgi complex were both visible on the TIR-FM.

To quantify surface arrival of CAV1, images acquired on the TIR-FM were analyzed using ImageJ. Three homogeneously illuminated areas (12,572 pixels) per cell (n = 6–15 for each data point) were chosen randomly, and the number of CAV1 positive spots counted. From one dataset, the visible area of all cells by TIR-FM was measured and averaged to be 671 ± 228 µm² (n = 60) disregarding indentations and nonflat areas of the PM. This was used as an average visible area of a cell. The number of caveolae per visible area by TIR-FM was thus calculated.
2.5 Movies

Movie 2.1 | **CAV1 leaving from the Golgi complex**

Cav1-GFP-labeled vesicles leaving the Golgi complex in CV-1 cells expressing Cav1-GFP as in Figure 2.1C, imaged at 5 Hz using spinning disconfocal microscopy. Displayed at 2x real-time.

[http://jcb.rupress.org/cgi/content/full/jcb.200506103/DC1/11](http://jcb.rupress.org/cgi/content/full/jcb.200506103/DC1/11)

Movie 2.2 | **Post-Golgi trafficking of CAV1-GFP**

Cav1-GFP-labeled vesicles leaving the Golgi complex and arriving at the PM in CV-1 cells expressing Cav1-GFP as in Figure 2.2A. The vesicles were imaged at 1 Hz on the TIR-FM for 300 frames with an incident angle that gave deeper penetration depth of the evanescent field and allowed visualization of a Golgi region close to the cell surface. Acquired at 1 Hz, displayed 10x real-time.

[http://jcb.rupress.org/cgi/content/full/jcb.200506103/DC1/12](http://jcb.rupress.org/cgi/content/full/jcb.200506103/DC1/12)

Movie 2.3 | **Post-Golgi trafficking of VSVG-EGFP**

VSVG-GFP-labeled vesicles leaving the Golgi complex and arriving at the PM in CV-1 cells expressing VSVG-EGFP as in Figure 2.2D. The vesicles were imaged at 1 Hz on the TIR-FM for 300 frames with an incident angle that gave deeper penetration depth of the evanescent field and allowed visualization of a Golgi region close to the cell surface. Acquired at 1 Hz, displayed at 10x real-time.
Chapter 3

Caveolae assembly as a multistep process during transit of caveolin-1 through the secretory pathway

Arnold Hayer, Christin Bissig and Ari Helenius
3.1 Introduction

Caveolae are invaginated plasma membrane domains, enriched in cholesterol and sphingolipids, present on many mammalian cell types. They are involved in a variety of cellular functions including endocytosis, regulation of lipid homeostasis, signaling, and cancer. The major structural protein of the caveolar coat is caveolin-1 (CAV1, Fra et al., 1995), a 22 kDa 178 amino acids protein that assumes an unusual hairpin-topology in the membrane with both amino- and carboxy terminus facing the cytoplasm. CAV1 can directly bind cholesterol and cholesterol is required for caveolae structure and function (Murata et al., 1995). In clear contrast to the classical vesicular membrane coats such as clathrin, COPI, and COPII, the caveolar coat represents a stable scaffold that, once assembled, does not undergo cycles of assembly and disassembly during transport (Tagawa et al., 2005).

Current models of caveolae biogenesis propose that assembly is linked to biosynthetic trafficking of CAV1 (Kirkham et al., 2008; Parton and Simons, 2007; Tagawa et al., 2005). Newly synthesized CAV1 inserts into the ER membrane in an SRP-dependent manner, where it forms SDS-resistant homooligomers of 10-14 CAV1 molecules (Monier et al., 1995; Scheiffele et al., 1998). ER-derived CAV1 homooligomers are transported to the Golgi complex, where they increase in size but remain detergent-soluble (Pol et al., 2005; Scheiffele et al., 1998). Several mutants of CAV1 have been shown to accumulate in the Golgi, indicating an important role of this organelle in maturation of the CAV1 complex (Luetterforst et al., 1999; Machleidt et al., 2000; Ren et al., 2004).

CAV1 is then delivered to the plasma membrane, where it is part of detergent resistant membranes (DRMs). Whether CAV1 acquires detergent-insolubility during Golgi-exit or only after arrival at the plasma membrane is unknown. In a previous study we have shown using live-cell imaging of newly synthesized, GFP-tagged CAV1 (CAV1-GFP) that Golgi-derived, caveolar exocytic carriers can directly dock to the plasma membrane, where they remain as stable units (Tagawa et al., 2005). This observation indicated that the caveolar coat was already stable upon arrival at the plasma membrane, suggesting assembly during a late stage of
Golgi passage or upon Golgi exit. Homooligomerization of CAV1 in the ER is mediated by its oligomerization domain spanning amino acids 61-101 (Sargiacomo et al., 1995). How these oligomers then form the caveolar coat is less clear and assembly intermediates have not been identified. The fact that mutations at various positions within CAV1 prevent caveolae formation has led to the conclusion that CAV1 is in a highly optimized conformation, but did not help the development of structural models on how the caveolar coat assembles (Machleidt et al., 2000; Ren et al., 2004). However, morphological studies using caveolin chimeras constructed from assembly-competent human CAV1 and assembly-incompetent *C. elegans* caveolin have identified the minimum sequence requirement for caveolae formation to amino acids 49-147 of human CAV1 (Kirkham et al., 2008).

How additional proteins are involved in formation of caveolae is just beginning to be understood. Polymerase I and transcript release factor (PTRF, also called PTRF-cavin or cavin) is associated with plasma membrane caveolae (Aboulaich et al., 2004; Liu and Pilch, 2008), but not with the Golgi-pool of CAV1 (Hill et al., 2008). siRNA-mediated knockdown of PTRF in tissue culture cells or gene disruption in mice causes a global loss of caveolae and degradation of CAV1, suggesting a role for PTRF in caveolae assembly and/or stability (Hill et al., 2008; Liu et al., 2008a; Liu and Pilch, 2008). PTRF does not directly interact with CAV1, but it can bind to phosphatidylserine and is associated with DRMs in cells expressing CAV1. The mechanism of PTRF association to caveolae may therefore depend on the lipid environment stabilized by the CAV1 network (Hill et al., 2008).

In this study, we have analyzed caveolae assembly using sucrose gradient centrifugation, fluorescence recovery after photobleaching (FRAP), and live-cell imaging. We have identified a novel oligomeric complex of CAV1 that sediments at around 70S. The emergence of this complex correlated with the formation of caveolae from newly synthesized CAV1. We show that assembly-competent CAV1 loses diffusional mobility in the Golgi, supporting our previous observations that caveolae assemble in the Golgi. Furthermore, our data indicate that Golgi-exit of CAV1 is dynamin-2 independent and distinct from the exocytic
CHAPTER 3

pathway used by tsO45-VSVG. Newly assembled caveolar exocytic carriers were capable of incorporating GFP-GPI as exocytic cargo that was released upon arrival at the plasma membrane. Finally, we provide evidence that PTRF preferentially associates with newly assembled caveolae at the plasma membrane, indicating a remarkably high stability of the PTRF-caveolae interaction.

3.2 Results

3.2.1 Identification of a biochemical correlate of assembled caveolae

In order to monitor caveola assembly from CAV1 monomers, we chose a sucrose velocity gradient approach. By testing a number of solubilization- and centrifugation conditions, we found that when cell lysates were prepared using mild detergent conditions, 0.5% Triton X-100 at 25°C, CAV1 resolved into two major peaks on sucrose gradients, consistent with two major oligomeric species containing CAV1 (Figure 3.1A). The two particle forms of CAV1 were observed in a variety of cell lines including HeLa, CV1, 3T3-L1, and HEK-293T.

Comparison with soluble proteins with known molecular weights (MW) on sucrose velocity gradients containing Triton X-100 revealed that the smaller species corresponded to a previously observed, early biosynthetic homo-oligomer of CAV1, as it peaked between the 150 and 200 kDa MW markers (Monier et al., 1995; Sargiacomo et al., 1995; Scheiffele et al., 1998). As its sedimentation coefficient $S_{20,w}$ was 8 we will call it the 8S complex (Supplementary Figure 3.1). Since the shape and detergent/lipid content of the complex remain unknown, the actual molecular weight is not clear.

The larger species by far exceeded the range of available MW markers (> 669 kDa). When we calibrated our gradients using simian virus 40 (SV40) and proteins, whose sedimentations constants are well established, we found that the larger complex sedimented around 70S (Supplementary Figure 3.1). The caveolar component PTRF displayed a partial overlap with the 70S-peak, but not with the 8S complex, suggesting that the 70S peak contained a fraction of CAV1 specific for
the assembled/caveolar form of CAV1, with which PTRF is known to associate at the plasma membrane (Figure 3.1A).

We found that the choice of detergent was critical for the preservation of the 70S complex and associated PTRF in cell lysates. When either combined 0.2% Triton X-100 and 0.4% SDS, or octyl β-D-glucopyranoside (octylglucoside) were used instead of 0.5% Triton X-100, the total cellular CAV1 resolved into the 8S/150-200 kDa species and PTRF was found at the top of the gradient, in monomeric form (Figure 3.1B and 3.1C). That the 70S complex was only partially stable even in 0.5% Triton X-100 was demonstrated by running the fractions containing the 70S complex from a first gradient over a second sucrose velocity gradient, where CAV1 again sedimented as 8S and 70S species (Supplementary Figure 3.2A).

To address whether the 70S peak of CAV1 represented bulk detergent-resistant membranes (DRMs) to which CAV1 was associated, rather than a CAV1 assembly of defined size, fractions containing the 70S complex were loaded on a sucrose density flotation gradient. As shown in Figure 3.1D, CAV1 remained at the bottom of the gradient, indicating that it was not associated with buoyant membrane lipids characteristic for DRMs. As a control, CAV1 from lysates prepared using 0.5% Trition X-100 at 4°C to preserve DRMs were observed to readily float (Figure 3.1E).

The 70S complex may therefore represent an assembly of 8S CAV1-complexes, PTRF, bound detergent and a small amount of lipids such as cholesterol known to bind to CAV1 and be needed for caveolar assembly. Under harsh detergent conditions, the 70S complex dissociates into its building blocks, the 8S CAV1 oligomers, monomeric PTRF, and possibly some lipids.

If the 70S complex represents the caveolar form of CAV1, then it should be sensitive to cholesterol depletion, since caveolae flatten out and disassemble upon cholesterol depletion (Rothberg et al., 1992; Tagawa et al., 2005). Consistent with this, we found a dramatic loss of 70S-CAV1 in cells treated with methyl-beta-cyclodextrin (MBCD, Supplementary Figure 3.2B). We further expressed an amino-terminal truncation mutant of CAV1 (CAV1<sub>60-178</sub>) that is incapable of
Figure 3.1 | A novel oligomeric form of CAV1. (B) Analysis of CAV1 oligomers by sucrose velocity gradient centrifugation. (10-40%, SW55, 42'000 rpm, 10h), migration of molecular weight
Dissecting caveolae assembly

forming caveolae (CAV1-ΔN-mEGFP, Kirkham et al., 2008) to test whether it could form the 70S complex. Sucrose gradient centrifugation indicated that CAV1-ΔN-mEGFP was capable of forming the 8S-equivalent complex, but not the 70S complex (Figure 3.1F).

To visualize the 70S complex by fluorescence microscopy, we made use of a CV1 cell line that stably expresses CAV1-mEGFP (CV1-CAV1-mEGFP). Gradient fractions containing the 70S complex were prepared as above and mounted on microscopy slides. Confocal microscopy revealed that those fractions contained subresolution CAV1-mEGFP positive units of homogenous intensity. When we compared the intensity profiles of the visualized 70S complex with caveolar spots in CAV1-mEGFP-expressing cells we found a striking similarity, suggesting that the 70S complex may represent mature caveolae (Figure 3.1G, H).

(Figure 3.1 continued) standards is indicated (kDa). Cell lysates prepared from CV1 cells using either a combination of 0.2% TX100 and 0.4% SDS or 60 mM octylglucoside dissociated CAV1 into a complex migrating between 150 and 200kDa standards. Extracts prepared using 0.5% TX-100 preserved a higher (Figure 3.1 continued) oligomeric structure. (A) Shorter run-times (4h15min) revealed the existence of two distinct oligomeric species of CAV1 in 0.5% TX100 lysates. The sedimentation coefficients of the two oligomers were estimated to 8S and 70S (Supplementary Figure 3.1). PTRF, a component of the caveolar coat comigrates with CAV1 in high-molecular weight fractions. (C) When cell lysates were prepared using 0.2% TX100 and 0.4% SDS, PTRF was no longer present in high-molecular weight fractions, but remained at the top of the gradient, in monomeric form. (D) The 70S complex does not represent bulk detergent resistant membranes (DRMs). Fractions from a velocity run (5-20% sucrose) containing the 70S CAV1-complex were adjusted to 40% sucrose and loaded at the bottom of a 5-35% sucrose gradient for a flotation run (42’000rpm, 16h), note that the 70S complex did not float but remained at the bottom of the gradient. (E) In a control experiment, cells were lysed in 0.5% TX100 at 4°C (DRM preparation) and extracts loaded at the bottom of a floatation gradient as in (D). Most of CAV1 equilibrated to light, buoyant fractions in the top-third of the gradient, calnexin (CNX) served as a marker not associated with DRMs. (F) A mutant of CAV1 lacking amino acids 1-60 (CAV1-ΔN-mEGFP) was incapable of forming the 70S complex. (G) and (H) Confocal fluorescence imaging of CAV1-mEGFP in cells and in gradient fractions. (G) Caveolae appear as punctuate structures at the surface in CV1 cells stably expressing CAV1-mEGFP (CV1-CAV1-mEGFP). (H) Gradient fractions containing the 70S complex were mounted on a microscopy slide and viewed on a confocal microscope. Note the similarity between fluorescence intensity (FI) profiles of caveolae in cells and the 70S complex in gradient fractions. Scale bars, G, H, 5 µm.
3.2.2 Sequential oligomerization of CAV1 during caveolae assembly

The above results prompted us to examine the oligomerization of CAV1 during passage through the secretory pathway. We transfected a suspension of CV1 cells with a construct encoding epitope-tagged CAV1 (CAV1-HA) using electroporation. Cells were subsequently incubated in presence of cycloheximide (CHX) to prevent protein synthesis until they had firmly attached to the culture dish (2 h). Coordinated CAV1-HA expression was induced by washout of CHX, and lysates prepared at defined time-points. Sucrose gradient centrifugation was performed as above and CAV1-HA detected by SDS-PAGE/Western blot. In parallel, cells treated identically, but seeded on coverslips, were fixed and processed for immunofluorescence staining to monitor intracellular distribution of newly synthesized CAV1-HA.

Profiles of CAV1-HA distribution in the gradients from lysates prepared at 30 min, 60 min and 120 min are shown in Figures 3.2A-C. At 30 min post CHX washout, most CAV1-HA was in the top fractions, thus monomeric, and in 8S/150-200 kDa oligomers (Figure 3.2A). However, a small fraction had already assembled into 70S oligomers. Immunofluorescence microscopy indicated that most of the CAV1-HA localized to the Golgi complex at this time-point (not shown). At 60min and 120min post CHX washout, CAV1-HA was distributed between the 8S and 70S oligomers, and an increasing amount of CAV1 had reached the cell surface (Figures 3.2B, C, F, G).

We next asked whether CAV1-HA in the 70S-complex could be enriched upon clearance of the biosynthetic pool. A 90 min CHX-treatment of cells that had been expressing CAV1-HA for 120 min efficiently depleted the Golgi pool of CAV1-HA. On the gradient, the CHX treatment reduced monomeric CAV1-HA to background levels (Figure 3.2D). However, although the peaks became sharper, the distribution between 8S and 70S complexes remained largely unchanged, with a profile qualitatively similar to endogenous CAV1 in CV1 cells at steady state (Figure 3.1A). Therefore, either 8S and 70S complexes coexist in cells, or the 70S complex is partly disassembled during sample processing (Supplementary Figure 3.2A).
The results so far are consistent with assembly of the 70S complex in a Golgi- or post-Golgi step. To confirm this, we expressed CAV1-HA in cells pretreated with brefeldin A (BFA) to disrupt Golgi function. CAV1-HA accumulated in the ER in BFA-treated cells (Figure 3.2H). Fractionation by sucrose gradient centrifugation indicated that the 8S, but not the 70S complex could assemble in BFA-treated cells (Figure 3.2E).

Our results thus confirm the formation of an 8S/150-200 kDa complex early after synthesis of CAV1 monomers in the ER. The 70S complex was detected as early as 30 min after CHX washout when most CAV1-HA localized to the Golgi. The relative amount of 70S-CAV1 increased as caveolar spots accumulated at the cell surface over time. Assembly of the 70S complex was sensitive to BFA, consistent with a Golgi-dependent step.

### 3.2.3 Assembly of caveolae correlates with loss of diffusional mobility of CAV1 in the Golgi complex

The above results and our previous findings (Tagawa et al., 2005) suggested that CAV1 assembles into stable domains in the Golgi complex. We hypothesized that such assembly may correlate with loss of diffusional mobility of CAV1. We thus developed a protocol that allowed us to assess mobility of GFP-tagged CAV1 (CAV1-mEGFP) in the Golgi complex of living cells by fluorescence recovery after photobleaching (FRAP, Figure 3.3A).

CV1 cells were transfected with CAV1-mEGFP using electroporation, and FRAP experiments were performed 30-90 min after synthesis of CAV1-mEGFP was induced. At this time, the cells displayed a predominant Golgi localization of CAV1-mEGFP. Alternatively, CAV1-mEGFP was expressed in presence of BFA to perform FRAP experiments on the ER-pool of CAV1. The FRAP experiments on the Golgi pool of CAV1 were technically challenging and required post-acquisition alignment of images due to dynamic movement of the Golgi. Since light microscopy is not sufficient to resolve the connectivity between Golgi subdomains and therefore the total diffusible CAV1-mEGFP in the bleached subcompartment was unknown, we limited our analysis to determine the half-
Figure 3.2 | Sequential oligomerization of CAV1 during caveolae assembly. CV1 cells were transiently transfected with CAV1-HA by electroporation in suspension and allowed to attach in presence of cycloheximide (CHX). Expression of CAV1-HA was induced by CHX washout and cell lysates were prepared at indicated time-points. Extracts were run through 10-40% sucrose velocity gradients, fractions TCA-precipitated and proteins resolved by SDS-PAGE followed by Western blot detection of the HA-epitope and densitometry. (A-C) Time-course of CAV1-HA oligomerization. (D) Addition of CHX at 2h eliminates the monomeric pool of CAV1, but not the
time of recovery ($t_{1/2}$). We first compared mobilities of CAV1-mEGFP in the ER of BFA-treated cells and in the Golgi of untreated cells (Figure 3.3B). We observed a dramatic loss of CAV1-mEGFP between ER and Golgi, with recovery half-times ($t_{1/2}$) of 14±1.7 s in ER and 145±16.5 s in the Golgi, indicating that loss of diffusional mobility might correlate with a process related to assembly.

We next investigated the diffusional mobility of caveolin-2 (CAV2) in the Golgi using a similar experimental strategy. CAV2 is incapable of forming caveolae on its own and accumulates in the Golgi complex when expressed in cells lacking CAV1 (Mora et al., 1999). In cells expressing CAV1, however, CAV2 associates with CAV1 in a stoichiometry of 1:2 to 1:4, is transported to the cell surface, and localizes to caveolae (Mora et al., 1999; Scheiffele et al., 1998). We expressed CAV2-GFP alone in CV1 cells and found that it was highly mobile in the Golgi with $t_{1/2}$ of 40 ± 6.4 s (Figure 3.3C). When CAV2-GFP was coexpressed with CAV1-mCherry and the mobility of CAV2-GFP assessed by FRAP, CAV2 became immobile to a similar extent as CAV1 with $t_{1/2}$ of 131 ± 28.2 s (Figure 3.3D). This indicated that CAV2 becomes immobile in the Golgi complex only when CAV1 is coexpressed to similar levels, and that loss of immobility of CAV2 is CAV1-dependent as is surface targeting of CAV2 and recruitment of CAV2 to caveolae.

In order to address whether expression of CAV1 had a general effect on mobility of membrane proteins in the Golgi complex, we tested whether the mobility of a GPI-anchored GFP (GFP-GPI) was affected by coexpression of CAV1. GFP-GPI was highly mobile in the Golgi complex with $t_{1/2}$ of 37 ± 7.6 s (Figure 3C). Even when CAV1-mCherry was coexpressed the mobility of GFP-GPI remained unchanged ($t_{1/2}$ of 32.6 ± 10.9s), indicating that CAV1 specifically

(Figure 3.2 continued) 8S peak. (E) Formation of the 70S complex is BFA-sensitive. (F) The emergence of the 70S complex is BFA-sensitive. (G) The emergence of the 70S complex correlates with the arrival of newly assembled caveolae at the plasma membrane (from Tagawa et al., 2005). (G) Arrival of newly assembled caveolae monitored by TIR-FM of CV1 cells expressing CAV1-EGFP. (H) CAV1-HA is trapped in the ER when expressed in presence of BFA (confocal micrograph). Migration of molecular weight standards in the gradient is indicated (kDa). Scale bars, G, H 10 µm.
**Figure 3.3 | CAV1 loses lateral mobility in Golgi complex.** (A) Setup of the FRAP experiments. CV1 cells transfected with GFP-tagged caveolin variants and EGFP-GPI were allowed to express GFP-tagged proteins for 40-90 min. A small fraction of the Golgi associated pool (2x2 µm) was photobleached by high-intensity laser irradiation and recovery over time was measured. Half-times of recovery (t\(_{1/2}\)) were derived from recovery curves. (B) CAV1-mEGFP loses mobility in the Golgi. CAV1-mEGFP was highly mobile in the ER of BFA-treated cells but lost diffusional mobility in the Golgi as indicated by a dramatic increase in t\(_{1/2}\). (C) Both CAV2-EGFP and EGFP-GPI are...
affects mobility of CAV2 but not of a caveolae-unrelated protein (Figure 3.3E).

Several mutants of CAV1 have been characterized with defects in either surface delivery or caveolae assembly. We tested the mobility of two mutants, the breast cancer-related CAV1-P132L (CAV1-P132L-mEGFP) and an N-terminal truncation mutant (CAV1-ΔN-mEGFP). CAV1-P132L-mEGFP accumulated in ER and Golgi complex as previously shown by others (Lee et al., 2002). CAV1-ΔN-mEGFP was efficiently delivered to the cell surface, but distributed uniformly in the plasma membrane and failed to form punctuate caveolae (not shown). Both mutants, CAV1-P132L-mEGFP and CAV1-ΔN-mEGFP were significantly more mobile in the Golgi than wild-type CAV1 with $t_{1/2}$ of 60 ± 8.5 s and 75 ± 14.9 s, respectively (Figure 3.3E).

Stability of caveolae requires cholesterol, and cholesterol is required for caveolae function and trafficking (Pol et al., 2005; Rothberg et al., 1992). Treatment of cells with the amphiphilic amine U18666A phenocopies the lipid-storage disease Niemann-Pick type C (NPC) by inducing cholesterol accumulation in late endosomes/lysosomes. The drug also inhibits cholesterol synthesis and was shown to deplete cholesterol from the Golgi complex (Cubells et al., 2007). We treated cells with U18666A to investigate whether cholesterol depletion in the Golgi had an effect on loss of diffusional mobility of CAV1. CV1 cells were pretreated with 5 µM U18666A for 16 h prior to transfection with CAV1-mEGFP. When mobility of CAV1 in the Golgi was measured by FRAP we found that in U18666A-treated cells CAV1 was significantly more mobile ($t_{1/2} = 74 ± 8.5$ s) than in untreated cells, consistent with a cholesterol-dependent step in the maturation of the complex (Figure 3.3E). Drug-treated cells displayed a uniform,
diffuse distribution of CAV1-mEGFP at the cell surface and the caveolar spots were reduced in number (not shown).

Together, these findings suggested that loss of diffusional mobility of CAV1 in the Golgi represents a critical step in assembly of CAV1 domains. This assembly step, hereafter referred to as ‘secondary oligomerization’, requires cholesterol and specifically immobilizes CAV2, a component of the caveolar coat. Since the requirements for loss of diffusional mobility correlate with the requirements for the formation of the 70S complex, our results are consistent with assembly of CAV1 domains in the Golgi complex.

3.2.4 Dynamin-independent Golgi exit of CAV1 and GFP-GPI in common vesicles

We next investigated Golgi-exit of CAV1 domains. We have previously shown by live-cell imaging that CAV1-mEGFP leaves the Golgi in spherical, non-tubular vesicles that follow curvilinear tracks towards the cell periphery. As these vesicles arrived at the cell surface, they behaved distinct from VSVG-loaded vesicles in that they were stable and the CAV1 did not diffuse apart (Tagawa et al., 2005). We now asked whether secretory cargo could be sorted into CAV1-positive post-Golgi transport carriers. To address this question we made use of two well studied secretory cargo proteins, tsO45-VSVG-GFP (VSVG-GFP) and GPI-anchored GFP (GFP-GPI). GFP-GPI and VSVG-GFP have been shown to be sorted into distinct post-Golgi transport vesicles in MDCK and PTK2 cells (Keller et al., 2001). GFP-GPI was moreover shown to be associated with lipid-microdomains and transported to the apical surface of polarized MDCK cells, whereas VSVG was transported to the basolateral surface and was not associated with raft-lipids (Keller et al., 2001).

We first coexpressed CAV1-mRFP and VSVG-GFP in CV1 cells, accumulated cargo in the Golgi complex by a 20°C block and assessed colocalization between CAV1 and VSVG by confocal microscopy of fixed cells. As shown in Figure 3.4A, CAV1 and VSVG resided in distinct subdomains of the Golgi complex with hardly any overlap. When CAV1-mRFP and GFP-GPI were coexpressed, however,
Figure 3.4 | CAV1 partitions with EGFP-GPI in the Golgi complex. Confocal fluorescence micrographs of Golgi complexes only. (A) Coexpression of VSVG-GFP with CAV1-mRFP and accumulation of cargo in the Golgi by a 20°C block showed a low degree of colocalization, as seen in the fluorescence intensity profiles. (B) EGFP-GPI and CAV1-mRFP, when coexpressed, displayed a high degree of overlap in the Golgi. (C) CAV1-mEGFP in the Golgi colocalizes with the cis/medial Golgi marker Giantin whereas VSVG-GFP is excluded from Giantin-positive domains (D). Scale bars, 5 µm.
Figure 3.5 | **CAV1 and GFP-GPI leave the Golgi together.** (A-D) CV1 cells transfected with CAV1-mCherry/mRFP and EGFP-GPI were prepared for TIR-FM imaging 40-90 min post induction of protein expression (see Materials and Methods). The evanescent field was adjusted such that both cell surface and partly the Golgi were illuminated. (B) One frame of a time-lapse series acquired at 1 Hz with 300ms integration time per channel is shown. (A) The enlargement shows that many post-Golgi carriers are positive for both markers, EGFP-GPI and CAV1-mCherry (arrowheads). GFP- and mCherry channels were acquired sequentially, consequently there is a spatial shift between channels for moving objects. (C) 50 frames from each channel of the time-lapse sequence were first subtracted from each other to eliminate stationary objects and then...
we observed extensive colocalization between the two cargoes (Figure 3.4B). Of note, CAV1 localized in the Golgi to ring-like structures positive for the cis-medial Golgi marker Giantin (Figure 3.4C) while VSVG was largely excluded from those structures (Figure 3.4D).

Due to the extensive overlap between CAV1-mRFP and GFP-GPI, we speculated that nascent CAV1-domains might incorporate GFP-GPI for transport in common vesicles to the plasma membrane. We undertook live-cell experiments making use of a spinning disc confocal and total internal reflection (TIR) microscopy to follow secretory vesicles. When CAV1-mCherry and GFP-GPI were coexpressed in CV1 cells, many of the visualized post-Golgi vesicles were positive for both markers (Figure 3.5A, Movies 3.1 and 3.2). When these vesicles arrived at the plasma membrane, the GFP-GPI was released from the vesicle and diffused laterally into the plasma membrane while the CAV1 domain remained stably assembled, consistent with our previous observations (Figure 3.5D, F; Movies 3.3 and 3.4).

We next asked whether CAV1 was associated with post-Golgi carriers loaded with VSVG-GFP. We coexpressed VSVG-GFP and CAV1-mRFP in CV1 cells, accumulated cargo in the Golgi at 20°C and imaged post-Golgi trafficking after shifting cells to 37°C. VSVG-loaded vesicles arriving at the plasma membrane did not contain detectable levels of CAV1-mRFP, consistent with the lack of colocalization between the two cargoes in the Golgi complex (Figure 3.5E, G; Movies 3.5 and 3.6).

(Figure 3.5 continued) superimposed to produce traces of moving objects. Post-Golgi carriers positive for both EGFP-GPI and CAV1-mCherry follow curvilinear tracks. Again, the traces are sequentially interrupted in alternating channels due to their sequential acquisition. See also Movie 3.1. (D) An EGFP-GPI and CAV1-mRFP-positive vesicle arriving at the cell surface (TIR-FM). Note that while GFP-GPI and CAV1-mRFP arrived together, GFP-GPI diffused laterally into the plasma membrane while the CAV1-mRFP domain remained as stable unit. Fluorescence intensity profiles for both channels are shown in (F). See also Movie 3.2. (E) In a cell coexpressing VSVG-GFP and CAV1-mRFP, a VSVG-GFP-loaded vesicles arriving and releasing its cargo is shown. Note that there was no CAV1-mRFP associated with the VSVG-GFP-positive post-Golgi carrier. Fluorescence intensity profiles are shown in (G). See also Movie 3.3. Scale bar, B 10 µm; D, E, frame size is 2 µm.
Because CAV1 apparently used an exocytic pathway distinct from VSVG, but common with GFP-GPI, we aimed at identifying molecular requirements for their Golgi exit. Dynamin-2 has been implicated in the Golgi exit of p75 neurotrophin receptor (Kreitzer et al., 2000; Liu et al., 2008b) and, although controversial, of VSVG (Bonazzi et al., 2005; Cao et al., 2005). To perturb Dynamin function we used a dominant-negative Dynamin-2 lacking the proline-rich domain (Dyn2-ΔPRD, (Cao et al., 2005) and the small-molecule inhibitor of dynamin family GTPases dynasore (Macia et al., 2006). We then tested whether the two putative pathways of Golgi exit were equally sensitive against the perturbations.

HeLa cells were cotransfected with VSVG-mRFP and Dyn2-ΔPRD-GFP, incubated at 39.5°C for 16 h, then shifted to 20°C to accumulate cargo in the Golgi. Efficiency of Golgi exit was then measured after shifting cells to 37°C by scoring fluorescence intensity in the Golgi region. In Dyn2-ΔPRD expressing cells Golgi exit of VSVG-mRFP was blocked with a 1.6-fold accumulation of VSVG 1h after release from the temperature block (Figure 3.6A, B). A similar result was obtained when we used dynasore that was added 15 min prior to the release of cells from the 20°C block (not shown). Thus, perturbing dynamin function had a severe effect on Golgi exit of VSVG in HeLa cells.

To test whether Golgi exit of XFP-GPI and CAV1 was equally sensitive towards dynamin perturbation we also made use of Dyn2-ΔPRD-GFP and dynasore. HeLa cells were transfected with either mRFP-GPI or CAV1-mCherry by electroporation and incubated in presence of CHX for 2 h until cells had attached to the coverslips. CHX was then washed out and mRFP-GPI and CAV1-mCherry were then expressed for 1 h, this lead to a prominent Golgi-staining of the cargoes. CHX was subsequently added to the culture media and efficiency of Golgi exit measured in presence/absence of dynasore by fluorescence intensity measurements in the Golgi area.

The effect of Dyn2-ΔPRD-GFP on Golgi exit of mRFP-GPI and CAV1-mCherry was tested in a similar experiment, but the dominant negative construct was expressed for 16 h before cells were transfected with the cargos mRFP-GPI or CAV1-mCherry. Neither dynasore treatment nor expression of Dyn2-ΔPRD-GFP had significant impact on Golgi-exit of CAV1-mCherry or mRFP-GPI (Figure 6A,
Figure 3.6 | Dynamin-2 independent Golgi-exit of CAV1 and GFP-GPI. The requirement of PI4P and dynamin-2 for Golgi exit of three cargo proteins, CAV1-mCherry, mRFP-GPI and VSVG-mRFP, was tested in HeLa cells. PI4P requirement was tested by overexpressing FAPP1-PH-EGFP to mask PI4P, dynamin-2 dependence by overexpressing a dominant negative version of dynamin-2 lacking the proline-rich domain (DYN2-ΔPRD-GFP). Cargo proteins were accumulated in the Golgi as detailed in Materials and Methods and “chased” either by adding 1 mM CHX in case of CAV1-mCherry and mRFP-GPI or by a temperature shift (20°C to 37°C) in case of VSVG-EGFP. 90 min later cells were fixed, processed for fluorescence microscopy, and perinuclear fluorescence quantified. Results are expressed as perinuclear fluorescence relative to control. (A, B) Golgi exit of VSVG-mRFP was sensitive towards overexpression of both FAPP1-PH and DYN2-ΔPRD-GFP. (C, D) Golgi exit of CAV1 was attenuated by overexpression of FAPP1-PH-EGFP, but not of DYN2-ΔPRD-GFP. (E, F) mRFP-GPI equally accumulated in the Golgi in presence of FAPP1-PH-EGFP, but not of DYN2-ΔPRD-GFP. In A, C, E, * denotes cells with accumulated cargo. In B, D, F bars represent means ± S.E.M. (n=48-74 per condition, from independent experiments). Statistical significance according to Mann-Whitney-U-test is indicated by ** p<0.001. Scale bars, 10 µm.
B, C, D), in contrast to Golgi exit of VSVG, which was strongly dependent on dynamin function (Figure 6E, F). Therefore, not only did CAV1 and XFP-GPI seem to use distinct post-Golgi transport carriers than VSVG, also the dependence on dynamin function was different.

Phosphatidylinositol-4-phosphate (PI4P) is the main phosphoinositide in the Golgi complex. Four phosphate adapter protein 1 and -2 (FAPP1 and FAPP2) both bind to PI4P and are essential for generation of post-Golgi carriers (Godi et al., 2004; Vieira et al., 2005). Overexpression of the pleckstrin-homology (PH-) domain of FAPP1 acts as a dominant-negative by masking PI4P at the Golgi to block exocytosis of apical and basolateral cargo in polarized MDCK cells (Vieira et al., 2005). To test whether post-Golgi trafficking of CAV1, mRFP-GPI and VSVG were affected by FAPP1-PH overexpression, we performed post-Golgi trafficking transport assays as with the dominant-negative dynamin-2 construct. As shown in Figure 3.6, FAPP1-PH overexpression inhibited exocytosis of all exocytic cargoes tested; VSVG-mRFP, CAV1-mCherry and mRFP-GPI.

That CAV1 and XFP-GPI were transported from the Golgi to the plasma membrane in a common pathway was supported by several observations. The two cargoes showed strong overlap in the Golgi complex, they were sorted together into post-Golgi carriers and their Golgi exit was dynamin-2 independent. VSVG, on the other hand, was sorted into and transported within distinct vesicles and required dynamin function for efficient Golgi exit.

3.2.5 Association of PTRF with CAV1 domains requires newly synthesized caveolin and occurs at the plasma membrane

The cytosolic protein PTRF has recently been shown to be a component of caveolar coats, required for caveolae stability and/or assembly (Hill et al., 2008; Liu et al., 2008a; Liu and Pilch, 2008). Several questions regarding the relationship of PTRF to caveolae remain open. At what stage during caveolae biogenesis is the cytosolic protein PTRF recruited to caveolar membranes? Is the association of PTRF to caveolae transient or stable and how is the interaction regulated?

We studied the nature of how PTRF associates with caveolar membranes by
making use of fluoresceinly tagged versions of CAV1 and PTRF expressed in tissue culture cells. We first coexpressed PTRF-mEGFP and CAV1-mCherry using our CHX washout protocol. 30 min post CHX washout, we found CAV1-mCherry primarily localized in the Golgi complex, whereas PTRF was in the cytoplasm (Figure 7A). 60 min post CHX washout and later CAV1-mCherry and PTRF-mEGFP colocalized at the cell surface in subresolution spots typical for caveolae (Figure 7A). Colocalization was also observed in larger intracellular structures of endosomal morphology. As reported previously, PTRF was never detected in the Golgi complex (Hill et al., 2008), suggesting that recruitment of PTRF to caveolar membranes occurs during a post-Golgi step.

We further noticed that most PTRF-positive spots at the cell surface colocalized with CAV1-mCherry, i.e. with newly synthesized CAV1, despite abundant endogenous CAV1 in CV1 cells. We thus hypothesized that PTRF associates preferentially with newly synthesized CAV1. To test this, we expressed PTRF-mEGFP either alone or together with CAV1-mCherry in CV1 cells and compared the number of PTRF foci appearing at the cell surface 90 min post CHX washout in a similar experimental setup as above. Cells coexpressing PTRF-mEGFP and CAV1-mCherry produced abundant PTRF-mEGFP-positive surface spots (Figure 7B). Strikingly, cells expressing PTRF-mEGFP alone displayed a primarily cytosolic distribution of PTRF with less than 20% of PTRF-foci compared to cells coexpressing CAV1 (Figure 7B and 7C). Similar results were obtained when PTRF-mEGFP and CAV1-mCherry were coexpressed for 60 min in presence of brefeldin A (BFA) to block the secretory pathway at the level of the ER. CAV1-mCherry was trapped in the ER while PTRF-mEGFP localized to the cytoplasm with no detectable PTRF-mEGFP surface spots (Figure 7D).

Together, these data suggested that PTRF associates with caveolar membranes during a post-Golgi step and that this association depends on the generation of newly synthesized CAV1. PTRF association with CAV1 domains may therefore be stable once caveolae have assembled. However, this model does not exclude regulated dissociation of PTRF from caveolae under certain conditions. PTRF coexpression, on the other hand, had no effect on the abundance of plasma membrane caveolae in CV1 cells (our unpublished observations). This could
Figure 3.7 | Association of PTRF with newly synthesized CAV1. CV1 cells were transfected with PTRF-mEGFP alone or with CAV1-mCherry by electroporation, incubated in presence of CHX during cell attachment and expression was induced by CHX washout. (A) Cells coexpressing PTRF-mEGFP and CAV1-mCherry were fixed at indicated time-points after CHX washout. PTRF colocalized with plasma membrane caveolae, but not with Golgi CAV1. (B) CV1 cells either
either indicate that endogenous PTRF is present in sufficient amounts, that additional components are needed for domain assembly, available at limiting amounts, or that CAV1 domain assembly is PTRF independent at the observed time-scales.

3.3 Discussion

3.3.1 Summary

In this study, we have investigated trafficking of CAV1 along the secretory pathway to elucidate the mechanism of caveolar domain assembly. Our experimental approach allowed us to induce expression of fluorescently or epitope-tagged variants of CAV1 at a defined time-point and follow newly synthesized protein along the secretory pathway, both by biochemical and morphological techniques.

Consistent with previous reports, we found that soon after synthesis CAV1 formed a homo-oligomeric complex of estimated 150-200 kDa in a BFA-insensitive manner. Using sucrose velocity gradient ultracentrifugation, we showed that this complex had a sedimentation coefficient $S_{20,w}$ of 8 and we thus termed it 8S complex. As caveolae began arriving at the plasma membrane, a second CAV1 complex emerged sedimenting at around 70S. This 70S complex formed in a BFA-sensitive manner and was resistant to solubilization in Triton X-100, but not to SDS or octylglucoside. With a similar sedimentation coefficient,

(Figure 3.7 continued) coexpressing CAV1-mCherry and PTRF-mEGFP or (C) expressing PTRF-mEGFP alone were fixed 90 min post CHX washout. PTRF-mEGFP was mostly cytoplasmic in cells expressing PTRF-mEGFP alone, but associated with CAV1-spots in cells coexpressing CAV1-mCherry. (D) Quantification of PTRF-mEGFP foci at the ventral cell surface of cells expressing PTRF-mEGFP alone or coexpressing CAV1-mCherry. Quantification was based on confocal fluorescence images and automated spot detection. (E) CAV1-mCherry and PTRF-mEGFP were expressed either in presence or absence of BFA for 60 min before fixation. PTRF-mEGFP remained cytoplasmic in absence of newly synthesized CAV1-mCherry at the plasma membrane. All images are confocal images. Scale bars, 10 µm.
the recently discovered caveolar protein PTRF-cavin co-sedimented with the high-molecular weight CAV-1 complexes. Under detergent conditions that did not preserve the 70S CAV1 complexes, PTRF sedimented close to the top of the gradient suggesting that PTRF and CAV1 where both associated with the 70S complexes or at least with part of them. The 70S complex therefore likely represents mature caveolae. This was further substantiated by isolation of the 70S complex from CAV1-mEGFP-expressing cells and visualization by fluorescence microscopy, where 70S complexes resembled caveolar spots at the plasma membrane of cells from which they were isolated.

In a previous study we had shown that caveolar domains budding from the Golgi remained stable after arrival at the plasma membrane, indicating that stable caveolar domains are formed in the Golgi (Tagawa et al., 2005). Consistent with this, we found that wild-type, but not assembly-incompetent CAV1 lost diffusional mobility in the Golgi as assessed by FRAP. This loss of mobility was specific to CAV1, as CAV2 only became immobile when CAV1 was present. We further found that the Golgi-pool of CAV1 colocalized with the lipid-raft marker EGFP-GPI, but not with the non-raft marker VSVG-GFP and was sorted with EGFP-GPI into common post-Golgi transport vesicles. Upon arrival of these vesicles at the cell surface EGFP-GPI was released whereas the CAV1-coat remained as stable unit at the site of fusion. This indicated that the Golgi-derived caveolar exocytic carriers were capable of incorporating cargo such as GPI-anchored proteins that were released into the plasma membrane upon fusion. Other than exocytosis of VSVG, surface delivery of CAV1 and mRFP-GPI was dynamin-2-independent, consistent with a previous study reporting differential requirements for syntaxin-6 function in exocytosis of VSVG and the raft-associated proteins CAV1 and EGFP-GPI (Choudhury et al., 2006). Finally, we show that PTRF associated preferentially with newly assembled caveolar domains at the plasma membrane. Our results furthermore indicate that once recruited to caveolae PTRF remains stably associated as we observed little or no exchange between caveolar and cytoplasmic pools for at least 1.5 h.
3.3.2 Model of caveolar domain assembly

Our findings argue for the existence of at least four different assembly states of CAV1 that sequentially form during passage of CAV1 through the secretory pathway.

The monomer (assembly state 1) cotranslationally inserts into the ER membrane where it rapidly oligomerizes to form the 8S complex (assembly state 2). Oligomerization does not seem to be required for ER-export since the oligomerization-incompetent mutant CAV1-P132L is efficiently delivered to the Golgi complex (Lee et al., 2002). In the Golgi, another round of oligomerization takes place that is dependent on cholesterol and characterized by loss of diffusional mobility of CAV1 as observed by FRAP experiments. 8S complexes may assemble into a relatively loose network whose stability depends on cholesterol (assembly state 3). The N-terminus of CAV1 (residues 1-59) moreover seems critical for stabilizing this putative network, since the mutant CAV1-ΔN (CAV1_{60-178}) failed to become immobile in the FRAP experiment. Although engaged in a cholesterol-stabilized network, CAV1 is likely to be still detergent-soluble at this point (Pol et al., 2005). However, the observation that EGFP-GPI partitions into the Golgi subdomain populated by CAV1 suggests that the CAV1 network already stabilizes a lipid environment that is favored by a GPI-anchored protein. Caveolar exocytic carriers are then capable of incorporating cargo such as EGFP-GPI and remain as stable units after fusion with the plasma membrane. CAV1 has become detergent-insoluble at this stage and sediments as a 70S complex (assembly state 4).

How caveolar exocytic carriers are formed and when exactly the stable coat emerges, whether in the Golgi or during Golgi-exit, remains unclear. In a possible scenario, stable caveolar units are formed as inhomogenities from within the putative CAV1-network in the Golgi and rapidly exported for surface delivery. Support for this model is provided by time-lapse series of CAV1-mEGFP expressing cells. We occasionally observed multiple globular CAV1 domains connected in linear arrangements leaving the Golgi for delivery to the plasma membrane. They often separated from each other on the way between Golgi and plasma membrane or after arrival at the cell surface (our unpublished results). The
observation that apparently multiple caveolar units can leave the Golgi in linear arrays argues that individual units were formed before their export.

3.3.3 The role of PTRF in caveola formation

PTRF has been implicated in caveole assembly as caveolae and CAV1 are lost when PTRF is absent. This was demonstrated both by RNAi- knockdown in cultured cells and gene-disruption in mice (Hill et al., 2008; Liu et al., 2008a; Liu and Pilch, 2008). Because loss of PTRF in mice caused concomitant loss of CAV1 in most tissues (Liu et al., 2008a) PTRF could be directly involved in caveola formation or alternatively function in stabilization and maintenance of preformed caveolae.

Our results indicate that PTRF is recruited to stable caveolar units soon after their arrival at the plasma membrane. Consistent with previous reports we found that PTRF did not associate with the Golgi pool of CAV1 (Hill et al., 2008). How does PTRF specifically bind to caveolar domains at the cell surface? PTRF can bind to phosphatidylserine (PS) in vitro and PS is present on the cytoplasmic leaflet of the plasma membrane (Hill et al., 2008). PS may diffuse into newly assembled caveolar domains after their arrival at the plasma membrane and together with other properties of the caveolar coat attract PTRF. Whether caveolar domains are flat or invaginated before association of PTRF is not known. PTRF may therefore either induce membrane curvature or support existing membrane curvature. Once associated with caveolae, PTRF likely supports their stability by preventing disassembly and degradation of CAV1. Because PTRF was shown to be phosphorylated at several residues (Aboulaich et al., 2004; Guha et al., 2008) the association between PTRF and caveolae could be dynamically regulated. An attractive prediction from this model is that cells can regulate the abundance of caveolae in response to extracellular stimuli by signal-induced recruitment or dissociation of PTRF. Whether PTRF is involved in regulating endocytic activity of caveolae remains to be determined.
3.3.4 Newly assembled caveolar domains in exocytic traffic

We found that newly assembled caveolar domains incorporated GPI-anchored GFP (EGFP-GPI) during transport from the Golgi complex to the cell surface, while CAV1 was excluded from VSVG-containing exocytic vesicles. This raised the possibility that CAV1 supports sorting events in the Golgi complex and serves as a scaffold for exocytic vesicles. A role for CAV1 in sorting and polarized exocytosis of GPI-anchored proteins has been suggested (Dupree et al., 1993; Kurzchalia et al., 1992; Zurzolo et al., 1994). However, later studies indicated that CAV1 was dispensable for sorting and polarized surface delivery in polarized MDCK cells (Manninen et al., 2005). Nevertheless, several exocytic cargoes have been shown to depend on caveolins for efficient surface delivery. These include Influenza-HA, the muscle repair protein dysferlin, (Hernandez-Deviez et al., 2005), the angiotensin receptor (Wyse et al., 2003), the insulin receptor (Cohen et al., 2003) and stretch-activated channel short transient receptor potential channel-1 (TRPC1, Brazer et al., 2003). Either downregulation of caveolin or overexpression of Golgi-trapped mutants of caveolin caused defects in surface targeting of these proteins. An interesting question is how transmembrane proteins are transported to the cell surface in caveolar exocytic carriers and whether the caveolar coat permits their diffusion into the surrounding plasma membrane after fusion. If CAV1 supports sorting and formation of exocytic carriers at the Golgi, then compensatory mechanisms must take over these functions in absence of CAV1, given the relatively mild phenotype of CAV1 -/- mice. In analogy to the plasma membrane, the role of CAV1 in the Golgi complex might be similar to its role in caveolar/raft-dependent endocytosis, where CAV1 is thought to add a regulatory level to an existing lipid-raft-dependent pathway (Damm et al., 2005).

3.3.5 Conclusion

A combination of biochemical and light-microscopy approaches allowed us to follow the maturation of CAV1 from monomer to caveolae and identify processes critical for assembly of CAV1 into domains. Our findings underscore the
importance of the Golgi complex in caveolar domain formation. Finally, the identification of a biochemical correlate of caveolae will aid solving the central question of how CAV1 monomers are arranged in the caveolar coat.

3.4 Materials and methods

Cell culture and transfections

CV1 cells (ATCC) were grown in DMEM (Invitrogen) supplemented with 10% FCS and 5% Glutamax (Invitrogen), HeLa (ATCC) cells in MEM-alpha supplemented with 10% FCS and 5% MEM-non essential amino acids (Invitrogen). CV1 cells stably expressing CAV1-mEGFP (CV1-CAV1-mEGFP) were generated using the parental cell line CV1-Flp-In (Invitrogen) and the plasmid CAV1-mEGFP/FRT/TO, following the manufacturer’s protocol for Flp-In recombination (Invitrogen). CV1-CAV1-mEGFP cells were cultivated as CV1 cells but in presence of 150 µg/mL hygromycin. 3T3-L1 pre-adipocytes (ATCC) were grown in DMEM (Invitrogen) supplemented with 10% bovine serum and 5% glutamax (Invitrogen). CV1 and HeLa cells were transfected by electroporation (AMAXA) or FuGene (Roche) according to manufacturer’s recommendation.

Plasmid constructs

CAV1-mEGFP refers to canine CAV1 tagged by monomeric EGFP, a version of EGFP with the dimer-interface destroyed by L221K mutation (Zacharias et al., 2002). CAV1-mEGFP was obtained by swapping of EGFP against mEGFP (from pmEGFP-N1, Jennifer Lippincott-Schwartz) as BamHI/NotI fragments in CAV1-EGFP (Pelkmans et al., 2001). To generate CAV1-mEGFP/FRT/TO, the coding sequence for CAV1-mEGFP was excised from the plasmid CAV1-mEGFP as HindIII/NotI fragment and ligated into appropriately digested pcDNA5/FRT/TO (Invitrogen). For CAV1-mCherry mEGFP in CAV1-mEGFP was replaced by mCherry as AgeI/BsrGI fragment, as obtained by PCR from pRSET-B-mCherry (from Roger Tsien) using primers sense 5'-ATAAACCGGTCGCCACCATGG-TGAGCAAGGGCGAG and antisense 5'-AATATGTACAGCTCGTCCATGCCC. For CAV1-HA a carboxy-terminal HA-tag was introduced by PCR using CAV1-
mEGFP as template and primers sense 5’-CCAAAATGTCGTAACACTCCG and antisense 5’-GTGCGGCCGCTAGCTAGCTGGGACGTCGTATGGTATGTTTCTTTCTCGATGTTGATGCCG. The EcoRI/NotI-digested PCR product was ligated into EcoRI/NotI digested pEGFP-N1 (Clontech), yielding CAV1-HA. To generate the amino-terminal truncation **CAV1-ΔN-mEGFP**, the coding sequence corresponding to CAV1 amino acids 60-178 was PCR amplified from CAV1-mEGFP using primers sense 5’-GAATTCATGAACGACGGTCAAGA and antisense 5’-CGGACACGCTGAACTTGTGG. The EcoRI/BamHI digested product was ligated into appropriately digested pmEGFP-N1. The point mutant **CAV1-P132L-mEGFP** was obtained by Quick Change mutagenesis of CAV1-mEGFP using primers sense 5’-CCTGCACATCTGGGCAGTGTGCTGATAGTTTCTCG and antisense 5’-CAGGAAAC-TCTTAATGCACAGCAACTGCCCAGAT-GTGCAGG. To prepare PTRF-mEGFP, PTRF was PCR amplified from FLAG-PTRF (mouse, from Ingrid Grummt) using primers sense 5’-TAAGCTTCCGCATGGAGGATGTCGCTCCATAT and antisense 5’-TGGATCCGCGTCGCTGTCCTTG-TCCAC. The HindIII/BamHI digested PCR product was ligated into HindIII/BamHI digested CAV1-mEGFP/FRT/TO, yielding PTRF-mEGFP. EGFP-CAV2, mRFP-GPI and VSVG-mRFP were kindly provided by Kai Simons prior to publication. Other constructs have been described: Dyn2-wt-EGFP (Cao et al., 1998), Dyn2-ΔPRD-EGFP (Lee and De Camilli, 2002), GFP-GPI and VSVG-GFP (Keller et al., 2001), FAPP1-PH-EGFP (Balla et al., 2005), and CAV1-mRFP (Tagawa et al., 2005).

**Antibodies and other reagents**

Rabbit pAb anti-CAV1 (N20) was from Santa Cruz (sc-894), rabbit pAb anti-PTRF from Abcam (ab48824), rabbit pAb anti-Giantin from Covance (PRB-114C), mAb anti-HA from Covance (MMS-101P). Alexa Fluor conjugated secondary antibodies for immunofluorescence were from Invitrogen. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.
CHAPTER 3

Velocity gradient centrifugation

To preserve large CAV1 complexes, 2x10^6 CV1 or 3T3- L1 cells were scraped into PBS, pelleted and lysed at room temperature for 20 min in 330 µl 0.5% Triton X-100 in TNE (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA), supplemented with “Complete” protease inhibitors (Roche). Post nuclear supernatants (PNS) were prepared by a 5 min centrifugation at 1100 g. 300 µl of PNS were loaded onto linear 10-40% or 5-20% linear sucrose gradients containing 0.5% Triton-X100, 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM EDTA, and protease inhibitors. Gradients were prepared using a Gradient Master device (Biocomp). After centrifugation in a SW55Ti rotor (Beckman) for 255 min (10-40% gradients) or 60 min (5-20% gradients) at 50,000 rpm (237,020 g) and 4°C, fourteen 360 µL fractions were collected from top. For Western blot analysis, proteins were TCA precipitated and resolved by SDS-PAGE. To disassemble large CAV1 complexes, cells were lysed in either 0.2% Triton X-100 and 0.4% SDS in TNE (Scheiffele et al., 1998) or in 60 mM (1.75%) octyl β-D-glucopyranoside (octylglucoside) in 25 mM MES pH 6.5, 150 mM NaCl. Gradient media consistently had the same composition as lysis buffers except that they contained sucrose. Ultracentrifugation was performed as above. To follow the formation of CAV1 complexes during passage of CAV1 through the secretory pathway, 2x10^6 CV1 cells per time-point were transfected with CAV1-HA by electroporation (AMAXA) and allowed to attach in presence of 1 mM cycloheximide (CHX). Protein synthesis was induced by CHX washout and lysates prepared in 0.5% Triton X-100/TNE lysis buffer at indicated time-points. Gradients were prepared and run as described above.

Density gradient centrifugation

Gradient fractions from a velocity gradient containing the large CAV1 complexes were pooled and adjusted to 40% sucrose by addition of 60% sucrose in 20 mM Tris pH 7.4, 100 mM NaCl, 5 mM EDTA (TNE). 1 mL was underlayered a 5-35 % linear sucrose gradient in TNE + protease inhibitors. Centrifugation was performed in a SW55Ti rotor (Beckman) for 16h at 40,000 rpm (151,693 g) and 4°C. Fractionation and analysis of fractions were done as above.
Immunofluorescence imaging

Cells grown on coverslips were fixed in 4% formaldehyde in PBS, permeabilized by 0.05% Saponin, 1% BSA in PBS, incubated with appropriate primary and secondary antibodies and coverslips mounted using Immumount (Thermo Shandon). Imaging was performed on a Zeiss LSM 510 meta system using a 100× 1.4 NA objective.

FRAP

For FRAP experiments, CV1 cells were transfected by electroporation (AMAXA), seeded on 18 mm coverslips and allowed to attach in presence of 1 mM cycloheximide (CHX) for 2 h. FRAP experiments were performed 45-90 min post CHX washout, when the expressed GFP-chimera localized predominantly to the Golgi complex. Coverslips with cells were transferred to a custom-built metal microscope coverslip chamber in CO₂-independent medium (Invitrogen), supplemented with 10% FCS. FRAP analyses were performed at 37°C on an inverted Zeiss LSM 510 meta confocal microscope system, equipped with a temperature-controlled stage and a 63× 1.4 NA objective. A defined region of interest (ROI, 2 × 2 μm) was bleached using the 488 nm line of a 30 mW Argon laser at high laser intensity (100% power, 100% transmission, 50 iterations) and fluorescence recovery was recorded by scanning at low laser intensity (100% power, 10% transmission). Images were acquired as 12 bit LSM files at 512 × 512 pixels/frame and 0.14-μm/pixel lateral resolution. Image series with little or no apparent motion of the Golgi were imported into ImageJ (http://rsb.info.nih.gov/ij/) and automatically aligned using the TurboReg plugin (http://bigwww.epfl.ch/thevenaz/turboreg/). FRAP curves from n=7-21 cells from at least cells 3 independent experiments fulfilled these conditions. The average fluorescence intensity of the ROI was determined after background subtraction and normalization according to (Phair and Misteli, 2000). Half-times of recovery (t₁/₂) were derived from hyperbolic fits of recovery curves.

Live-cell fluorescence imaging

CV1 cells expressing fluorescently tagged constructs and seeded on 18 mm coverslips were transferred to a custom-built metal microscope coverslip chamber
in CO₂-independent medium (Invitrogen), supplemented with 10% FCS. Time-lapse series were acquired on an Olympus IX71 microscope equipped with a TILL IMAGO QE camera (TILL Photonics), TILL TIR condenser (TILL Photonics), an Argon-Krypton laser (Spectra Physics, Acousto-Optic Tunable Filters (Opto-electronic), and a 60× 1.45 NA objective, a temperature-controlled incubation chamber, and using Metamorph (Molecular Devices). Alternatively, a Leica AM TIRF system was used, equipped with a 100× 1.46 NA objective, an EM-CCD camera (C9100-02, Hamamatsu) and a temperature-controlled stage. The depth of the evanescent field was adjusted such that both ventral cell surface and parts of the Golgi complex were illuminated. EGFP- and mRFP/mCherry channels were acquired sequentially at an overall frequency of 1 Hz.

Quantification of post-Golgi trafficking

To quantify VSVG-transport, HeLa cells were cotransfected with tsO45-VSVG-mRFP (VSVG-mRFP) and dominant-negative or control constructs using FuGene (Roche), incubated for 16 h at 39.5°C to accumulate VSVG-mRFP in the ER. Cells were then shifted to 20°C for 2 h to accumulate VSVG-mRFP in the Golgi. Cells were fixed 90 min post shifting cells to 37°C. To quantify transport of CAV1-mCherry or mRFP-GPI, HeLa cells were first transfected with dominant-negative or control constructs. 16 h later, the same cells were transfected with CAV1-mCherry or mRFP-GPI using electroporation (AMAXA) and seeded on extracellular matrix coated coverslips, incubated in 1 mM cycloheximide (CHX) for 2h for cell attachment. To accumulate cargo in the Golgi complex CHX was washed out and CAV1-mCherry and mRFP-GPI expressed for 1h. CHX was readded to chase cargo out of the Golgi for 90min before fixation. Fluorescence images (n=48-74 per condition, from 3 independent experiments) were acquired using a Zeiss LSM 510 Meta system, imported into ImageJ (http://rsb.info.nih.gov/ij/), perinuclear fluorescence quantified and compared between cells expressing dominant-negative and control constructs.
3.5 Acknowledgements

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3.6 Movies

Movie 3.1 | CAV1-mCherry and EGFP-GPI leaving the Golgi together (I)

A CV1 cell coexpressing CAV1-mCherry and EGFP-GPI was imaged on a Leica TIRF setup with the evanescent field adjusted such that surface and partly the Golgi were visible. mCherry and EGFP channels were acquired sequentially with 300 ms integration time per channel and an overall imaging frequency of 1 Hz. Numerous vesicles positive for both CAV1-mCherry and EGFP-GPI can be observed budding off the Golgi and moving towards the cell periphery. Displayed at 10x real-time

Movie 3.2 | CAV1-mCherry and EGFP-GPI leaving the Golgi together (II)

A zoom-in from Movie 3.1. A train of vesicles is observed moving along a linear track from the Golgi (left) to the cell periphery (right). Displayed at 10x real-time

Movie 3.3 | Surface arrival of CAV1-mRFP and GFP-GPI (I)

A CV1 cell coexpressing CAV1-mRFP and EGFP-GPI was imaged on an Olympus TIRF setup. mRFP and EGFP channels were acquired sequentially at an overall 1 Hz acquisition frequency (400 ms integration time per frame). Secretory vesicles positive for both CAV1-mRFP and EGFP-GPI arriving at the plasma membrane can be observed. EGFP-GPI diffuses laterally into the plasma membrane upon
fusion while CAV1-mRFP foci remain stable. Stable caveolar domains may therefore serve as post-Golgi vesicles for EGFP-GPI. Displayed at 5x real-time.

Movie 3.4 | **Surface arrival of CAV1-mRFP and EGFP-GPI (II)**
Detail form Movie 3.3 with a zoom-in illustrating a fusion event of an EGFP-GPI loaded CAV1-mRFP domain. Displayed at 5x real-time.

Movie 3.5 | **Surface arrival of CAV1-mRFP and VSVG-GFP (I)**
A CV1 cell coexpressing CAV1-mRFP and VSVG-GFP was imaged using an Olympus TIRF setup after release from a 20°C block that accumulates secretory cargo in the Golgi complex. mRFP and EGFP channels were acquired sequentially at an overall 1 Hz acquisition frequency (400 ms integration time per frame). VSVG-loaded vesicles can be observed arriving and releasing their cargo into the plasma membrane. Note that no CAV1-mRFP is associated with VSVG-GFP-positive vesicles. Displayed at 10x real time.

Movie 3.6 | **Surface arrival of CAV1-mRFP and VSVG-GFP (II)**
Detail form Movie 3.5 with a zoom-in showing fusion events of VSVG-GFP loaded vesicles devoid of CAV1-mRFP. Displayed at 10x real-time.
Supplementary Figure 3.1 | Sedimentation coefficients of CAV1-oligomeric complexes. Molecular weight standards (gel filtration standards, Sigma) with known sedimentation coefficients (in Svedbergs, 10^-13 s) were used to calibrate a 5-20% sucrose velocity gradient run (SW55, 4°C, 50,000 rpm). (A) Sedimentation behavior of the standards at 45min, 90min and 135min. Peak positions of the standards are plotted over time. (B) The smaller CAV1 complex sedimented similarly as ADH (150kDa, 7.7S) and β-amylase (200kDa, 8.9S). The smaller CAV1 complex was therefore defined as “8S complex”. Since the larger CAV1 did not sediment within the range of available sedimentation coefficient standards we used two complementary approaches. (C) First, we performed linear regression with available standards to extrapolate the sedimentation coefficient of the large CAV1 complex. Sedimentation values obtained for 90min run time were used, where the large CAV1 complex had just pelleted. The extrapolation yielded a sedimentation coefficient of around 70S. (D) Second, comparison with sedimentation of SV40 (245S). CAV1 was run through a gradient with established sedimentation behavior of SV40 (SW41, 5-20% sucrose, 4°C, 36,000rpm, 75min) (Schelhaas et al., 2007). Calibration with thyroglobulin and SV40 equally yielded a sedimentation coefficient for the large CAV1 complex of around 70S. The large CAV1 complex was therefore defined as “70S complex”.
Supplementary Figure 3.2

(A) The 70S complex is only partially stable in 0.5% Triton X-100. Gradient fractions containing the 70S complex from a first gradient run (fractions 9 and 10, “run 1”) were loaded on top of a second gradient and run under identical conditions. On the second gradient (“run 2”) CAV1 resolved again in two distinct peaks, indicating dissociation of 70S CAV1 into 8S CAV1.

(B) The 70S complex is sensitive to cholesterol-depletion. To test whether the 70S complex was sensitive to acute cholesterol depletion 3T3-L1 cells were either incubated in presence of 20 mM methyl-beta-cyclodextrin (MBCD) for 90 min at 37°C, or left untreated. 0.5% TX100 - cell extracts were run through 10-40% sucrose velocity gradients as detailed in Materials and Methods. MBCD treatment caused a redistribution from the 70S to the 8S form.
Chapter 4

Degradation of caveolin-1 in the endo/lysosomal pathway

Arnold Hayer and Ari Helenius
4.1 Introduction

Caveolae are small plasma membrane invaginations found on the surface of many mammalian cell types. They have been implicated in various physiological processes including endocytosis, pathogen entry, transcytosis, lipid regulation, signaling, and cancer (Parat, 2009; Razani et al., 2002). Caveolae can bud into the cell to form endocytic vesicles, dock to endosomal organelles for cargo delivery and recycle back to the plasma membrane (Pelkmans et al., 2004). During transport, the caveolar coat remains tightly associated with the vesicle, in striking contrast to other coated vesicle transport like clathrin or COPI/II, in cells (Tagawa et al., 2005).

Caveolin-1 (CAV1) is the major structural protein of the caveolar coat in non-muscle cells (Fra et al., 1995). CAV1 is an integral membrane protein with a central hydrophobic domain that inserts into the cytoplasmic leaflet of membranes. With both amino and carboxy-termini facing the cytoplasm, CAV1 assumes a hairpin topology (Dupree et al., 1993; Monier et al., 1995). Assembly of the caveolar coat is linked to biosynthetic trafficking of CAV1. CAV1 is cotranslationally inserted into the ER membrane, where it rapidly forms homooligomeric complexes of 150-200 kDa sedimenting at 8S (Monier et al., 1995; Hayer et al., in preparation). In the Golgi, 8S complexes are thought to associate with each other in cholesterol-dependent manner into a network that is characterized by loss of lateral mobility in fluorescence recovery after photobleaching (FRAP) experiments (Hayer et al., in preparation). Caveolar domains then bud off the Golgi and are transported to the cell surface as stable units, consistent with their formation in the Golgi (Hayer et al., in preparation; Tagawa et al., 2005). Caveolar CAV1 is detergent insoluble and sediments as a complex of 70S (Hayer et al., in preparation). Structural details of the 8S complexes and how they are associated in the caveolar coat remain elusive.

PTRF (polymerase I and transcript release factor, also called PTRF-cavin or cavin) is a cytosolic protein essential for caveolae formation and/or stability (Hill et al., 2008; Liu et al., 2008; Liu and Pilch, 2008). PTRF is a phosphatidylycerine (PS-) binding protein that likely associates with caveolar domains after their
arrival at the cell surface and may support membrane curvature (Hayer et al., in preparation; Hill et al., 2008). Knockdown of PTRF in tissue culture cells or gene disruption in mice causes loss of caveolae and downregulation of CAV1 (Hill et al., 2008; Liu et al., 2008; Liu and Pilch, 2008). PTRF may therefore be involved in regulating cellular levels of CAV1.

Little is known about how caveolae disassemble and how CAV1 is degraded. Mutants of CAV1 that fail to assemble into caveolae and remain trapped in the Golgi, or CAV2 expressed in the absence of CAV1 are known to be degraded by the proteasomal pathway (Galbiati et al., 2000; Razani et al., 2001). Wild-type CAV1 has an estimated half-life in the range of 5-10 h (Conrad et al., 1995; Forbes et al., 2007). However, it remains unknown whether mature caveolae are degraded as entire structures, whether they are disassembled into smaller units, or whether CAV1 is even extracted from membranes for degradation. Downregulation of PTRF was recently shown to shorten the half-life of CAV1. The fact that this accelerated degradation was sensitive to lysosomal inhibitors raised the possibility that CAV1 enters the endosomal/lysosomal pathway for degradation (Hill et al., 2008). In HepG2 cells, CAV1 has moreover been observed to localize to multivesicular bodies following albumin uptake (Botos et al., 2008).

In this study we found that when caveolae assembly was saturated by either cholesterol depletion, overexpression of CAV1, or knockdown of PTRF, CAV1 localized to a diffusely-distributed, non-caveolar pool at the plasma membrane. Saturation of caveolae assembly moreover caused increased targeting of CAV1 to RAB5-positive early endosomes and RAB7/LAMP1-positive late endosomes. By coexpressing mCherry-tagged and GFP-tagged CAV1 in cells and live-cell imaging of endosomes, we found that the pH-sensitive fluorescence of GFP was quenched during progression of CAV1 through the endosomal pathway, while the less pH-sensitive mCherry remained brightly fluorescent. Our findings thus suggested that CAV1 is targeted to the intraluminal space of multivesicular endosomes for subsequent degradation in lysosomes.
4.2 Results

4.2.1 Saturation of caveolae assembly causes dispersed surface distribution of CAV1

Our finding that cholesterol depletion induced by U18666A caused a defect in secondary oligomerization of CAV1 in the Golgi complex (Hayer et al., in preparation) raised the question whether CAV1 could exit the Golgi and reach the plasma membrane under conditions where cholesterol in the Golgi was reduced.

We used a CV1-based cell line expressing CAV1-mEGFP under a tetracyclin-inducible promoter (CV1-CAV1-mEGFP-tTR cells). Addition of doxycyclin, a tetracycline analogue, to the culture media induced robust expression of CAV1-mEGFP (Supplementary Figure 4.1A). CV1-CAV1-mEGFP-tTR cells were pretreated with U18666A for 16 h before expression of CAV1-mEGFP was induced for 8 h. Cells were subsequently fixed and the cellular distribution of CAV1-mEGFP analyzed by confocal microscopy. CAV1-mEGFP was efficiently targeted to the cell surface with no apparent accumulation in the Golgi complex in both U18666A-treated and control cells. (Figures 4.1A, 1B). However, there were two striking differences between drug-treated and untreated cells. First, surface distribution of CAV1-mEGFP was dispersed and uniform in U18666A-treated cells with few, if any caveolar spots. Second, there was accumulation of CAV1 in larger intracellular organelles of endosomal morphology (Figure 4.1B). Similar results were obtained when CV1 cells constitutively expressing CAV1-mEGFP (CV1-CAV1-mEGFP) were treated with U18666A for 16h (Figures 4.1C, D) or when parental CV1 cells were drug treated and endogenous CAV1 stained with antibody (Supplementary Figure 4.1B).

The dispersed surface distribution was quantified as intensity variation within a defined field in the periphery of cells excluding intracellular organelles from analysis. U18666A-treated cells displayed an almost 50% reduced intensity variation (from 21.3 ± 2.1 to 11.7 ± 1.0) with no significant difference in average intensity within the fields analyzed (Figures 4.1F, G).
Homo-oligomerization of CAV1 occurs soon after synthesis of CAV1-monomers in the ER (Scheiffele et al., 1998) and is thought to be cholesterol-independent as bacterially expressed or in-vitro translated CAV1 spontaneously form oligomers (Monier et al., 1995; Sargiacomo et al., 1995). To rule out that U18666A treatment impaired primary homo-oligomerization in our system we analyzed CAV1 oligomers by SDS-PAGE. Oligomers of CAV1 derived from cell lysates are stable in SDS sample buffer unless boiled and resolve into multiple bands > 200 kDa. Lysates from drug treated and control cells (CV1) were loaded on 4-12% SDS-PAGE and analyzed by Western blot probing endogenous CAV1. CAV1 resolved in four major bands > 200 kDa in both drug-treated and control cells with no monomeric CAV1 (Figure 4.1E). U18666A therefore did not affect primary oligomerization of CAV1 but likely a later step in CAV1 domain assembly.

Surface caveolae are rather immobile unless cells are activated by SV40, vanadate or okadaic acid (Tagawa et al., 2005; Thomsen et al., 2002). To investigate whether the dispersed surface pool of CAV1-mEGFP in U18666A-treated cells was more mobile than caveolar spots in untreated cells we undertook FRAP experiments in CV1-CAV1-mEGFP cells and derived two kinetic parameters, the mobile fraction ($M_f$) and half-time of recovery ($t_{1/2}$). Mobility of GFP-GPI at the surface of CV1 cells was also tested for comparison (Figure 4.1H). As shown in Figure 4.1I, there was no significant difference between $t_{1/2}$ of U18666A-treated (35.4 ± 2.0 s) and control cells (29.6 ± 3.2 s). GFP-GPI recovered significantly faster with $t_{1/2}$ 6.1 ± 0.4 s. The mobile fraction ($M_f$) of CAV1-mEGFP in untreated cells was 0.41±0.04 and 0.76 ± 0.01 for GFP-GPI in CV1 cells. U18666A treatment almost doubled the mobile fraction of CAV1-mEGFP to 0.71±0.04, to a value close to $M_f$ of GFP-GPI (Figure 4.1J). The change in surface mobility of CAV1-mEGFP in U18666A-treated cells is therefore reflected in a dramatic increase in the mobile fraction ($M_f$), but not in the half-time of recovery ($t_{1/2}$).

The dispersed surface pool of CAV1 in U18666A-treated cells could originate from CAV1 domains that disassembled rapidly after their arrival at the plasma membrane due to the lack of cholesterol. Alternatively, CAV1 could be delivered
Figure 4.1 | Non-caveolar surface CAV1 in U18666A treated cells. CV1-CAV1-mEGFP-tTR cells were left untreated (A) or were treated with 5 µg/ml U18666A for 16 h (B) before expression of CAV1-mEGFP was induced by addition of 5 µg/ml doxycyclin for 8 h. Fixed cells were imaged by confocal microscopy. CAV1-mEGFP was efficiently delivered to the cell surface both in U18666A-treated and untreated cells. CV1-CAV1-mEGFP cells were left untreated (C) or were treated with 5 µg/ml U18666A for 16 h (D), fixed, and viewed by confocal microscopy. U18666A treatment caused caveolar spots to disperse into a non-caveolar pool of CAV1 at the plasma membrane. (E) Lysates of CV1 cells, U18666A-treated (5 µg/ml, 16 h) and control, were resolved by 4-12% SDS-PAGE without boiling of samples (see methods). U18666A treatment did not affect

untreated

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from the Golgi complex to the plasma membrane in unassembled form, as already suggested by the higher mobility of CAV1-mEGFP in the Golgi in U18666A-treated cells (Hayer et al., in preparation). To discriminate between these two possibilities we performed live-cell microscopy to follow newly synthesized CAV1 during post-Golgi trafficking and surface arrival. We hypothesized that unassembled CAV1-mEGFP would diffuse out of secretory vesicles when delivered to the plasma membrane and not remain stably assembled as in untreated cells (Hayer et al., in preparation; Tagawa et al., 2005).

CV1 cells were treated with U18666A for 16 h before transfection with CAV1-mEGFP. Golgi to plasma membrane transport of CAV1-mEGFP was visualized using a TIRF setup as previously described (Hayer et al., in preparation; Tagawa et al., 2005). In control cells, CAV1-mEGFP was delivered to the cell surface in subresolution vesicles that budded off the Golgi and were transported radially to the cell surface. Upon arrival at the surface the CAV1-mEGFP in these vesicles remained as a stable unit and did not diffuse apart (data not shown, Tagawa et al., 2005). Strikingly, in U18666A-treated cells, we observed numerous large, tubular post-Golgi carriers loaded with CAV1-mEGFP moving from the perinuclear area to the cell periphery. As these carriers fused with the ventral plasma membrane their cargo was released, visible as a burst of fluorescence that rapidly distributed laterally into the surrounding plasma membrane (Figures 4.2A, B; Movie 4.1). Occasionally, we observed that stable caveolar domains were delivered to the

(Figure 4.1 continued) oligomerization of CAV1 into >200 kDa oligomers. (F) Image-based quantification of the non-caveolar surface pool of CAV1-mEGFP by intensity variation analysis. Variation of pixel intensity (S.D.) was scored in 200x200 pixel fields in the periphery of cells (n=30 per condition). (G) Variation of pixel intensity was plotted against image intensity for each field. While intensity variation was significantly lower in U18666A treated cells compared to control, there was no significant difference in image intensity. (H-J) FRAP analysis of the non-caveolar surface pool of CAV1. 4x4 µm squares were photobleached in the periphery of either CV1-CAV1-mEGFP cells, (U18666A-treated, 5 µg/ml 16 h and untreated), or CV1 cells expressing GFP-GPI. Recovery was recorded (H) and the parameters mobile fraction (M) and half-time of recovery (t_1/2) determined (I and J, respectively). U18666A-treatment caused a significant increase in mobile fraction of CAV1-mEGFP to the extent of GFP-GPI. Half-time of recovery of CAV1-mEGFP was not significantly affected by U18666A. Scale bars, 10 µm.
Figure 4.2 | Delivery of Golgi-derived, non-caveolar CAV1 to the plasma membrane. CV1 cells treated with U18666A (5 µg/ml, 16 h) were transfected with CAV1-mEGFP and post-Golgi trafficking imaged by TIRF time-lapse imaging (1Hz). The evanescent field was adjusted such that plasma membrane and partly the Golgi were illuminated (B). (A) tubular carriers arriving at the surface and releasing CAV1-mEGFP upon fusion with the plasma membrane. Note that CAV1-mEGFP diffused laterally and no caveolar spots were left behind. (C) A post-Golgi carrier apparently delivering both caveolar and non-caveolar CAV1 to the plasma membrane. Similar to (A), the tubular transport carrier released cargo that rapidly diffused laterally. However, a caveolar spot matching the intensity of a stationary surface caveolae remained stable for 2.5 min. Movies are available in supplementary data (Movie 4.1). Scale bars, A, 2 µm; B, 10 µm; C, 2 µm.

surface together with diffusible CAV1-mEGFP, as these domains remained stable even long after tubular carriers had released their CAV1-mEGFP (Figure 4.2C and Movie 4.1, left insert).

These observations demonstrate that CAV1-mEGFP can indeed be delivered from the Golgi to the plasma membrane in unassembled form and that unassembled CAV1-mEGFP is not selectively retained in the Golgi in U18666A-treated cells. Moreover, assembled and non-assembled CAV1 can be transported to the cell surface in the same transport vesicles.
Together, our results indicated that cellular cholesterol imbalance induced by U18666A caused severe defects in caveolae assembly and distribution. While there was no effect of the drug on primary homo-oligomerization of CAV1 and its surface targeting, most of the protein failed to assemble into stable domains. This was reflected in a lack of immobility of CAV1 in the Golgi (Hayer et al., in preparation) and delivery of non-assembled CAV1 to the plasma membrane. There, CAV1 was localized to a mobile and homogenously distributed pool rather than to punctuate, immobile domains as observed in untreated cells. Importantly, these findings underscore that loss of diffusional mobility in the Golgi complex is a crucial step in CAV1 domain assembly. Additionally, CAV1 was observed in large intracellular organelles. These organelles were investigated in the following section.

4.2.2 CAV1 is targeted to LE/LYS

The identity of the large intracellular organelles to which CAV1 was redistributed during U18666A-treatment was investigated by immunofluorescence. We found that many of these organelles were positive for LAMP1, a marker for late endosomes/lysosomes (LE/LYS, Figures 4.3A, B).

Knockdown of the caveolar protein PTRF was shown to equally cause dispersal of caveolar domains at the plasma membrane with subsequent degradation, apparently by the endo/lysosomal pathway (Hill et al., 2008). To further clarify whether CAV1 was targeted to LE/lysosomes even under the condition of PTRF knockdown, we performed siRNA-mediated knockdown of PTRF in CV1-CAV1-mEGFP cells. In analogy to U18666A treatment, PTRF knockdown caused redistribution of CAV1-mEGFP to a non-caveolar surface pool and to LE/lysosomes, as revealed by LAMP1 staining in IF (Supplementary Figure 4.2A). These observations indicated that the non-caveolar plasma membrane pool of CAV1 was rapidly removed from the cell surface to enter the endosomal/lysosomal pathway for degradation.

We next asked whether CAV1 was targeted to the endo/lysosomal pathway also when caveolae assembly was not saturated by either cholesterol depletion or PTRF
Figure 4.3 | **CAV1 in the endo/lysosomal pathway.** (A-C) CAV1-mEGFP targeting to a LAMP1-positive compartment of CV1-CAV1-mEGFP cells in response to U18666A (5 µg/ml, 16 h). (A) In control cells, fixed and stained with anti-LAMP1 (LE/LYS) and anti-Giantin (Golgi), no significant overlap between CAV1-mEGFP and LAMP1 was observed. (B) In U18666A treated cells, CAV1-mEGFP was targeted to a LAMP1-positive compartment. (C) CAV1-mEGFP in the LE/LYS is recognized by anti-CAV1(2297), an antibody that otherwise recognizes the Golgi- but not the surface pool of CAV1. (D-E) CV1 cells stably expressing moderate levels of CAV1-mEGFP (CV1-CAV1-mEGFP) were treated with NH₄Cl (20 mM, 12 h) to neutralize acidic endosomes. (D) In control cells, CAV1-mEGFP localized primarily to the cell surface (caveolae). (E) NH₄Cl treatment caused visualization and/or accumulation of CAV1-mEGFP in endosomal organelles, presumably LE/LYS. All images are single confocal sections. Scale bars, 10 µm.
knockdown. In otherwise unperturbed cells, inhibition of lysosomal degradation should then lead to accumulation of CAV1 in LE/lys. To test this, we used NH₄Cl to neutralize acidic intracellular organelles and thus inhibit lysosomal degradation. CV1-CAV1-mEGFP cells treated with NH₄Cl (20 mM, 12h) and imaged live displayed dramatic accumulation of CAV1-mEGFP in endosomal organelles, indicating that indeed CAV1-mEGFP was degraded in LE/lys (Figure 4.3D, E). We concluded that CAV1 was delivered to LE/lys for degradation when caveolae assembly was saturated, but also in unperturbed cells. Additional experiments are planned to test the half-lives of CAV1 in presence/absence of lysosomal inhibitors to further clarify lysosomal degradation of CAV1.

4.2.3 CAV1 in the endosomal pathway

The above results prompted us to further investigate CAV1 trafficking through the endosomal pathway. We first tested colocalization of CAV1 with GFP-fusions of the classical endosomal markers Rab5, Rab7 and LAMP1. CV1 cells were cotransfected with CAV1-mCherry and GFP-tagged endosomal markers. Cells moderately expressing CAV1-mCherry were imaged by confocal microscopy either live or following fixation and immunofluorescence staining. As shown in Figures 4.4A-D, CAV1-mCherry colocalized with all markers tested, EGFP-Rab5, EGFP-Rab7 and LAMP1-EGFP, suggesting a passage of CAV1 from early endosomes (EE) to LE/lys. Interestingly, while the pool of CAV1-mCherry colocalizing with EGFP-Rab5 could be fixed by formaldehyde-based fixative, we were not able to fix in unperturbed cells the late endosomal pool of CAV1 colocalizing with EGFP-Rab7 or LAMP1-EGFP. In U18666A-treated cells, however, CAV1 colocalizing with LAMP1 could be fixed without difficulty (Figure 4.3B).

We next addressed whether CAV1 underwent changes in antigenicity while passing through the endosomal pathway. Certain antibodies raised against CAV1 recognize specific cellular pools of CAV1 in immunofluorescence indicating compartment-specific conformational states of the CAV1 molecule (Pol et al., 2005). We used two antibodies, anti-CAV1(N20), raised against an N-terminal
Antigenicity of cellular pools of CAV1

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peptide, and anti-CAV1(2297) that recognizes an internal stretch of CAV1, including the oligomerization domain (CAV1$_{61-101}$). Anti-CAV1(2297) was previously shown to detect a detergent-soluble pool of Golgi CAV1 (Pol et al., 2005). We found that anti-CAV1(N20) recognized all cellular pools tested i.e. ER, Golgi, PM caveolae, PM non-caveolar, EE, except the LE/LYS pool (Figures 4.4B, E and data not shown). That LE/LYS CAV1 was not detected by anti-CAV1-N20 may indicate N-terminal degradation or modification of CAV1, since anti-CAV1(N20) detects the N-terminus of CAV1. Anti-CAV1(2297) on the other hand, recognized the early biosynthetic pools, i.e. ER/Golgi, and the LE/LYS pool in U18666A treated cells (Fig. 4.3C, Fig. 4.4A, E and data not shown), suggesting that CAV1 in LE/LYS might be in a similar conformational state as in ER/Golgi.

Our findings thus demonstrated trafficking of CAV1 through the endosomal pathway. Two observations suggested that CAV1 underwent a conformational change during progression from EE to LE/LYS. First, CAV1 could be fixed in EE, but not in LE/LYS in untreated cells. Second, CAV1 changed antigenicity between EE and LE/LYS. That the LE/LYS pool was recognized by an antibody that otherwise recognized ER/Golgi CAV1 indicates that CAV1 in LE/LYS was, at least in part, in disassembled state.

4.2.4 CAV1 is targeted to the acidic lumen LE/LYS

CAV1 is embedded in the cytoplasmic leaflet of membranes with both N- and C-termini facing the cytoplasm. In order to be degraded in lysosomes, CAV1 would need to be exposed to the lumen of lysosomes. In analogy to degradation of

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Figure 4.4 | **CAV1 in the endosomal pathway.** (A-B) CAV1-mCherry localizes to early endosomes marked by EGFP-Rab5. CV1 cells transfected with CAV1-mCherry and EGFP-Rab5, fixed and immunostained with either anti-CAV1(2297) or anti-CAV1(N20). The early endosomal pool of CAV1 is recognized by anti-CAV1(N20), but not by anti-CAV1(2297). (C-D) CAV1-mCherry localizes to late endosomes marked by EGFP-Rab7 and LAMP1-EGFP. CV1 cells expressing CAV1-mCherry and either EGFP-Rab7 or LAMP1-EGFP were imaged live. (E) Antigenicity of cellular pools of CAV1. Anti-CAV1(2297) recognized ER, Golgi, and LE/LYS pool of CAV1, while anti-CAV1(N20) recognized all but the LE/LYS pool. Scale bars, 10 µm.
cell-surface receptors, this could occur by inward budding of the limiting endosomal membrane during intra-luminal vesicle formation.

To address whether CAV1 was exposed to the acidic lumen of LE/LYS we took advantage of the differential pH sensitivity of mCherry and EGFP. While EGFP fluorescence is quenched at pH below 6 (pK<sub>a</sub> 6.0), mCherry remains brightly fluorescent in acidic environment down to pH 4.5 (pK<sub>a</sub> <4.5, Kneen et al., 1998; Shaner et al., 2004; Shaner et al., 2005). The pH in the endosomal pathway ranges from pH 5.5-6.1 in EE and pH 5.5 in late endosomes to pH 4.7 in lysosomes (Kielian and Cohn, 1982; Zen et al., 1992). Acidification of luminal endosomal pH can be followed using a luminally targeted protein with an EGFP-mCherry tandem-tag because EGFP fluorescence is quenched in lysosomes, while mCherry remains fluorescent (Pankiv et al., 2007).

Following a similar strategy, we coexpressed CAV1-mEGFP and CAV1-mCherry in CV1 cells and performed live-cell confocal imaging. CAV1-mEGFP and CAV1-mCherry co-assembled into “yellow” caveolar domains at the plasma membrane and colocalized in round shaped, limiting membranes of endosomes (Fig 4.5A, dashed insert). The diffuse stain representing free CAV1 was also yellow. However, many endosomal structures displayed bright red color of mCherry-, but no green of mEGFP fluorescence. This was consistent with pH-dependent quenching mEGFP in LE/LYS. To test whether CAV1-mCherry-positive endosomes had acidic luminal pH we loaded cells expressing only CAV1-mCherry with LysoTracker (green, 100 nM, 1 h) and imaged them live. Most, if not all acidic organelles marked by LysoTracker were also positive for CAV1-mCherry (Fig. 4.5B).

To further clarify whether CAV1 was targeted to the lumen of LE/LYS we treated cells coexpressing CAV1-mCherry and CAV1-mEGFP with bafilomycin A<sub>1</sub> (0.2 µM, 12 h), an inhibitor of the vacuolar ATPase (v-ATPase), to neutralize luminal pH of acidic organelles and prevent quenching of mEGFP-fluorescence. Endosomes of bafilomycin A<sub>1</sub>-treated were, consistent with our hypothesis, brightly fluorescent both in mCherry and mEGFP channels (Figure 4.5C). Similar results were obtained when NH<sub>4</sub>Cl (20 mM, 12 h) was used instead of bafilomycin A<sub>1</sub> (data not shown).
We next set out to follow quenching of CAV1-mEGFP-fluorescence in individual endosomes in living cells. CV1 cells coexpressing CAV1-mEGFP and CAV1-mCherry were imaged for 45 min at 0.2 Hz using an epi-fluorescence microscope. Numerous endosomes initially positive for both mEGFP and mCherry fluorescence gradually lost their mEGFP fluorescence over time while their mCherry fluorescence remained constant (Figure 4.5D-F, Movie 4.2), in agreement with quenching of mEGFP fluorescence in the acidic lumen of LE/LYS. Importantly, the loss of mEGFP fluorescence was not due to photobleaching as the overall perinuclear mEGFP fluorescence remained constant (Figure 4.5D).

Finally, we took advantage of our observation that CAV1 in LE/LYS could not be fixed efficiently in formaldehyde to learn about the oligomeric state of CAV1 in this compartment. Cells expressing CAV1-mCherry were imaged “live” and formaldehyde (to final 4%) was added to the culture medium while time-lapse series were acquired. Within seconds after addition of formaldehyde individual CAV1-mCherry-positive endosomes were observed bursting and releasing CAV1-mCherry into the cytosol. The released CAV1-mCherry appeared as diffusible fluorescence rather than as punctuate caveolar domains, suggesting that the LE/LYS pool of CAV1 was, at least in part, in disassembled form (Supplementary Figure 4.2B, Movie 4.3).

Together, our data were consistent with a model in which CAV1 present on early endosomes is targeted to the acidic lumen by inward budding during intra-luminal vesicle formation. Subsequent acidification and lysis of intra-luminal vesicles causes quenching of mEGFP fluorescence and eventually degradation of CAV1.
Figure 4.5 | CAV1 is exposed to the acidic lumen of late endosomes/lysosomes. (A) When CV1 cells coexpressing CAV1-mEGFP and CAV1-mCherry were imaged live, the two caveolar markers colocalized in caveolar spots at the plasma membrane (dashed insert, contrast settings adjusted), but not in many of the intracellular organelles. (B) Colocalization between CAV1-mEGFP and CAV1-mCherry in endosomes was restored by treatment of cells with an inhibitor of the vacuolar ATPase, bafilomycin A (12 h, 0.2 µM), indicating acid-quenching of mEGFP-fluorescence. (C) CV1 cells expressing CAV1-mCherry were stained with Lysotracker green (100 nM, 1 h) and imaged live to identify acidic organelles. Most acidic organelles were also positive for CAV1-mCherry. (A-C), single confocal sections of live cells, scale bar, 10 µm. (D-E) Live-cell time-lapse imaging of CV1 cells co-expressing CAV1-mEGFP and CAV1-mCherry to monitor acid-dependent fluorescence quenching of CAV1-mEGFP fluorescence. (D) An endosome initially positive for CAV1-mEGFP and CAV1-mCherry and tracked over 42 min as outlined in (E) and fluorescence intensity profiles plotted against time are shown. CAV1-mEGFP fluorescence decayed exponentially over time.
4.3 Discussion

4.3.1 Summary

In this study, we have focused on the fate of CAV1 when expressed under conditions when caveolae assembly was either saturated or compromised. We found three different conditions that resulted in the elevation of a pool of CAV1 in the plasma membrane that was not present in caveolar structures. Instead of forming stable spots of uniform size, the non-caveolar CAV1 was homogenously distributed, and displayed high lateral mobility as determined by FRAP.

One of the conditions that led to elevation of the non-caveolar complexes involved over-expression of CAV1 either in its wt or GFP-tagged forms. Presumably such over-expression resulted in exhaustion of other necessary factors such as PTRF, a protein required to stabilize assembled caveolae at the plasma membrane. This was consistent with the observation that silencing of the PTRF gene using siRNA resulted in an elevation in the pool of free CAV1. A reduction in cellular cholesterol using U18666A had a similar effect; the number of caveolae was reduced and the level of free CAV1 increased in the plasma membrane.

Whereas these three conditions elevated free CAV1 in the plasma membrane, they also had another effect: They caused the accumulation of CAV1 in a LAMP1-positive compartment. We found, using a dual-tag strategy and live-cell imaging, that a large fraction of the CAV1 was, in fact, targeted into the acidic lumen of these vacuoles during progression of endosomal maturation. Although CAV1 was not normally observed in such large amounts in late endosomes and lysosomes there was reason to believe that this represented the normal pathway of CAV1 degradation.

(Figure 4.5 continued) while CAV1-mCherry fluorescence was stable. To rule out photo-bleaching of mEGFP, the total perinuclear mEGFP fluorescence was measured and plotted (blue dashed outline in (E) and intensity profile in (D)). Time-lapse series were recorded with epi-fluorescence illumination at 0.2 Hz. (F) Stills of the CAV1-mEGFP and CAV1-mCherry-positive endosome tracked in (D). Movie is available in supplementary data (Movie 4.2). Scale bars, (A-C) 10 µm, (E) 5 µm, (F) 0.5 µm.
4.3.2 The non-caveolar plasma membrane pool of CAV1

The free CAV1 in the plasma membrane was likely to originate in part from dissociation of caveolae destabilized by the lack of sufficient PTRF, cholesterol, or other critical caveolar factors. However, our results indicated that free CAV1 could also be delivered to the plasma membrane from the Golgi complex. This was particularly evident when cells were treated with U18666A to deplete the Golgi of cholesterol, an essential component during assembly of 70S caveolar assemblies from 8S subunits (Hayer et al., in preparation). In U18666A-treated cells, CAV1-mEGFP could be seen to leave the Golgi complex in elongated tubular carriers that, with regard to their morphology, resembled VSVG-loaded post-Golgi carriers (Tagawa et al., 2005). They were distinct from the small vesicles that carry CAV1 and GFP-GPI to the plasma membrane in untreated cells (Hayer et al., in preparation).

That CAV1 can be delivered to the plasma membrane by two different types of exocytic vesicles has previously been shown in polarized epithelial cells (MDCK). Interestingly, the CAV1 oligomers found in the apical, lipid-raft dependent pathway were larger than the oligomers present in basolateral exocytic vesicles (Scheiffele et al., 1998). Importantly, our observations suggested that non-assembled CAV1 can be transported from the Golgi complex to the cell surface.

Does non-caveolar CAV1 have functions in the plasma membrane? In neurons that express caveolins but lack caveolae (Head and Insel, 2007), CAV1 was shown to be involved in regulating synaptic transmission. The scaffolding domain of CAV1 (CSD) can interact with glutamate receptors and regulate 2-amino-5-hydroxy-3-methyl-4-isoxazole propionate (AMPA-) receptor activity by inhibiting phospholipase A2 (PLA2, Gaudreault et al., 2004). Functions of non-caveolar CAV1 have also been documented in non-neuronal cells. In focal adhesions, CAV1 has been proposed to localize to membrane domains distinct from caveolae (Gaus et al., 2006) and to promote, when tyrosine-phosphorylated and together with the galectin lattice, focal adhesion turnover (Goetz et al., 2008). Non-caveolar CAV1 was, moreover, shown to act as a membrane adaptor linking the integrin αv subunit to the tyrosine kinase Fyn (Wary et al., 1998).
4.3.3 CAV1 in the endo-lysosomal pathway

We found that CAV1 entered the endo-lysosomal pathway where it colocalized with the classical markers RAB5, RAB7 and LAMP1. During endosomal maturation, CAV1 became exposed to the acidic lumen of LE/LYS, consistent with its targeting to the lumen of multi-vesicular bodies and degradation in lysosomes.

Several studies have proposed that CAV1, which has a half life of 5-10 h (Conrad et al., 1995; Forbes et al., 2007), might be degraded in the endo/lysosomal pathway. Stimulation of Hep2G cells with albumin results in increased endocytosis of CAV1 and subsequent targeting to multi-vesicular bodies as seen in immuno-electron microscopy (Botos et al., 2008). Other reports showed that either siRNA-knockdown of flotillin-1 or treatment of cells with PDGF results in accelerated degradation of CAV1 that could be reversed by incubation of cells with inhibitors of lysosomal degradation (Peterson et al., 2003; Vassilieva et al., 2009).

How does CAV1 enter the endosomal/lysosomal pathway and how are caveolae disassembled for degradation? It seems likely that CAV1 can reach early endosomes by two different pathways. The first pathway involves caveolar endocytosis. Caveolar endocytic vesicles have been shown to associate with early endosomes, where they serve as stable scaffolds and from where they can recycle back to the plasma membrane (Pelkmans et al., 2004). The second pathway involves endocytosis of non-caveolar CAV1, as observed when caveolae assembly is saturated. Here, CAV1 is likely not involved in the formation of the endocytic vesicle but rather endocytosed as cargo by another mechanism. Whether this pathway involves clathrin remains unknown.

That progression of CAV1 from the RAB5- to the LAMP1 compartment was associated with a change in antigenicity, suggested that CAV1 underwent a conformational change; the caveolar domains may for example disassemble, in transit between these two compartments. Whether disassembly of stable caveolar domains takes place before or after targeting to intraluminal vesicles needs to be determined.
4.3.4 Caveosomes and caveolar endocytosis of SV40

Earlier studies from our laboratory proposed that during cellular entry Simian virus 40 (SV40) uses CAV1-rich intracellular organelles distinct from endosomes, so called caveosomes, as intermediate endocytic organelles during transit to the ER. Caveosomes were characterized by their neutral luminal pH, the presence of CAV1-GFP, and accumulation of SV40 during entry. Immunofluorescence showed that they did not contain markers of early endosomes (Pelkmans et al., 2001). More recent reports showed that productive entry of SV40 can occur independently of CAV-1, that it requires the function of RAB5 and RAB7, and that an acid-dependent step is involved (Damm et al., 2005; Engel et al., manuscript in preparation). Furthermore it was found that, consistent with transit through the endo/lysosomal pathway, the incoming SV40 particles co-localized with early- and late endosomal markers (Engel et al., manuscript in preparation).

Together with the data presented in this study, it seems likely that the caveosomes originally described were in fact late endosomes/lysosomes, in which over-expressed CAV1-EGFP had accumulated. The observation that over-expression of constitutively active RAB5 caused a redistribution of CAV1 from caveosomes to early endosomes is consistent with this model because the mutant likely prevented progression of cargo from early to LE/LYS by inducing homotypic fusion of early endosomes (Pelkmans et al., 2004; Stenmark et al., 1994).

We have shown here that EGFP-fluorescence was quenched in LE/LYS when CAV1-mEGFP was expressed at moderate levels (Figures 4.3D, 4.5). However, CAV1-mEGFP remained fluorescent in LE/LYS when cells were treated with U18666A, likely due to a rise of luminal pH due to cholesterol accumulation (Lafourcade et al., 2008). We also observed that CAV1-mEGFP remained fluorescent in LE/LYS when heavily overexpressed, suggesting that LE/LYS loading with CAV1-mEGFP caused an increase in luminal pH and perturbed LE/LYS function (our unpublished observations). Although the neutral pH of caveosomes was determined in virus-loaded organelles independent of CAV1-EGFP expression (Pelkmans et al., 2004; Pelkmans et al., 2001) it now seems unlikely that caveosomes represent organelles independent of LE/LYS. We propose that the term caveosome no longer be used.
4.3.5 Perspectives

In this study we have found that CAV1 enters the endo/lysosomal pathway, where it is likely targeted to intraluminal vesicles for degradation in lysosomes. Further studies should address what machinery is required for endocytosis of non-caveolar CAV1 from the plasma membrane and aim at identifying the mechanism by which CAV1 is targeted to intraluminal vesicles. The dual-tag strategy employed here may be useful in studies on intraluminal vesicle formation and endosomal maturation.

4.4 Materials and methods

Cell culture and transfections

CV1 cells (ATCC) were grown in DMEM (Invitrogen) supplemented with 10% FCS and 5% Glutamax (Invitrogen). CV1 cells stably expressing CAV1-mEGFP (CV1-CAV1-mEGFP) were generated using the parental cell line CV1-Flp-In (Invitrogen) and the plasmid CAV1-mEGFP/FRT/TO, following the manufacturer’s protocol for Flp-In recombination (Invitrogen). In a similar way, CV1-GFP-GPI cells were generated, using the plasmid GFP-GPI/FRT/TO. Both recombinant cell lines were cultivated as CV1 cells but in presence of 150 µg/ml hygromycin. CV1-CAV1-mEGFP-tTR cells were generated by stably transfecting CV1-CAV1-mEGFP cells with a vector encoding HA-tagged tet-repressor (tTR-HA). CV1-CAV1-mEGFP-tTR cells were grown as CV1 cells but in presence of 40 µg/ml hygromycin and 0.5 µg/ml puromycin. Expression of CAV1-mEGFP was induced by adding of 0.5 µg/ml doxycyclin to the culture media. Cells were transfected by electroporation (AMAXA) according to manufacturer’s recommendation (Kit V, program A24).

Plasmid constructs

CAV1-mEGFP refers to canine CAV1 tagged by monomeric EGFP, a version of EGFP with the dimer-interface destroyed by L221K mutation (Zacharias et al., 2002). CAV1-mEGFP was obtained by swapping of EGFP against mEGFP (from pmEGFP-N1, Jennifer Lippincott-Schwartz) as BamHI/NotI fragments in CAV1-
EGFP (Pelkmans et al., 2001). To generate **CAV1-mEGFP/FRT/TO**, the coding sequence for CAV1-mEGFP was excised from the plasmid CAV1-mEGFP as HindIII/NotI fragment and ligated into appropriately digested pcDNA5/FRT/TO (Invitrogen). For **CAV1-mCherry**, mEGFP in CAV1-mEGFP was replaced by mCherry as AgeI/BsrGI fragment, as obtained by PCR from pRSET-B-mCherry (from Roger Tsien) using primers sense 5’-ATAAACCGGTGACCGACCATGGTGAGCAAGGGCGAG and antisense 5’-AATATGTACAGCTCGTCCATGCCG. **GFP-GPI/FRT/TO** was generated by subcloning GFP-GPI from EGFP-GL-GPI (Keller et al., 2001) into pcDNA5/FRT/TO (Invitrogen) as HindIII/NotI fragment. HA-tagged tet-repressor (**tTR-HA**) was constructed by first PCR amplifying the tet-repressor from pLVtTrkrabRed (Didier Trono) using primers sense 5’-ACGAATTCATGGCTAGATTAGATAAAAGTAAAGTGATT and antisense 5’-GTGCGGCCGCTAGCTAGCGTAGTCTGGGACGTCGTATGGGTACGACCCACTTTCACATTTAAGTTG, introducing a C-terminal HA-tag. The resulting PCR product was ligated as EcoRI/NotI fragment into appropriately digested pIRESpuro (Clontech).

**Antibodies and other reagents**

Rabbit pAb anti-CAV1 (N20) was from Santa Cruz (sc-894), mouse mAb anti-CAV1 (clone 2297) from BD Biosciences (610407), rabbit pAb anti-PTRF from Abcam (ab48824), rabbit pAb anti-Giantin from Covance (PRB-114C), mAb anti-HA from Covance (MMS-101P), and mouse mAb anti-LAMP1 from Santa Cruz (sc-20011). Alexa Fluor-conjugated secondary antibodies for immunofluorescence were from Invitrogen. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

**Analysis of primary CAV1 oligomers**

2x10⁶ CV1 cells, either untreated or treated with 5 µg/ml U18666A for 16 h were scraped into PBS, pelleted and lysed at room temperature for 20 min in 300 µL 0.2% Triton X-100 in TNE (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA), supplemented with “Complete” protease inhibitors (Roche). Post nuclear supernatants (PNS) were prepared by a 5 min centrifugation at 1100 g. SDS
sample buffer containing final 0.1% SDS was added and samples were not boiled, as this preserves oligomeric CAV1 (Monier et al., 1995; Sargiacomo et al., 1995; Scheiffele et al., 1998). Samples were resolved by 4-12% gradient SDS-PAGE, followed by Western blot and detection using anti-CAV1(N20).

**Immunofluorescence imaging**

Cells grown on coverslips were fixed using either 4% formaldehyde in PBS or 4% formaldehyde in 25 mM Pipes pH 6.9, 1 mM EGTA, 1 mM MgCl₂, 15% glycerol to better preserve endosomal structures. Cells were permeabilized using 0.05% Saponin, 1% BSA in PBS, incubated with appropriate primary (1:500) and secondary (1:1000) antibodies and coverslips mounted using Immunomount (Thermo Shandon). Imaging was performed on a Zeiss LSM 510 meta system using a 100× 1.4 NA objective.

**FRAP**

For FRAP experiments either CV1-CAV1-mEGFP cells, untreated or treated with U18666A (5µg/ml, 16h), or CV1-GFP-GPI cells were used. Coverslips with cells were transferred to a custom-built metal microscope coverslip chamber in CO₂-independent medium (Invitrogen), supplemented with 10% FCS. FRAP analyses were performed at 37°C on an inverted Zeiss LSM 510 meta confocal microscope system, equipped with a temperature-controlled stage and a 63× 1.4 NA objective. A defined region of interest (ROI, 4 × 4 µm) was bleached using the 488 nm line of a 30 mW Argon laser at high laser intensity (100% power, 100% transmission, 30 iterations) and fluorescence recovery was recorded by scanning at low laser intensity (100% power, 10% transmission). Images were acquired as 12 bit LSM files at 512 × 512 pixels/frame and 0.14-µm/pixel lateral resolution. Image series with little or no apparent motion of cells were imported into ImageJ (http://rsb.info.nih.gov/ij/) and automatically aligned using the TurboReg plugin (http://bigwww.epfl.ch/thevenaz/turboreg/). The average fluorescence intensity of the ROI was determined after background subtraction and normalization according to (Phair and Misteli, 2000). Half-times of recovery (t₁/₂) and mobile fraction (M₀) were derived from hyperbolic fits of recovery curves.
Live-cell fluorescence imaging

CV1 cells expressing fluorescently tagged constructs and seeded on 18 mm coverslips were transferred to a custom-built metal microscope coverslip chamber in CO$_2$-independent medium (Invitrogen), supplemented with 10% FCS. TIRF and epifluorescence time-lapse imaging was performed on an Olympus IX71 microscope equipped with a TILL.IMAGO QE camera (TILL Photonics), TILL TIR/EPI dual-condenser (TILL Photonics), an Argon-Krypton laser (Spectra Physics), Acousto-Optic Tunable Filters (Opto-electronic), a monochromatic light-source Polychrome IV (TILL Photonics), a Dual View beamsplitter (Optical Insights) in the detection lightpath, a 60× 1.45 NA objective, a temperature-controlled incubation chamber, and using Metamorph (Molecular Devices). For TIR illumination of CAV1-mEGFP, the 488nm laser line was used and the depth of the evanescent field was adjusted such that both ventral cell surface and parts of the Golgi complex were illuminated. For epi-illumination the monochromator was used at 488 nm and 568 nm and a beamsplitter was used in the detection lightpath to avoid crosstalk between mEGFP and mCherry signals. Confocal live-cell imaging was performed on a Zeiss LSM510 meta system (Zeiss) equipped with a 100× 1.4 NA objective and a temperature-controlled stage.

4.5 Acknowledgements

We thank for generous gifts of plasmids - Jean Gruenberg for EGFP-Rab7 and LAMP1-EGFP, Marino Zerial for EGFP-Rab5, Jennifer Lippincott-Schwartz for pmEGFP-N1, Kai Simons for EGFP-GL-GPI, Roger Tsien for pRSET-mCherry, and Didier Trono for pLVTTRkrabRed. We are grateful to Fabian Herzog for preparing the cell line CV1-GFP-GPI.
4.6 Movies

Movie 4.1 | **Surface-delivery of CAV1-mEGFP in U18666A-treated cells.** CV1 cells treated with U18666A (5 µM, 16 h) were transfected with CAV1-mEGFP using electroporation (AMAXA) and incubated in presence of CHX during cell attachment (2 h). Expression of CAV1-mEGFP was induced by washout of CHX and cells were imaged on a TIR-FM setup 60 min post washout. The evanescent field was adjusted such that both cell surface and partly the Golgi were visible. CAV1-mEGFP can be observed in tubular post-Golgi carriers. Upon arrival at the cell surface they release their cargo, visible as burst of fluorescence diffusing laterally into the plasma membrane. Time-lapse sequences were acquired at 1 Hz, with integration time of 500 ms per frame. Displayed at 15x real time.

Movie 4.2 | **Targeting of CAV1 to the acidic lumen of endosomes.** CV1 cells coexpressing CAV1-mEGFP and CAV1-mCherry (12 h) were imaged on an epi-fluorescence setup equipped with a dual-view beamsplitter at 0.2 Hz. The endosome marked by a circle is both red and green initially, but loses green fluorescence as the endosome progresses towards the perinuclear area. Since mEGFP fluorescence is sensitive towards acidic pH, the loss of mEGFP fluorescence is consistent with targeting of CAV1 to the acidic lumen of LE/lys (see also Figure 4.4). Displayed at 50x real time.

Movie 4.3 | **CAV1-mCherry in LE/lys cannot be fixed by formaldehyde.** CV1 cells expressing CAV1-mCherry were mounted in a live-cell chamber, formaldehyde was added to final 4% at t=0 s, and time-lapse imaging was performed on an epi-flourescence microscope at 0.2 Hz. Note the disappearance of numerous CAV1-mCherry-positive organelles within 6 min of fixation. Displayed at 15x real time.
Supplementary Figure 4.1

A  Induction of CAV1-mEGFP expression by addition of doxycyclin

(B) Effect of U18666A on cellular distribution of endogenous CAV1

Supplementary figure 4.1 | (A) Induction of CAV1-mEGFP expression by doxycyclin. Expression of CAV1-mEGFP in CV1-CAV1-mEGFP-tTR cells was induced by addition of 5µM doxycyclin. Cells were fixed at indicated time points and imaged by epi-fluorescence microscopy. (B) Effect of U18666A on endogenous CAV1 in CV1 cells. CV1 cells were incubated in presence of 5 µM U18666A for 16 h or left untreated, fixed and stained with anti-CAV1(N20) and anti-LAMP1. Confocal fluorescence microscopy showed that the drug caused a dispersed distribution of CAV1 at the cell surface. Scale bars, 10 µm.
Supplementary Figure 4.2

A siRNA-knockdown of PTRF causes targeting of CAV1-mEGFP to LE/lys.

Supplementary figure 4.2 | (A) siRNA-knockdown of PTRF causes targeting of CAV1-mEGFP to LE/lys. CV1-CAV1-mEGFP cells were transfected with either control siRNA (AllStarsNeg, Qiagen) or siRNA targeting PTRF (oligo # 6, Qiagen) at 20 nM using Lipofectamine RNAiMAX following manufacturer’s recommendation (Invitrogen). 72 h post transfection, cells were fixed and processed for immunofluorescence staining (anti-LAMP1). In PTRF-depleted cells, CAV1-mEGFP localized to a non-caveolar pool at the cell surface and was targeted to LE/lys. Scale bars, 10 µm.

B Release of CAV1-mCherry from LE/lys upon addition of formaldehyde

Supplementary figure 4.2 | (B) CV1 cells expressing CAV1-mCherry growing on coverslips were mounted in a live-cell chamber, formaldehyde was added to final 4% at t=0 min, and time-lapse imaging was performed on an epi-fluorescence microscope. Note the disappearance of numerous CAV1-mCherry-positive organelles within 6 min of fixation. Scale bar, 5 µm.
Chapter 5
Assembly and biosynthetic trafficking of flotillin-microdomains

Arnold Hayer and Ari Helenius
5.1 Introduction

Flotillin-1 and -2 are ubiquitously expressed, highly conserved integral membrane proteins belonging to the SPFH (stomatin/prohibitin/flotillin/HflK/C) domain protein family. In mammalian cells, flotillin-1 and -2 coassemble to form microdomains at the plasma membrane and they may induce membrane curvature (Frick et al., 2007; Solis et al., 2007). Flotillin-1 and flotillin-2, (also called reggie-2 and reggie-1, respectively) are, as caveolins, associated with detergent-resistant membranes (Bickel et al., 1997), however, microdomains formed by flotillins are distinct from caveolae (Langhorst et al., 2005).

Flotillins have been implicated in various cellular processes including axon regeneration (Schulte et al., 1997), signaling (Neumann-Giesen et al., 2007), secretion of morphogen in Drosophila (Katanaev et al., 2008) and cell adhesion (Langhorst et al., 2008a; Langhorst et al., 2007). Flotillins were furthermore shown to be involved in a clathrin-, caveolin-, and dynamin-independent endocytic pathway in mammalian cells for uptake of GPI-anchored proteins and glycosphingolipids (Frick et al., 2007; Glebov et al., 2006; Schneider et al., 2008). Endocytosis of flotillin-microdomains requires Fyn kinase and can be stimulated by EGF (Neumann-Giesen et al., 2007; Riento et al., 2009).

Flotillins have been suggested to assume a hairpin-membrane topology similar to caveolins and share other structural and functional properties with caveolins (Bauer and Pelkmans, 2006; Langhorst et al., 2005). In flotillin-microdomains, flotillin-1 and -2 are associated in homo- and heterotetrameric complexes that are stabilized by their C-terminal domains (Solis et al., 2007). They are moreover fatty acylated, (Morrow et al., 2002; Neumann-Giesen et al., 2004) and form microdomains ≤ 100 nm in size at the plasma membrane (Stuermer et al., 2001).

Biosynthetic trafficking of flotillins is controversial. Flotillin-1 was shown to pass through the Golgi complex in a variety of cell types (Gkantiragas et al., 2001). Consistent with a Golgi-dependent step during assembly and trafficking, mutants of flotillin-2 were shown to accumulate in the Golgi and surface targeting of flotillin-2 was BFA-sensitive (Langhorst et al., 2008b). However, a Sar1-independent, Golgi-independent pathway for delivery to the cell surface has been
Assembly and biosynthetic trafficking of flotillin-microdomains

proposed for flotillin-1 (Morrow et al., 2002). Assembly of caveolae and surface targeting of caveolin-1 (CAV1), the major structural component of caveolae, is strictly dependent on a functioning Golgi complex, as CAV1 is trapped in the ER and redirected to lipid-droplets in BFA-treated cells (Ostermeyer et al., 2001).

In this study, we set out to readdress the role of the Golgi complex in assembly and trafficking of flotillins. By comparing flotillins with CAV1 during biosynthetic trafficking, we found that flotillins can assemble into microdomains at the cell surface in a BFA-insensitive manner, independent of the Golgi complex. However, in presence of a functioning Golgi, flotillins associated with the Golgi and traveled in post-Golgi vesicles to the cell surface. When ER-to Golgi transport was disrupted by a selective inhibitor of COPII assembly, flotillins, but not CAV1 associated with the Golgi, suggesting that flotillins can enter the secretory pathway at the level of the Golgi. Finally, cell fusion experiments, where cells expressing either EGFP-tagged or mCherry-tagged flotillins were fused, revealed that flotillin microdomains were remarkably stable once assembled, with no detectable exchange of subunits between domains for many hours.

5.2 Results

5.2.1 Golgi-dependent and -independent biosynthetic trafficking of flotillins

We investigated flotillin-microdomain formation by expressing EGFP-tagged versions of flotillins in CV1 cells followed by fluorescence microscopy, where flotillin-microdomains appear as distinct subresolution punctae at the cell surface (Glebov et al., 2006; Stuermer et al., 2001). Since both flotillin-1 and flotillin-2 are required for efficient microdomain formation at the cell surface (Frick et al., 2007), we coexpressed EGFP-tagged flotillin-1 and flotillin-2 in all our experiments (F1/F2-EGFP).

We first addressed whether flotillins and the caveolar marker caveolin-1 (CAV1) formed independent domains at the plasma membrane when expressed together. CV1 cells were cotransfected with F1/F2-EGFP and CAV1-mCherry and
allowed to express the fluorescently-tagged proteins for 2 h before they were fixed and analyzed by confocal microscopy. As shown in Figure 5.1A, caveolin- and flotillin-positive domains had formed distinct foci distributed over the cell surface with no significant overlap between them. We also noted that the flotillin-domains generally displayed a lower fluorescent intensity and were more abundant than CAV1-domains. Identical results were obtained when mCherry-tagged flotillins and mEGFP-tagged CAV1 were coexpressed (not shown).

How do flotillins and caveolins differ in terms of biosynthetic trafficking and where after their synthesis do they get separated? We addressed this by making use of our established system to study biosynthetic trafficking of newly synthesized membrane proteins (Hayer et al., in preparation). We cotransfected CV1 cells with F1/F2-GFP and CAV1-mCherry using electroporation and incubated the cells in presence of protein synthesis inhibitor (cycloheximide, CHX) until they had attached, washed out CHX and followed the emerging newly synthesized proteins by fixing samples after various time-points and subsequent analysis by confocal microscopy. 30 min after CHX washout both CAV1 and F1/F2 localized almost exclusively to the Golgi complex, after 60 min and 120 min CAV1 and F1/F2-positive domains had appeared at the cell surface (Figure 5.1B). Timing and type of transport appeared similar for CAV1 and F1/F2. These results suggested that F1/F2 passed through the Golgi complex during biosynthetic transport, similar to CAV1.

Caveolae assembly and surface targeting of CAV1 depend on a functional Golgi complex, as CAV1 accumulates in the ER in presence of BFA (Ostermeyer et al.,

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Figure 5.1 | Cellular distribution and biosynthetic trafficking of flotillins. Cells expressing GFP-tagged flotillin 1 and flotillin 2 (F1/F2-GFP) display a punctuate pattern at the cell surface. (A) CV1 cells coexpressing F1/F2-GFP and CAV1-mCherry were fixed 2h post CHX washout. There was no detectable overlap between flotillin- and caveolar domains. Single confocal slices are shown (LSM). (B) CV1 cells were cotransfected with F1/F2-GFP and CAV1-mCherry and fixed 30 min, 60 min and 120 min post CHX washout to monitor biosynthetic trafficking of the proteins. Both F1/F2-GFP and CAV1-mCherry accumulated in the Golgi complex before distinct domains appeared at the cell surface. Shown are z-projections of confocal slices acquired on a spinning disc confocal system. Scale bars, 10 µm.
Figure 5.2 | **Surface targeting of flotillins is BFA insensitive.** (A) CV1 cells were cotransfected with F1/F2-GFP and CAV1-mCherry and incubated in presence of CHX until they had firmly attached to the coverslip. BFA (5 µg/ml) was added after CHX washout and cells were fixed 2 h later. F1/F2-GFP transport to the cell surface remained unaffected whereas CAV1-mCherry accumulated in the ER. **(B)** In control cells, both F1/F2-GFP and CAV1-mCherry reached the cell surface. Images are single confocal slices (LSM). Scale bars, 10 µm.
To test whether transport of F1/F2 was dependent on Golgi function as well we cotransfected CV1 cells with F1/F2-EGFP and CAV1-mCherry and expressed the proteins in presence of BFA for 2 h. BFA treatment caused a complete retention of CAV1-mCherry in the ER, as seen by its localization to a reticular pattern and the lack of surface spots (Figure 5.2A). However, surface targeting of F1/F2-EGFP was unaffected and microdomains had formed in the absence of a functioning Golgi complex (Figure 5.2A). In control cells, CAV1-mCherry and F1/F2 both had reached the plasma membrane and formed independent domains (Figure 5.2B).

Taken together, F1/F2 and CAV1 appeared to follow similar biosynthetic trafficking routes when a functioning Golgi was present, from soon after their synthesis until they appeared as distinct domains at the plasma membrane. However, in the absence of a functioning Golgi, flotillins could still reach the plasma membrane, suggesting a non-classical mechanism of membrane association in the secretory pathway.

5.2.2 COPII-independent association of flotillins with the Golgi complex

The above results prompted us to investigate the mode of how flotillins and CAV1 enter the secretory pathway.

Association of CAV1 with microsomal membranes in vitro was shown to occur co-translationally and to be SRP-dependent, suggesting that CAV1 uses the classical translocon machinery for membrane association (Monier et al., 1995). To visualize very early steps of biosynthetic trafficking of CAV1, we expressed CAV1 only for short times and visualized its distribution in cells. As early as 7 min post CHX washout, we found CAV1 to localize to vesicular structures throughout the cytoplasm and to the Golgi complex (Figure 5.3A). The vesicular structures were positive for Sec31A, a marker for ER exit sites, consistent with COPII-dependent export of CAV1 from the ER to the Golgi (Figure 5.3B). These findings suggested a highly efficient export from the ER exit sites and transport to the Golgi complex. That CAV1 was localized to Golgi complex only a few minutes after CAV1
CHAPTER 5

A

7 min

αGiantin              CAV1-HA            merge     αSec31            CAV1-mEGFP    merge

B

30 min

αGiantin              CAV1-HA         merge     αSec31            CAV1-mEGFP   merge

C

control, 45 min

D

CI-976, 45 min

E

control, 60 min

F

CI-976, 60 min
expression had begun could also be due to direct access of CAV1 to the Golgi complex.

To distinguish between these two possibilities, we used the compound CI-976, an inhibitor of COPII-mediated ER-Golgi transport (Brown et al., 2008). Importantly, brief treatments of cells with CI-976 efficiently inhibit COPII-dependent ER-Golgi transport without apparently affecting the Golgi complex (Brown et al., 2008). When CI-976 was added immediately after induction of CAV1-HA expression, CAV1-HA did not reach the Golgi complex marked by Giantin within 45 min (Figure 5.3C). Instead, CAV1-HA localized to vesicular structures distributed throughout the cytoplasm, likely ER exit sites (Figure 5.3D). Together, these findings demonstrated that CAV1 enters the secretory pathway at the level of the ER and requires COPII machinery to leave the ER and reach the Golgi complex.

We next investigated whether flotillins also required COPII transport to access the Golgi complex. To directly compare between trafficking of flotillins and of CAV1, we cotransfected CV1 cells with F1/F2-EGFP and CAV1-mCherry. When expression was induced by CHX washout, cells were either left untreated or incubated in presence of CI-976. In untreated cells, fixed at 60 min post CHX

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Figure 5.3 | Flotillins can access the Golgi complex independently of COPII transport. (A) CV1 cells were transfected with CAV1-HA and fixed 7 min after expression of CAV1-HA was induced. CAV1 localized to punctuate vesicular structures and to the Golgi complex as identified by Giantin staining. (B) In a similar experiment, CV1 cells were transfected with CAV1-mEGFP and fixed 30 min post CHX washout. Antibody staining with anti-Sec31, a COPII marker, revealed localization of CAV1-mEGFP to ER exit sites. (C) and (D) inhibition of COPII transport by the compound CI-976 blocked ER exit of CAV1. CAV1-mEGFP was transfected as above and CI-976 (50 µM) was added upon CHX washout. Cells were fixed 60 min later and stained with anti-Giantin. In control cells, CAV1-mEGFP colocalized with the Golgi marker Giantin, whereas in CI-976 treated cells, CAV1 remained trapped in the ER. Note that the Golgi morphology was not apparently affected by CI-976. (E) and (F) flotillins, but not CAV1 can access the Golgi in presence of CI-976. CV1 cells were cotransfected with F1F2-EGFP and CAV1-mCherry. Upon washout of CHX, CI-976 (50 µM) was added or cells were left untreated (control). Cells were fixed after 60 min and processed for fluorescence imaging. In control cells, F1/F2-EGFP colocalized with CAV1-mCherry in the Golgi. However, in CI-976 treated cells, F1/F2-EGFP, but not CAV1, localized to the Golgi. All images are single confocal slices (C-LSM). Scale bars, 10 µm.
washout, F1/F2-EGFP and CAV1-mCherry colocalized in the Golgi complex (Figure 5.3E). In CI-976-treated cells, flotillins localized the Golgi, while CAV1 was retained in vesicular structures as observed before (Figure 5.3F). Flotillins may therefore enter the secretory pathway at the level of the Golgi complex.

5.2.3 Flotillin-microdomains as stable units at the plasma membrane

We have previously shown using live-cell imaging of CV1 cells expressing CAV1-EGFP that Golgi-derived, CAV1-positive vesicles remained as stable caveolar units after arrival at the plasma membrane. We thus concluded that caveolar domains assembled in the Golgi complex (Tagawa et al., 2005). At the plasma membrane, caveolar units remained stably assembled for many hours indicating that once assembled, they do not undergo cycles of assembly and disassembly (Tagawa et al., 2005).

To address whether Golgi-derived, flotillin-positive vesicles equally represent stable structures, we performed live-cell imaging of cells expressing F1/F2-EGFP. When time-lapse sequences were acquired 40-60 min after expression was induced, we observed numerous flotillin-positive domains budding from the Golgi and moving to the cell periphery (Movie 5.1). The vesicles followed curvilinear tracks consistent with microtubule-dependent transport. Importantly, as these domains arrived at the plasma membrane, they did not diffuse apart, but instead remained as stable units, suggesting that they had assembled into stable structures in the Golgi complex (Figure 5.4A, B, Movie 5.1).

We next investigated the stability of flotillin-microdomains at the plasma membrane by a cell fusion approach that we have previously developed to demonstrate the stability of the caveolar coat (Tagawa et al., 2005). We transfected two pools of CV1 cells with either GFP- or mCherry- tagged F1/F2, mixed the two pools and seeded them together on glass coverslips. After cells had attached we induced cell fusion using UV-inactivated Semliki Forest virus (SFV) by brief acidification of the medium. Immediately post cell fusion, CHX was added to prevent further protein synthesis and cells were analyzed by confocal microscopy at several time-points. In case of rapid assembly and disassembly of flotillin-
positive domains, red and green domains would disappear and yellow domains would form through mixing of subunits over time.

Our first observation was that red and green F1/F2 domains crossed the former boundaries between the fused cells only slowly (Figure 5.4C). In many cells the boundary was clearly visible up to 3 h, indicating that F1/F2 domains display a relatively immobile behavior at steady-state. The second observation was that individual F1/F2-GFP and –mCherry labeled foci remained either green or red, whether they had crossed the boundary or not. This indicated that once formed F1/F2 domains were stable for at least several hours with no detectable exchange of flotillin-subunits between them. Importantly, we found that when F1/2-GFP and F1/F2–mCherry were coexpressed in the same cells, the color of flotillin-domains was nearly uniformly yellow (Figure 5.4D).

Our results strongly suggest that surface domains defined by flotillins exhibit similar stability as caveolar domains in that once formed they retain structural stability over a long period of time.

5.3 Discussion

5.3.1 Summary

In this study, we compared biosynthetic trafficking of flotillins and caveoliln-1 to investigate assembly of flotillins into microdomains that are found on the surface of many cell types. By coexpressing F1/F2-EGFP with CAV1-mCherry, we first confirmed that CAV1 and flotillins formed independent domains at the plasma membrane. We next found that although flotillin-microdomains could form at the surface of BFA-treated cells, flotillins seemed to transit through the Golgi during biosynthetic trafficking in unperturbed cells. Using a specific inhibitor of COPII transport we found that CAV1, but not flotillins required COPII machinery to reach the Golgi complex, suggesting that flotillins can directly access the Golgi complex. Live-cell imaging of F1/F2-EGFP expressing cells revealed that Golgi-derived F1/F2-EGFP-positive domains represented stable units that remained assembled after arriving at the plasma membrane. Finally, by
CHAPTER 5

A

F1/F2-EGFP

B

F1/F2-EGFP

maximum projection

C

F1/F2-mCherry

F1/F2-EGFP

merge

D

F1/F2-mCherry

F1/F2-EGFP

merge
fusing cells that expressed either F1/F2-EGFP or F1/F2-mCherry, we found that flotillin microdomains represent remarkably stable structures that remain stably assembled at the plasma membrane for several hours.

5.3.2 Assembly of flotillin-microdomains

Assembly of caveolae requires the transit of CAV1, the major structural protein of caveolae, through the secretory pathway. Maturation of CAV1 into domains involves a first homo-oligomerization step of CAV1 into SDS-stable complexes that occurs soon after synthesis in the ER (Scheiffele et al., 1998). In the Golgi, CAV1 undergoes a second round of oligomerization that requires cholesterol and that is characterized by loss of diffusional mobility of CAV1, as observed in fluorescence recovery after photobleaching (FRAP) experiments (Hayer et al., in preparation). Golgi-derived caveolar exocytic carriers then reach the plasma membrane, where they remain stably assembled for several hours (Hayer et al., in preparation; Tagawa et al., 2005).

Flotillin-1 and Flotillin-2 associate into homo- and heterotetramers that are resistant to high concentration of urea (Solis et al., 2007). Whether this oligomerization requires membrane association is unknown. The number of

Figure 5.4 | Flotillin-domains are stable and do not exchange subunits. (A) and (B) live cell imaging of F1/F2-EGFP-expressing CV1 cells by TIR-FM showed that numerous vesicles budded off the Golgi and traveled to the cell periphery where they fused with the plasma membrane (Movie 5.1). The angle of the incident laser beam was adjusted such that surface and partly the Golgi were illuminated. Time-lapse sequences were acquired at 1 Hz with 500 ms integration time per frame. (A) A single frame of the image sequence presented in Movie 5.1 is shown. For (B), 100 consecutive frames were each subtracted from each other to yield an image sequence of only moving objects. A maximum projection was then generated to show tracks of moving objects, indicated by arrowheads. (C) To test the stability of flotillin-microdomains at the cell surface, cells expressing F1/F2-GFP were fused with cells expressing F1/F2-mCherry using UV-inactivated Semliki Forest virus (SFV). Following fusion, cells were incubated in the presence of CHX for 3 h and then fixed. Surface associated F1/F2 domains remained either red or green and crossed the former fusion boarder only slowly, indicating immobility and stability of flotillin-domains. (D) In control cells coexpressing F1/F2-GFP and F1/F2-mCherry most surface domains were uniformly stained with both F1/F2-GFP and F1/F2-mCherry. Scale bars, 10 µm.
F1/F2 molecules in flotillin-microdomains was estimated to be around 175, requiring in analogy to caveolae formation a second oligomerization step (Frick et al., 2007). Our results suggest that in unperturbed cells domain assembly occurs in the Golgi complex. However, the Golgi complex seemed dispensable as flotillin-microdomains could also form at the plasma membrane of BFA-treated cells.

The role of cholesterol and other lipids in flotillin-domain assembly and stability is unclear. However, there are indications that cholesterol is important for flotillin-microdomain structure and function. Cholesterol extraction by methyl-β-cyclodextrin was shown to shift flotillin-1 from low- to high density fractions in sucrose density gradients (Westover et al., 2003). Also, trafficking of flotillin-microdomains is stimulated by cholesterol-loading and inhibited by cholesterol depletion (Langhorst et al., 2008b).

A major difference between CAV1 and flotillins during biosynthetic trafficking was that flotillins seemed to directly associate with Golgi membranes, without prior membrane insertion in the ER. Golgi-independent plasma-membrane association of flotillin-1 is known to require the amino-terminal prohibitin (PHB-) domain and palmitoylation at Cys34, a residue that is highly conserved among PHB-containing proteins (Morrow et al., 2002). Whether the same features of flotillins are required for their association with Golgi membranes remains to be shown.

5.3.3 Stability of flotillin-microdomains

We have previously shown that caveolar domains are remarkably stable structures at the cell surface that, once assembled, represent stable structures that do not exchange CAV1 protein between each other or with non-caveolar pools (Tagawa et al., 2005). Fusion of CV1 cells expressing either F1/F2-EGFP or F1/F2-mCherry revealed that flotillin-microdomains are also remarkably stable structures at the surface of cells. That they may also remain stable during flotillin-domain mediated trafficking is supported by live cell experiments monitoring kiss-and-run-dynamics of flotillin-domains. Lipid-dye loaded, flotillin-positive vesicles were observed to release dye upon fusion with the plasma membrane,
while the associated flotillin-domain remained as stable unit (Langhorst et al., 2008b). We propose the flotillin mediated transport follows similar functional principles as caveolar transport.

5.3.4 Conclusion

Our study provides evidence that assemblies of CAV1 and flotillins both represent scaffolds that stabilize lipid-raft domains at the plasma membrane and during membrane transport. Likely, the emerging concept is not limited to caveolae and flotillin-microdomains, but applicable to additional, yet unknown, cell-type and tissue specific scaffolds regulating signaling at the plasma membrane and membrane transport.

5.4 Materials and methods

Cell culture and transfections

CV1 cells (african green monkey kidney cells, ATCC) were grown in DMEM (Invitrogen) supplemented with 10% FCS and 5% Glutamax (Invitrogen). Cells were transfected by electroporation, following manufacturer’s instructions (AMAXA) and using kit V, program A-24. For time-course and live-cell experiments, transfected cells in suspension were plated in full media containing 1 mM cycloheximide (CHX) until cells had firmly attached to the coverslips (2h). Expression was then induced by washout of CHX. Flotillin-1 and flotillin-2 were coexpressed in order to induce efficient formation of flotillin microdomains at the plasma membrane (Frick et al., 2007).

Antibodies and other reagents

Rabbit pAb anti-Giantin was from Covance (PRB-114C), mAb anti-HA from Covance (MMS-101P), and mAb anti-Sec31A from BD Biosciences (612350). AlexaFluor-labeled secondary antibodies were from Invitrogen. BFA and CI-976 were purchased from Sigma.
DNA constructs

CAV1-mCherry, CAV1-mEGFP, and CAV1-HA have been described (Hayer et al., in preparation). C-terminally EGFP-tagged versions of human flotillin-1 and flotillin-2 in pEGFP-N vectors (F1-EGFP, F2-EGFP) were generous gifts from Claudia Stürmer (University of Konstanz). F1-mCherry and F2-mCherry were generated based on F1-EGFP and F2-EGFP by swapping EGFP against mCherry as AgeI/BsrGI fragments.

Cell fusion

For cell fusion, 10^6 CV1 cells were transfected in separate cuvettes with either 1 µg of F1-EGFP + F2-EGFP or F1-mCherry + F2-mCherry using electroporation and plated together. After cells had attached (4-6 h) fusion was induced using UV-inactivated Semliki Forest virus (SFV) as previously described (White et al., 1981). In brief, SFV was UV inactivated for 3 min in a laminar flow hood, and 7 x 10^7 particles in 20 µl were bound to 2 x 10^5 cells on 18 mm coverslips on ice for 1 h. Cell fusion was induced at 37°C by a transient (1 min) change of pH from 6.8 to 5.0 in DME-medium containing 10 mM MES, 10 mM HEPES, and 0.2% BSA. SFV itself did not induce any detectable change in flotillin distribution and/or behavior.

Immunofluorescence imaging

Cells grown on coverslips were fixed in 4% formaldehyde/PBS, permeabilized by 0.05% Saponin, 1% BSA in PBS, incubated with appropriate primary and secondary antibodies and coverslips mounted using Immumount (Thermo Shandon). Imaging was performed on a Zeiss LSM 510 Meta system using a 100× 1.4 NA objective. Alternatively, images were acquired using a spinning disc confocal microscope (Axiovert 200M, Zeiss), equipped with a QLC100 spinning disc confocal scanning system (VisiTech), a 100× 1.4 NA Objective, an ORCA ER camera (Hamamatsu), and Orbit AOTF and controllers (Improvision).

Live-cell imaging

Time-lapse series were acquired on an Olympus IX71 microscope equipped with a TILL IMAGO QE camera (TILL Photonics), a TILL TIR condenser (TILL Photonics), an Argon-Krypton laser (Spectra Physics, Acousto-Optic Tunable Filters (AOTF, Opto-electronic), and a 60× 1.45 NA objective, a temperature-
controlled incubation chamber, and using Metamorph (Molecular Devices). The depth of the evanescent field was adjusted such that both ventral cell surface and parts of the Golgi complex were illuminated. Image series were acquired at 1 Hz.

5.5 Acknowledgements

We thank Claudia Stürmer (University of Konstanz) for generously providing flotillin constructs.

5.6 Movie

Movie 5.1 | Post-Golgi trafficking of flotillins. CV1 were transfected with F1/F2-EGFP and imaged 45 min after expression was induced (see also Figure 5.4). The image sequence was acquired on a TIRF microscope with the evanescent field adjusted such that both cell surface and partly the Golgi were illuminated. Numerous vesicles positive for F1/F2-EGFP are observed budding from the Golgi and moving towards the cell periphery. Time-lapse sequence was acquired at 1 Hz with 400 ms integration time per frame. Displayed at 10x real-time.
Chapter 6

General discussion
6.1 Outline

In this thesis, assembly and disassembly of caveolae were studied using a variety of biochemical and cell biological approaches. The initial motivation for this project was the observation that the caveolar coat is remarkably stable and thus its mode of function in membrane transport fundamentally different from other known coated vesicle transport mechanisms (Tagawa et al., 2005). The stability of the caveolar coat raised the question as to where and how the coat initially assembles, how it disassembles, and how it is degraded. This chapter highlights the key findings and integrates them into existing models of CAV1 trafficking and caveolae assembly (Figure 6.1). Furthermore, a model for disassembly and degradation of caveolae is presented (Figure 6.2) and future challenges in caveolae research are discussed.

6.2 Assembly of caveolae

Association of CAV1 with the ER

Assembly of caveolae is linked to biosynthetic trafficking of CAV1 and begins with the association of CAV1 with the ER-membrane. CAV1 does not encode a cleavable amino-terminal signal sequence but is thought to insert into membranes by a central hydrophobic stretch. The mechanism of CAV1 membrane association was studied by in vitro transcription/translation in presence of microsomes. CAV1 is cotranslationally inserted into microsomes and requires signal-recognition particle (SRP) and Sec61, components of the classical translocon machinery (Monier et al., 1995). How CAV1 then assumes the hairpin-topology with both amino- and carboxy-termini in the cytoplasm is not known. Consistent with entry of CAV1 into the secretory pathway at the level of the ER, we found that a few minutes after synthesis, CAV1 colocalized with the ER exit site marker Sec31 (Chapter 5).
Figure 6.1 | **Model of caveolae assembly and biosynthetic trafficking of CAV1.** Assembly of caveolae involves transit of CAV1 through the secretory pathway and multiple oligomerization steps. Lipid-dependent oligomerization in the Golgi precedes budding of nascent domains. Two alternative models are shown for the role of PTRF in caveolae assembly. In model (1), invaginated caveolae are not induced, but stabilized by PTRF. In model (2), PTRF associates with previously flat caveolar domains to induce membrane curvature. See also main text.
Oligomerization of CAV1 into 8S complexes

Soon after synthesis, CAV1 forms SDS-stable homo-oligomers in the ER that are considered as the building blocks of caveolar domains. The molecular weight of the homo-oligomers was estimated in the range of 150-400 kDa and may therefore incorporate 7-18 CAV1 monomers (Monier et al., 1995; Sargiacomo et al., 1995; Scheiffele et al., 1998). As long as samples are not boiled, these oligomers can be observed in SDS-PAGE, where they resolve in multiple bands with apparent molecular weights of 200, 400 and 600 kDa (Scheiffele et al., 1998, Chapter 4). Oligomerization is mediated by a 41 amino-acid stretch located in the amino-terminal cytoplasmic domain (CAV1\textsubscript{61-101}, Sargiacomo et al., 1995). An amino-terminal fragment (CAV1\textsubscript{1-101}), when expressed in bacteria, spontaneously formed oligomers and biophysical characterization suggested that these were heptamers (Fernandez et al., 2002). The smallest complex observed in SDS-PAGE (\textasciitilde150kDa) may therefore be a heptamer and the additional bands multimers of the heptamers. The sedimentation coefficient S\textsubscript{20,w} of the complex prepared from cell lysates was estimated to 8S and we thus termed it “8S complex” (Chapter 3).

In cells, 8S complexes are rapidly exported from the ER and transported to the Golgi. In fact, CAV1 was already observed in ER exit sites and the Golgi complex a few minutes after its synthesis (Chapter 5). ER- to Golgi transport of CAV1 depends on COPII machinery, as CAV1 colocalized with COPII component Sec31 and ER-export was sensitive to the COPII-inhibitor CI-976 (Chapter 5).

Secondary oligomerization in the Golgi complex

In the Golgi, the newly arrived CAV1 oligomers are exposed to a new lipid environment with higher concentrations of cholesterol as compared to the ER, and to glycosphingolipids. Transit of CAV1 through the Golgi is relatively slow and a steady-state pool of Golgi-CAV1 can readily be observed in many cell types (Machleidt et al., 2000; Ren et al., 2004). The relatively high density of CAV1 together with the membrane properties in the Golgi are thought to allow for a second round of oligomerization, in which 8S complexes associate with each other.
Evidence for this secondary oligomerization comes from FRAP experiments on the Golgi pool of cells expressing CAV1-mEGFP. Wild-type CAV1 was highly mobile in the ER, but lost diffusional mobility when in the Golgi (Chapter 3). Mutants of CAV1 that fail to form caveolae at the plasma membrane also failed to become immobile in the Golgi, consistent with immobility representing an essential step during CAV1 complex maturation and caveola formation (Chapter 3). Immobility was specific for CAV1, as CAV2 became immobile only when coexpressed with CAV1 (Chapter 3). That immobility of CAV1 was sensitive to depletion of cholesterol from the Golgi argues for a cholesterol-dependent oligomerization step (Chapter 3).

CAV1 can directly bind cholesterol and encodes a CRAC motif (cholesterol recognition/interaction amino acid consensus), located proximal to the central hydrophobic stretch (CAV1_94-101, Epand et al., 2005; Murata et al., 1995). Additionally, phosphorylation of CAV1 at Ser80 was shown to regulate cholesterol binding of CAV1 (Fielding et al., 2004). What is the potential architecture of immobile CAV1 oligomers in the Golgi? 8S complexes are likely to be laterally packed into a relatively loose network that is held together by cholesterol.

Whether CAV1 is already part of detergent-resistant membranes (DRMs) at this point is controversial (Gkantiragas et al., 2001; Pol et al., 2005). Possibly after arrival from the ER, CAV1 is first detergent soluble and then becomes increasingly detergent-insoluble as it progresses through the Golgi complex. In the Golgi, CAV1 preferentially localizes to the cis-Golgi and co-partitions with lipid-raft markers (Chapter 3, Luetterforst et al., 1999). It is unclear whether caveolar domains can bud off from the cis-Golgi or require to progress to the TGN for Golgi exit.

Using sucrose velocity gradient centrifugation, we have identified a novel oligomeric form of CAV1. The sedimentation coefficient $S_{20,w}$ of the complex was estimated to be 70 and we have thus termed it “70S complex”. The 70S complex was resistant to Triton X-100 at room temperature but dissociated in presence of octyl-glucoside and SDS (Chapter 3). The formation of the 70S complex was BFA-sensitive and correlated with the arrival of newly assembled caveolar domains at the cell surface (Chapter 3). In detergent conditions that preserved the 70S
complex, we found that the caveolar protein PTRF was recruited to high-molecular weight fractions. Accordingly, when the 70S complex was dissociated by harsh detergent conditions, PTRF was found in monomeric form at the top of the gradient. Confocal fluorescence microscopy of 70S complexes isolated from CAV1-mEGFP-expressing cells revealed that the complexes were homogenous in fluorescence intensity with similar fluorescence intensity profiles as caveolae in intact cells. Together, the 70S complex is likely to represent the mature form of caveolar domains as they bud off from the Golgi or as they are observed at the cell surface.

Domain assembly and exocytic transport

In cells expressing CAV1-mEGFP, caveolar exocytic carriers can be observed by live-cell imaging. Golgi-derived carriers remained stably assembled after arrival at the cell surface (Chapters 2 and 3, Tagawa et al., 2005), implying that the stable structure of the caveolar coat had assembled in the Golgi. The formation of stable caveolar domains may take place either in the Golgi or immediately upon exit of domains from the Golgi. Whether the budding process itself is driven by conformational changes from within the CAV1 oligomeric network in the Golgi or imposed by cytoplasmic budding factors remains to be determined. Also, whether the budding process itself represents the transition of CAV1 into a stable coat is unknown at this point.

Interestingly, the Golgi and surface pools of CAV1 are differentially recognized by certain antibodies, indicating a conformational change during assembly of caveolae. Epitopes in the oligomerization domain (CAV1_{61-101}) and the C-terminus (CAV1_{161-178}) are no longer accessible in caveolae-associated CAV1 (Dupree et al., 1993; Pol et al., 2005, and data not shown). Based on this change of antigenicity between Golgi and surface caveolae and the observation that the C-terminal domain of CAV1 can interact with both the N- and C-termini, a model was proposed for how 8S complexes could be packed in caveolar domains (Song et al., 1997). In this model, every 8S complex interacts with multiple surrounding 8S complexes.
Exocytic transport of lipid-raft associated cargo.

In the Golgi, the network of oligomerized CAV1 probably generates an environment that attracts lipid-raft associated cargo such as GPI-anchored proteins (Chapter 3). CAV1 may therefore support sorting of exocytic cargo at the level of the Golgi. EGFP-GPI was found to colocalize with CAV1 in the Golgi and in exocytic vesicles (Chapter 3). Mutants of CAV1 that fail to form 8S complexes, such as the breast-cancer associated P132L mutant, fail to exit the Golgi and can act as dominant-negative by trapping wild-type CAV1 and other cargo in the Golgi complex (Hernandez-Deviez et al., 2005; Lee et al., 2002).

Golgi exit of both EGFP-GPI and CAV1 was dynamin-2-independent, whereas VSVG, which is not lipid-raft associated, required dynamin-2 function for efficient Golgi exit (Chapter 3). Furthermore, a previous study has shown that EGFP-GPI and CAV1, but not VSVG, require syntaxin-6 for surface delivery (Choudhury et al., 2006). Besides differential functional requirements between these two pathways, we found that their post-Golgi carriers had distinct morphologies. VSVG post-Golgi carriers were elongated and tubular, whereas EGFP-GPI and CAV1- vesicles spherical and small (Chapters 2, 3).

Whether CAV1 is required for the lipid-raft exocytic pathway is unclear. A role for CAV1 in sorting and polarized exocytosis of GPI-anchored proteins has been suggested soon after the discovery of CAV1 (Dupree et al., 1993; Kurzchalia et al., 1992; Zurzolo et al., 1994). However, later studies indicated that CAV1 was dispensable for sorting and polarized surface delivery in MDCK cells (Manninen et al., 2005). Nevertheless, several exocytic cargoes have been shown to depend on caveolins for efficient surface delivery. These include Influenza-HA (Scheiffele et al., 1998), the muscle repair protein dysferlin, (Hernandez-Deviez et al., 2005), the angiotensin receptor (Wyse et al., 2003), the insulin receptor (Cohen et al., 2003) and stretch-activated channel short transient receptor potential channel-1 (TRPC1, Brazer et al., 2003). Either downregulation of caveolin or overexpression of Golgi-trapped mutants of caveolin caused defects in surface targeting of these proteins. If CAV1 supports sorting and formation of exocytic carriers at the Golgi, then compensatory mechanisms must take over these functions in absence of CAV1, given the relatively mild phenotype of CAV1 -/- mice. In analogy to the plasma
membrane, the role of CAV1 in the Golgi complex might be similar to its role in caveolar/raft-dependent endocytosis, where CAV1 is thought to add a regulatory level to an existing lipid-raft-dependent pathway (Damm et al., 2005).

The role of PTRF in caveola formation

The cytoplasmic protein PTRF is associated with caveolae at the cell surface and in its absence caveolae are lost and CAV1 is degraded (Hill et al., 2008; Liu et al., 2008; Liu and Pilch, 2008). Our results indicate that PTRF associates with newly assembled caveolar domains after their arrival at the plasma membrane. How does PTRF specifically bind to caveolar domains at the plasma membrane and not to the Golgi pool of CAV1? PTRF can bind to phosphatidylserine (PS) in vitro and PS is present on the cytoplasmic leaflet of the plasma membrane (Hill et al., 2008). PS may diffuse into newly assembled caveolar domains after their arrival at the plasma membrane and together with other properties of the caveolar coat attract PTRF.

PTRF has been proposed to induce membrane curvature in caveolae (Chadda and Mayor, 2008; Hill et al., 2008). Because it is unclear whether newly assembled caveolar domains arriving from the Golgi at the plasma membrane are flat or invaginated, we propose two alternative models for the role of PTRF in caveolae formation. In model (1) PTRF associates with invaginated caveolar domains and stabilizes the coat. In model (2), newly assembled caveolar domains are flat after their arrival from the Golgi complex and only after recruitment of PTRF they become invaginated caveolae (Figure 6.1). Provided PTRF further proves to stabilize caveolae and thus prevents them from disassembly and degradation, then regulated dissociation of PTRF from caveolae could be a way for cells to regulate the number of caveolae present on their surface.

Saturation of caveolae assembly

We found that under certain conditions caveola formation was saturated or compromised, resulting in the appearance of a non-caveolar pool of CAV1 at the cell surface. Overexpression of CAV1, siRNA mediated knockdown of PTRF, or
cholesterol depletion all caused a uniform distribution of CAV1 at the cell surface instead of a punctuate pattern typical for caveolae (Chapter 4). We concluded that all three components are required for efficient assembly and stability of caveolae.

When cells were depleted of cholesterol by U18666A, we observed that CAV1 was delivered to the surface in tubular carriers instead of small spherical post-Golgi vesicles (Chapter 4). Upon surface arrival, these carriers released non-caveolar CAV1 that rapidly diffused laterally into the surrounding plasma membrane, demonstrating that non-caveolar CAV1 is not retained in the Golgi, but can be delivered to the cell surface. Based on the morphology of the post-Golgi carriers, non-caveolar CAV1 was likely delivered to the plasma membrane by the non-raft pathway otherwise used by VSVG.

6.3 Disassembly of caveolae and degradation of CAV1

Disassembly of caveolae

We propose two non-exclusive models for caveolae disassembly (Figure 6.2). In the first model, caveolae are disassembled at the plasma membrane. This could occur by signal-induced dissociation of PTRF and subsequent disassembly of the of the caveolar coat. Non-caveolar caveolin is then no longer stabilized in the plasma membrane and internalized to be delivered to early endosomes. During its endocytosis, CAV1 is then probably not involved in the formation of the endocytic vesicle, but rather endocytosed as cargo by an as yet unknown mechanism.

The second model predicts disassembly of the caveolar coat in early endosomes. Caveolar endocytic vesicles have been shown to associate with early endosomes, where they can serve as stable scaffolds and from where they can recycle back to the plasma membrane (Pelkmans et al., 2004). However, the coat may disassemble in early endosomes, either through dissociation of PTRF or through altered membrane environment.
PM

caveola

1

PTRF

domain disassembly either in the PM or in EE

2

caveolar endocytosis

EE

uptake of CAV1 as ligand

RAB4/11/COP/ARF1

RAB5/EEA1

MVB/LE

RAB7/LAMP1

CAV1 oligomeric complex (8S)

PTRF

cholesterol

LYS

LAMP1/LBPA

exposure to acidic pH and degradation of CAV1

targeting of CAV1 to intraluminal vesicles. ESCRT components involved?

Figure 6.2 | Model of caveolae disassembly and degradation. Caveolae disassemble either in the plasma membrane (1) or following caveolar endocytosis (2) in the early endosomes. In either case, dissociation of PTRF from the caveolar domain destabilizes the coat. Disassembled CAV1 in the plasma membrane is endocytosed as ligand. In endosomes, CAV1 is targeted to intraluminal vesicles. Signals controlling this process and possible machinery are currently unknown. In LE/lysosomes CAV1 is exposed to the acidic vacuolar pH and degraded by proteolytic cleavage. See also main text.
Degradation of CAV1

By using a dual-tag strategy and live-cell imaging, we found that during endosome maturation, CAV1 became exposed to the acidic lumen of LE/LYS (Chapter 4). This observation is consistent with targeting of CAV1 from the cytosolic face of the limiting endosomal membrane to the lumen of intraluminal vesicles. The potential involvement of ESCRT components such as Hrs, Tsg101 and Vps4 in this process should be addressed in further studies. That progression of CAV1 from the RAB5- to the LAMP1 compartment was associated with a change in antigenicity indicated that CAV1 underwent a conformational change, i.e. caveolar domains were disassembled between these two compartments. Finally, CAV1 is degraded in LE/LYS.

When lysosomal degradation was blocked, CAV1-mEGFP was observed to accumulate in LE/LYS, indicating a basal level of degradation in the endo/lysosomal pathway at steady state (Chapter 4). Several studies suggest, however, that stimulation of caveolar endocytosis increases degradation of CAV1. FRAP experiments of CAV1-EGFP in “caveosomes”, likely CAV1-positive LE/LYS, showed that recovery of CAV1 was increased when caveolar endocytosis was stimulated by either phosphatase inhibitors or SV40 (Tagawa et al., 2005). Stimulation of caveolar endocytosis by albumin in HepG2 cells caused accumulation of endogenous CAV1 in multi-vesicular bodies (Botos et al., 2008). Although caveolar endocytic carriers can serve as stable scaffolds that recycle from endosomes back to the plasma membrane (Pelkmans et al., 2004), it seems likely that stimulation of caveolar endocytosis also increases degradation of CAV1.

6.4 Perspectives

6.4.1 The structure of the caveolar coat

In this thesis, we have presented a refined model of the caveolae assembly pathway that involves several oligomerization steps and lipid-interactions occurring during transit of CAV1 through the secretory pathway. Although assembly intermediates in ER, Golgi and plasma membrane are now well
established, structural information about the architecture of the caveolar coat is still missing.

A key to a detailed understanding of caveolar structure might be the 8S complex, the building block of the caveolar coat. In the clathrin field, the discovery of the clathrin-triskelion was a major breakthrough in understanding structure and assembly of the clathrin coat (Ungewickell and Branton, 1981). Future studies should determine the precise number of CAV1 monomers incorporated in the 8S complex so that structural models can be developed. Cryo-electron microscopy of purified 8S complexes may reveal the structure of this basic assembly unit of the caveolar coat. How the 8S complexes are arranged in context of the caveolar coat should be addressed by structural analysis of 70S complexes.

6.4.2 Regulation of caveolae structure and function by the PTRF-family of proteins

The PTRF-family of proteins likely represents a set of important modulators of caveolae structure and function. Currently, PTRF is the best studied among the new caveolar coat proteins, as it seems to be an important factor during caveolae assembly and/or maintenance (Hill et al., 2008; Liu et al., 2008; Liu and Pilch, 2008). Because PTRF appears to stabilize caveolae and cellular levels of CAV1, regulated association and dissociation of PTRF to and from caveolae could allow the cell to control the number of caveolae, and levels of CAV1. Our results indicate that PTRF associates with newly assembled caveolar domains at the cell surface and remains rather stably associated with them. However, PTRF can be phosphorylated (Guha et al., 2008), pointing at the intriguing possibility that its state of phosphorylation controls localization to caveolae. It will now be important to identify the signaling pathways and their extracellular inputs that potentially regulate PTRF localization to caveolae. The identified components are likely to reveal the physiological relevance of PTRF-regulation in caveolae structure.

SDPR and SRBC were shown to bind to caveolae via a leucine zipper (LZ) motif and it is likely that PTRF is also recruited to caveolae by its leucine zipper
(McMahon et al., 2009). PTRF and SDPR both bind to PS, however, the relevance for targeting to caveolae is unclear. While PTRF seems to have a structural role in caveolae function, SRBC probably regulates caveolae dynamics (McMahon et al., 2009). SDPR and SRBC can both bind to PKC and may thus serve as adapters recruiting PKC and/or other signaling components to caveolae. In fact, PKC is known to be a critical factor in caveolar endocytosis (Anderson et al., 1996) and therefore SDPR and SRBC might regulate endocytic activity of caveolae.

While PTRF, SRBC and SDPR all bind to the cytoplasmic face of caveolae, they may each confer specific functions to caveolae. This raises the possibility that the function of a caveola is in part defined by the composition and abundance of its additional coat components. The CAV1-coat would then represent the basic morphological feature, to which specific functions are added in a modular fashion. In this scenario, caveolae become multifunctional units that exert various functions in membrane transport and signaling dependent on cell-type and tissue context. This modular control of caveolae could be investigated by two lines of research. First, the relative abundance of the additional coat components in caveolae could be determined in samples of tissue, where caveolae are believed to exert diverging biological functions, e.g. in adipose tissue and in endothelia. Second, caveolae dynamics could be studied by live-cell imaging in presence and absence of PTRF, SDPR and SRBC to determine their contribution to caveolae behavior in cells. A modular control could potentially explain why caveolae have been associated with so many and diverging biological functions.

### 6.4.3 Degradation of CAV1

Our finding that CAV1 enters the classical endosomal/lysosomal pathway and is targeted to intraluminal vesicles suggested degradation of CAV1 in LE/LYS. This now needs to be demonstrated by simple degradation time-courses of CAV1 in presence/absence of lysosomal inhibitors. Moreover, it will be interesting to identify the machinery used for intra-luminal targeting of CAV1. The involvement of Hrs, Tsg101 and Vps4 should be tested, also whether CAV1 is ubiquitylated during this process.
The dual-tag strategy where CAV1-mEGFP and CAV1-mCherry were coexpressed proved to be a powerful tool to follow endosomal maturation and degradation of CAV1. Fluorescence of mEGFP is more sensitive to acidic pH than mCherry and thus intraluminal targeting and endosomal maturation can be followed in real-time, provided GFP/mCherry-tagged CAV1 are targeted to the lumen of endosomes. During progression from early to late endosomes, mEGFP fluorescence is gradually quenched as the pH drops, while mCherry fluorescence is largely unaffected. While this strategy will certainly be useful for defining the molecular components required for intraluminal targeting of CAV1, it is likely to be a powerful tool applicable also in the virus entry field.

An important finding of this thesis is that previously described “caveosomes” most likely are late endosomes or lysosomes, in which CAV1 has accumulated, rather than independent, pH-neutral organelles. Consistent with this, endocytosis of SV40 has now been shown to enter the endosomal/lysosomal pathway for productive infection (Engel et al., manuscript in preparation). Other studies dealing with caveosomes should be carefully evaluated, whether they are also compatible with our new interpretation of the CAV1-positive, intracellular organelles.

6.4.4 Lipid-raft stabilizing scaffolds

Flotillins are lipid-raft associated proteins that have been suggested to share structural and functional properties with caveolins (Bauer and Pelkmans, 2006; Langhorst et al., 2005). We and others showed that flotillin-microdomains indeed share several key-properties with caveolae. Flotillins form small (≤ 100 nm) domains at the cell surface that stabilize a lipid-raft-type membrane environment (Stuermer et al., 2001). During biosynthetic trafficking, they transit through the Golgi, where they form stable domains that, once arrived at the plasma membrane, remain stable for many hours (Chapter 5). Finally, their endocytosis can be activated (Neumann-Giesen et al., 2007). The major difference between CAV1 and flotillins was that flotillins seemed to use, but did not require, the Golgi for
biosynthetic trafficking whereas surface delivery of CAV1 was strictly dependent on Golgi function (Chapter 5).

The underlying principle of protein scaffolds stabilizing lipid-raft domains might be of more general nature, as additional members of the SPFH (stomatin/prohibitin/flotillin/HflK/C)-domain protein family potentially form scaffolds with similar properties. Such assemblies may compartmentalize specific proteins and lipids at the cell surface, support molecular sorting in the Golgi complex and function in endocytosis of certain ligands in response to specific signals.

Future studies should aim at identifying discriminating properties of multiple lipid-raft stabilizing scaffolds with regard to sorting, clustering, and endocytic processes. In light of the relatively mild phenotype of CAV1 -/- mice, particular emphasis should also be placed on overlapping functions as these may point towards pleiotropy, i.e. to the existence of compensatory mechanisms where one particular scaffold can take over the function of another.
Abbreviations

AMPA 2-amino-5-hydroxy-3-methyl-4-isoxazole propionate
ARE apical recycling endosome
ARF1 ADP-ribosylation factor
BC breast cancer
BFA brefeldinA
cAMP cyclic adenosine monophosphate
CAV1 caveolin-1
CAV2 caveolin-2
CAV3 caveolin-3
CD36/FAT cluster of differentiation 36/fatty acid translocase
CHX cycloheximide
COPI coat protein complex I
COPII coat protein complex II
CRAC cholesterol recognition/interaction amino acid consensus
CRE common recycling endosome
CSD caveolin scaffolding domain
Csk C-terminal Src kinase
DM distal myopathy
DRM detergent resistant membrane
DYN2 dynamin-2
EE early endosome
EEA1 early endosomal antigen 1
EGFP enhanced green fluorescent protein
EGFR epidermal growth factor receptor
EMP2 epithelial membrane protein 2
eNOS endothelial nitric oxide synthase
ER endoplasmic reticulum
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ERES</td>
<td>ER-exit sites</td>
</tr>
<tr>
<td>ESCRT</td>
<td>endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>F1</td>
<td>flotillin-1 (also called reggie-2)</td>
</tr>
<tr>
<td>F2</td>
<td>flotillin-2 (also called reggie-1)</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FAPP1</td>
<td>four-phosphate adapter protein 1</td>
</tr>
<tr>
<td>FHCK</td>
<td>familial hyperCKaemia</td>
</tr>
<tr>
<td>FLOT1</td>
<td>flotillin-1 (also called reggie-2)</td>
</tr>
<tr>
<td>FLOT2</td>
<td>flotillin-2 (also called reggie-1)</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRT</td>
<td>Fisher rat thyroid</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosyl-phosphatidyl-inositol</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>Hrs</td>
<td>hepatocyte responsive serumphosphoprotein</td>
</tr>
<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LAMP</td>
<td>lysosomal membrane protein</td>
</tr>
<tr>
<td>LAMP1</td>
<td>late endosomal membrane protein 1</td>
</tr>
<tr>
<td>LE</td>
<td>late endosome</td>
</tr>
<tr>
<td>LGMD1C</td>
<td>limb girdle muscular dystrophy 1C</td>
</tr>
<tr>
<td>LSM</td>
<td>laser scanning microscope (confocal fluorescence - )</td>
</tr>
<tr>
<td>LYS</td>
<td>lysosome</td>
</tr>
<tr>
<td>MBCD</td>
<td>methyl beta cyclodextrin</td>
</tr>
<tr>
<td>mCherry</td>
<td>monomeric cherry fluorescent protein</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>mEGFP</td>
<td>monomeric enhanced green fluorescent protein</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor.</td>
</tr>
<tr>
<td>mRFP</td>
<td>monomeric red fluorescent protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>MVB</td>
<td>multi-vesicular body</td>
</tr>
<tr>
<td>MYO</td>
<td>myopathy</td>
</tr>
<tr>
<td>OG</td>
<td>octyl β-D-glucpyranoside (octylglucoside)</td>
</tr>
<tr>
<td>p75</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylen glycol</td>
</tr>
<tr>
<td>PEST</td>
<td>Polypeptide stretch enriched in proline (P), glutamate (E), serine (S) and threonine (T)</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin-homology (-domain)</td>
</tr>
<tr>
<td>PHB</td>
<td>prohibitin homology (-domain)</td>
</tr>
<tr>
<td>PI4P</td>
<td>phosphatidylinositol-4-phosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PTRF</td>
<td>polymerase I and transcript release factor, also called cavin or cavin-1</td>
</tr>
<tr>
<td>RAB</td>
<td>&quot;Ras-related in brain&quot;, small GTPase</td>
</tr>
<tr>
<td>RMD</td>
<td>rippling muscle disease</td>
</tr>
<tr>
<td>SDPR</td>
<td>serum-deprivation protein response, also called SDR, PS-p68 or cavin-2</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>SHCK</td>
<td>spontaneous hyperCKaemia</td>
</tr>
<tr>
<td>SPFH</td>
<td>stomatin/prohibitin/flotillin/HflK/C</td>
</tr>
<tr>
<td>SRBC</td>
<td>SDR-related related gene product that binds to c-kinase, also called PRKCDBP or cavin-3</td>
</tr>
<tr>
<td>SRP</td>
<td>signal recognition particle</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TGFβR</td>
<td>transforming growth factor beta receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>trans Golgi network</td>
</tr>
<tr>
<td>TIR-FM</td>
<td>total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>TRPC1</td>
<td>stretch-activated channel short transient receptor potential channel-1</td>
</tr>
<tr>
<td>Tsg101</td>
<td>Tumor susceptibility gene 101</td>
</tr>
<tr>
<td>tsO45-VSVG</td>
<td>temperature sensitive folding mutant of VSVG</td>
</tr>
<tr>
<td>TX100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>U18666A</td>
<td>2β-(2-diethylaminoethoxy)-androstenone HCl</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VIP21</td>
<td>vesicular integral membrane protein of 21 kDa</td>
</tr>
<tr>
<td>Vps4</td>
<td>vacuolar protein sorting 4</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular Stomatitis virus glycoprotein</td>
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References


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