

## Lipid rafts, ceramide and molecular transcytosis

Fang Bian<sup>1,2\*</sup>, Bin Xiong<sup>3\*</sup>, Xiaoyan Yang<sup>4</sup>, Si Jin<sup>1,4</sup>

<sup>1</sup>Department of Endocrinology, Institute of Geriatric Medicine, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, 430077, China, <sup>2</sup>Department of Pharmacy, Hubei University of Arts and Science Affiliated Xiangyang Central Hospital, Xiangyang, 441000, Hubei, China, <sup>3</sup>Department of Interventional Radiology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China, <sup>4</sup>Department of Pharmacology, Tongji Medical College, Huazhong University of Science and Technology. The Key Laboratory of Drug Target Research and Pharmacodynamic Evaluation of Hubei Province. Wuhan, 430030, China

## TABLE OF CONTENTS

1. Abstract	
2. Introduction	
2.1. Concept of transcytosis	
2.2. Types of transcytosis in human physiology	
2.2.1. Transport of Alb	
2.2.2. Transport of lipoprotein	
2.2.3. Transport of insulin	
2.2.4. Transport of iron	
2.3. Dysregulated caveolae-mediated transcytosis and human diseases	
2.3.1. Caveolae-mediated transcytosis and atherosclerosis	
2.3.2. Caveolae-mediated transcytosis and diabetes	
2.3.3. Caveolae-mediated transcytosis and Alzheimer's disease	
2.3.4. Caveolae-mediated transcytosis and lung diseases	
2.3.5. Caveolae-mediated transcytosis and tumors	
2.3.6. Caveolae-mediated transcytosis and inflammation	
2.4. Transcytosis and drug delivery	
3. Lipid rafts (Lrs)	
3.1. Origin and development of the concept	
3.2. LRs on cell membranes	
3.3. Ceramide and LRs	
4. Transcytosis Molecules Associated With Lrs And Ceramide	
4.1. Molecular machinery for LR-mediated endocytosis	
4.1.1. Caveolae-mediated endocytosis	
4.1.1.1. Receptor clusters in caveolae	
4.1.1.2. Budding and fission mechanisms	
4.1.2. Flotillin-mediated endocytosis	
4.1.3. GRAF1-regulated endocytosis	
4.1.4. Arf6- regulated endocytosis	
4.1.5. RhoA- regulated endocytosis	
4.1.6. Other molecular mediators for LRs-dependent endocytosis	
4.1.6.1. Annexins (Anxs)	
4.1.6.2. Syndecan (Syn)	
4.2. Trafficking motors	
4.3. Molecular machinery for LR-mediated exocytosis	
4.3.1. The SNARE hypothesis	
4.3.2. SNARE proteins	
4.3.3. NSF and SNAP	
4.3.4. Other regulators for vesicle exocytosis	
4.3.4. 1. The Rabs	
4.3.4. 2. Munc18	
4.3.4. 3. Munc13	
4.3.4. 4. Syt and CPX	

#### 4.4. Ceramide mediated transcytosis

### 5. Conclusions And Perspectives

### 6. Acknowledgement

### 7. References

## 1. ABSTRACT

Transcytosis, a widely described process concerning transport of macromolecules between the apical and basolateral sides in various cell types, is extremely important for multicellular organisms to selectively exchange materials in different microenvironments while maintaining cellular and body homeostasis. Uncontrolled transcytosis is involved in a wide range of pathophysiological processes. Lipid rafts (LRs), the sphingolipid and cholesterol-enriched membrane microdomains, enable to form different functional membrane macrodomains or platforms upon stimulations. In particular, ceramide-enriched membrane microdomains play extremely critical roles in LRs clustering or platform formations. Notably, various transcytosis-related molecules are tightly correlated with LRs and ceramide. We attempt to summarize the basic and advanced information about the roles of different types of transcytosis in human health and diseases, and the types and functions of LRs involved in transcytosis, as well as multiple transcytosis-related molecules associated with LRs and ceramide. It is hoped that all information and discussions could provide much more comprehensive insights into the understanding of the association of LRs with transcytosis, as well as shed some new light on the translational significance in this area.

## 2. INTRODUCTION

### 2.1. Concept of transcytosis

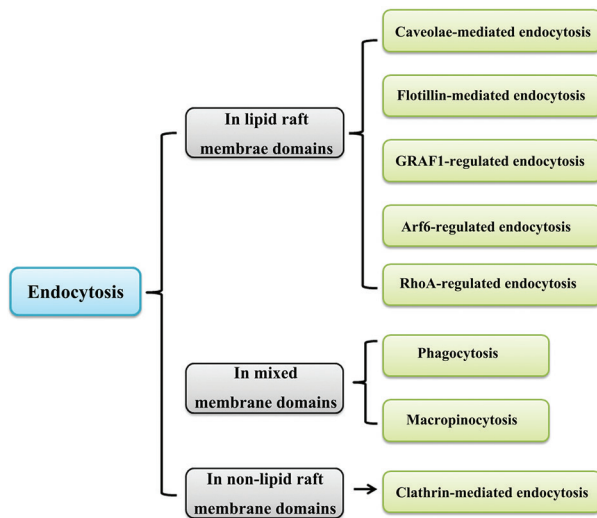
Transcytosis is the transport of macromolecules between the apical and basolateral surface (apical to basolateral or the reverse) in polarized cells. Historically, upon the discovery of plasma lemma vesicles in endothelial cells (ECs), the existence of transcytosis was first postulated in the 1950s by Palade (1). N.Simionescu first introduced the concept of transcytosis to portray the transcellular transport of macromolecules across capillary ECs to the interstitial of tissues via plasma lemma vesicles (2). During this same period, another type of transcytosis was being discovered. Comparing the different types of immunoglobulin found in various secretions (e.g. milk, the intestinal lumen, serum, saliva), immunologists hypothesized that the secretory IgA is selectively transported across epithelial cells barriers. Moreover, the secretory component in secretory IgA was identified as the ectoplasmic domain of the membrane receptor that binds dimeric IgA and induces transepithelial transport (3). Transcytosis is now recognized as a widespread physiological process and certainly occurs in various cell types including ECs, epithelial cells, neurons,

osteoclasts, fibroblasts *etc.* It is extremely important for multicellular organisms to selectively exchange materials in different microenvironments while maintaining cellular and body homeostasis.

### 2.2. Types of transcytosis in human physiology

Transcytosis is a complex multi-step process which includes endocytosis, intracellular trafficking and exocytosis. Varying in the particular cellular context, the cargos, and the molecular machinery and so on, different organ or tissue may exhibit different transcellular mechanisms. Transcytosis takes place either by a receptor-dependent process or a receptor-independent mechanism (4). Receptor-dependent transcytosis entails the binding of plasma molecules to the receptors localized on membrane vesicles and the recruitment of special proteins and lipids, and transport of cargos across the cytoplasm, as well as discharging cargos through the target plasma membrane. Receptor-independent process includes adsorptive transcytosis and a non-specific-fluid phase. Adsorptive transcytosis implies an electrostatic interaction between cargo molecules and vesicles. The fluid phase mechanism has been demonstrated for several molecular species, like glycogen, ferritin and dextran.

Current evidence suggests that the exocytotic machinery (targeting and fusion machinery) is almost similar in different tissues (5). However, endocytic pathways are tightly associated with cell types and conditions, the specialized cargo, the molecular machinery, cargo destination, *etc.* (6). On the basis of the size of cargos, the common classification of endocytic pathways divide them into phagocytosis and pinocytosis. Depending on the requirement for dynamin, endocytic pathways is also classified into dynamin-dependent and dynamin-independent endocytosis. Another classification system is based on the involvement of small GTPases: GTPase regulator associated with focal adhesion kinase (GRAF1)-regulated endocytosis, RhoA- and Adenosine diphosphate-ribosylation factor 6 (Arf6)-regulated endocytosis. Based on the membrane regions that make up the primary endocytic vesicles, endocytic routes have been classified into endocytosis occurring in lipid rafts (LRs) membrane domains, in non-LRs membrane domains, and in mixed membrane domains as illustrated in Figure 1, which is also the primary division in our literature. Endocytic routes in LRs include caveolae-mediated endocytosis, flotillin-mediated endocytosis and GTPases-regulated endocytosis (GRAF1-, RhoA-, Arf6-regulated endocytosis). Phagocytosis and



**Figure 1.** Classification of the endocytosis based on the dependence of membrane domains. Endocytic routes taking place in lipid raft (LR) include caveolae-mediated endocytosis, flotillin-mediated endocytosis and GTPases-regulated endocytosis (GRAF1-, RhoA-, Arf6-regulated endocytosis). Phagocytosis and macropinocytosis are endocytic pathways can be observed in both LR and non-LR membrane domains. Clathrin-mediated endocytosis is independent of lipid raft regions.

macropinocytosis are endocytic pathways which can be observed in both LRs and non-LRs membrane domains. Clathrin-mediated endocytosis (CME) is independent of LR regions.

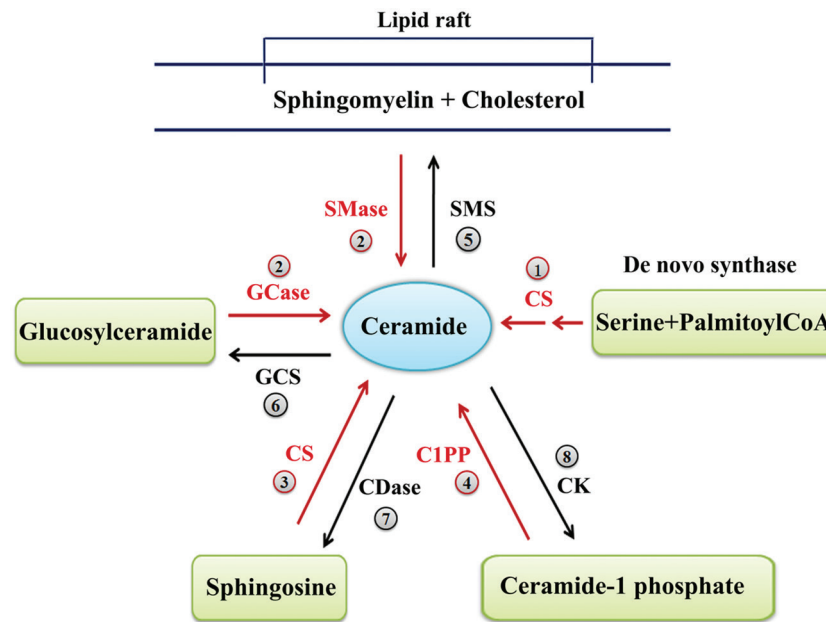
So far, CME is the most widely studied endocytic pathway and plays critical roles in human health and disease. What we now call “clathrin-coated pits” was first observed by Roth and Porter in studies of yolk-protein uptake by mosquito oocytes (7). This pathway encompasses the ubiquitous uptake of nutrient-receptor complexes, adhesion molecules, growth factors, membrane transporters, toxin and viruses, the recycling of synaptic vesicles and activation of signaling pathways those regulating development and immune responses (8). David J. Begley revealed that three major endocytic pathways have been definitively identified in brain endothelia (9): the most frequently CME, caveolae-mediated endocytosis and macropinocytosis. The process of CME involves the recruitment of clathrin from cytosol into the plasma membrane, that concentrate receptors, invaginating to clathrin-coated pits formation, then pinching off to form clathrin-coated vesicles (CCVs), and finally releasing the clathrin coat which fuse with the endosomal system (6). Cargos delivered via CME pathway are digested and degraded in the endolysosome (PH~4.5). Generally, receptor-mediated endocytosis via CME is for internal use.

Caveolae are specialized LRs that form 50-100nm flask-shaped invaginations in the plasma membrane, which are in most differentiated cell types

and are especially abundant in ECs, adipocytes, muscle and fibroblasts but are absent from platelets, red blood cells and lymphocytes. A variety of receptors (e.g. low density lipoprotein receptor/LDL-R, high density lipoprotein receptor/HDL-R, albumin receptor/Alb-R, transferrin receptor/Tf-R, insulin receptor/Ins-R etc), channels, and enzyme systems are located in caveolae microdomains (4). Caveolae greatly increase the surface area of the cell and are also involved in macromolecular transport, vesicular trafficking, signal transduction, mechanotransduction, and maintenance of plasma membrane integrity (10). Currently, numerous literatures pay more attention to transcytosis via caveolae. The latest data suggested that the fate of caveolae is dependent on the cell types in which endocytosis takes place. In ECs, cargos undergo transcytosis across ECs to the underlying tissues, generally bypassing the lysosomes. These cargos are made available to the adjacent tissues. By contrast, cargos taken up by non-ECs are destined for the endolysosomal system (11). Some evidence identified that caveolae exhibit their highest frequency in ECs (~10,000/cell) (4). It is suggested that the population of clathrin-coated pits and vesicles is relatively small, comprising about 3% of the total endothelial vesicles (12). In ECs, the frequency of caveolae varies depending on the organ from ~1,200/ $\mu\text{m}^3$  in endothelium of skeletal muscle to ~130/ $\mu\text{m}^3$  in pulmonary capillaries, and <100/ $\mu\text{m}^3$  in brain endothelia (5). Extensive data suggested that caveolae play a major role in the pathophysiology of the cardiovascular system. In fact, caveolae are present in essentially all cell types of the cardiovascular system, including ECs, smooth muscle cells, macrophages, cardiac myocytes and fibroblasts. Plasma proteins have various crucial functions including the maintenance of the oncotic pressure gradient of body fluids (e.g. Alb), carriers of metabolites (e.g. LDL, Tf, Alb, and ceruloplasmin), regulators of cell metabolism (e.g. insulin) and immune response (e.g. immunoglobulin). Furthermore, these circulating molecules are selectively taken up by ECs and further sorted and transported to the correct destination, which guarantee local physiological needs.

## 2.2.1. Transport of Alb

Transcytosis is a physiologically important process that accounts for much of Alb transport across the resting endothelium. Alb is transported predominantly via receptor-mediated transcytosis. That is, Alb transcytosis is initiated by cross-linking of the protein with Alb-binding glycoproteins (18, 31, 60, and 75 kDa) that localize on ECs caveolar membrane. The clues originally came from morphological studies by electron-microscopy examination that Alb-Au (Alb attached to gold particles) or radioiodinated monomeric Alb was concentrated in caveolae open to blood vessel lumens. The critical role for caveolae in Alb transcytosis has been reinforced by the study of *caveolin-1(Cav-1)*<sup>-/-</sup> mice. Razani B and co-workers observed that in cultured fibroblasts from



**Figure 2.** Ceramide-generating and -metabolism pathways. Ceramide-generating pathways primarily include: 1) De novo synthesis pathway: this process is conducted within ER and begins with the condensation of serine and palmitoylCoA; 2) Sphingolipid recycling pathway: glucosylceramidase (GCase) enables to hydrolyze glucosylceramide to regenerate ceramide and SMase, acid or neutral SMase, which hydrolyze sphingomyelin (SM) to ceramide. 3) Salvage pathway: salvage of free sphingosine by N-acylation by ceramide synthase (CS). 4) Ceramide-1 phosphate pathway: ceramide-1 phosphate can be catalyzed by ceramide-1 phosphate phosphatase (C1PP) to generate ceramide. Furthermore, ceramide-metabolism pathways primarily include: 5) SM synthase (SMS) catalyzes the transfer of choline phosphate groups from phosphatidylcholine to ceramide, generating both SM and diacylglycerol. 6) Glucosylceramide synthase (GCS) catalyzes ceramide to glucosylceramide. 7) Ceramidase (CDase) catalyzes ceramide to SM. 8) Ceramide kinase (CK) catalyzes ceramide to generate ceramide-1 phosphate.

*Cav-1*<sup>-/-</sup> mice, the uptake of FITC-Alb was inhibited whereas Tf uptake by CME was not affected (13). Concomitantly, in contrast to the wild-type, iodinated Alb transcytosis in aortic ring segments was significantly decreased in the *Cav-1*<sup>-/-</sup> mice. Blaine J and colleagues also reported that the majority of FITC-Alb entered podocytes is dependent on caveolae by inhibition of CME and caveolae-mediated endocytosis (14). However, the observation of Alb transcytosis in the choroid plexus in *Cav-1*<sup>-/-</sup> mice indicated alternative pathways for Alb transport. Recently, studies by Zhang and colleagues revealed that in addition to the caveolae-mediated Alb in pulmonary endothelium, a portion of Alb uptake is CME and macropinocytosis-like pathway (15). Interestingly, Alb uptake was also found to follow RhoA-dependent endocytosis in Chinese hamster ovary cells (16). By exploring Alb transport across microvascular endothelium, it was suggested that Cav-1-mediated transcytosis and paracellular permeability are co-regulated through a signaling pathway linking dynamin, Rac, and actin (17).

### 2.2.2. Transport of lipoprotein

Evidence has documented that Cav-1 play an important role in the regulation of lipoprotein metabolism by regulating plasma lipoprotein levels as well as lipid composition (18). Studies by Matveev and Phillips showed that Cav-1 negatively regulates scavenger

receptor class B type I (SR-BI)-mediated HDL-derived cholesteryl ester uptake and increases plasma HDL levels (19, 20). However, Cav-1 may not affect SR-BI-mediated cholesterol efflux or selective uptake of cholesteryl ester in HEK 293 and FRI cells. In contrast to wild-type mice, *Cav-1*<sup>-/-</sup> mice displayed increased plasma triglyceride levels and reduced hepatic very-low-density lipoprotein (VLDL) secretion (18).

LDL is the main plasma cholesterol carrier. The uptake of LDL is mediated by LDLR through CME and caveolae-dependent endocytosis. Caveolae are greatly associated with the transcytosis of LDL and oxidative LDL (ox-LDL) across human umbilical vein vessels (21-23). It is also reported that Cav-1 and Niemann-Pick C1 interact to regulate efflux of LDL-derived cholesterol ester from late endosomes/lysosomes by transfecting with Cav-1 siRNA into fibroblasts (24). In the lung microvasculature, transport of LDL occurs through both caveolae-mediated and receptor-independent mechanisms (25). Nonetheless, in the brain, LDL transport is virtually by a receptor-mediated mechanism without fluid-phase transcytosis (9). Based on a shear-dependent three-pore model, others suggested that the “leaky junction”, formed by detachment of adjacent ECs due to apoptosis and mitosis of the cell, is the most significant pathway of LDL uptake by comparison to the receptor-mediated



pathway (26). Recently, exploring the mechanisms of LDL and HDL across the peripheral vascular endothelium from plasma into interstitial fluid in healthy humans, Miller *et al.* revealed that the transport of LDL and HDL is by passive ultrafiltration through intercellular pores without an active process such as receptor-mediated transcytosis (27).

### 2.2.3. Transport of insulin

Insulin is an extremely important hormone in regulating targeting tissues (e.g. fat and muscle) physiological functions *in vivo*. To reach its cellular targets, insulin must first traverse the vascular endothelium to enter the tissue interstitium. Insulin transcytosis *in vitro* is saturable, temperature-sensitive, and receptor-mediated process rather than the passive pathway. Furthermore, the transport of insulin across the microvasculature endothelium is also a rate-limiting step in insulin availability. Since the tight nature of the microvascular endothelium, the dominant route for insulin transport is by transcytosis not by paracellular pathway. Using a novel single-cell assay and total internal reflection fluorescence (TIRF) microscopy, Warren L *et al.* demonstrated that insulin transcytosis across primary adipose microvascular endothelial cells is clathrin- and dynamin- but not Cav-1-dependent pathways (28), while insulin uptake by myoblasts is finally degraded in lysosomes. They highlighted that the initial uptake of insulin (insulin internalized for 1 min in human adipose microvascular endothelial cells) is clathrin dependent and caveolae independent.

Interestingly, the route of uptake is extremely different between macrovascular and microvascular ECs. Wang *et al.* observed that in bovine aortic ECs, insulin uptake via Cav-1-dependent mechanism (29). In attempt to analyze these different findings, Warren L *et al.* further explored the possible colocalization of insulin with caveolin or clathrin in human aortic ECs and found that insulin colocalized markedly more with Cav-1 than with clathrin (28). Studies by Malik summarized that the main route of alveolar epithelial protein transport is likely to be via transcytosis involving caveolae and clathrin coated pits (30). However, since insulin permeability across primary rat alveolar epithelial cell monolayers and its small diameter (~2.2 nm) relative to the estimated pore diameter (10-12 nm) of the alveolar epithelium, some evidence implied that transport of insulin across alveolar epithelial is thought to be predominantly paracellular pathway. Bahhady *et al.* demonstrated that paracellular transport and CME may be involved in insulin transport across rat alveolar epithelial cell monolayers (31). To conclude, these findings above illustrate the selective insulin uptake mechanisms that take place across ECs vary with different vascular beds.

### 2.2.4. Transport of iron

Iron transport is still controversial as to whether

it is delivered with or without Tf, the main plasma carrier of iron. It is appeared that Tf-iron transport takes place through different mechanisms due to various functions of the cell. Two receptors, TfR1 and TfR2, can interact with iron-bound Tf in the liver, which is recycled and responsible for iron homeostasis in cells and tissues. Within hepatocytes, evidence suggested that CME and caveolae-mediated endocytosis are involved in TfR2 internalized (32). Studies by Chang (33) revealed that upon binding with TfR on the ECs of the blood-brain barrier (BBB), Tf-iron is internalized by CME. However, caveolae-mediated transcytosis of Tf was also described *in vitro* model of the BBB (33). In addition, mammalian lactoferrin, a cationic iron-binding glycoprotein, could cross the BBB through receptor-mediated transcytosis by interacting with its receptor on the ECs of the BBB (34). Of note, studies by Roberts *et al.* indicated that transcytosis of Tf across the thymus capillary wall is independent of TfR, which is not expressed on the ECs, but that uptake of Tf by epithelial reticular cells is a receptor dependent process (clathrin- or caveolae-dependent mechanism) (35). In fibroblasts, TfR can be internalized not only by CME but also by caveolae-dependent pathway. Moreover, with a defect in CME, TfR internalization occurs by caveolae-mediated mechanism (36).

## 2.3. Dysregulated caveolae-mediated transcytosis and human diseases

### 2.3.1. Caveolae-mediated transcytosis and atherosclerosis

Currently, more and more evidences suggested that the subendothelial retention of apoB100 containing lipoproteins (e.g. LDL etc) is the initial step of atherogenesis, and is usually termed "response to retention hypothesis" in literatures (21, 22, 37). Following *in situ* perfusion of radioiodinated  $\beta$ -VLDL-induction of hypercholesterolemia in rabbits, Vasile *et al.* revealed that a large number of particles are transcytosed by plasmalemmal vesicles and accumulate within the subendothelial space in lesion prone areas (aortic arch, heart valves, and coronaries) (37). Early study has indicated that Cav-1 deletion could decrease the incidence of aortic atheromas, with about 70% reduction of atherosclerotic lesion area (38). Furthermore, studies by Nievelstein and Vasile separately found that injection of LDL to induce hypercholesterolemia in rabbits, the microaggregates of LDL in the arterial wall is the earliest detectable change that occurs within 2 h (39); *in situ* perfusion of arterial segments exhibits substantial accumulation of LDL within 5 minutes (40). Using  $^{125}\text{I}$ -LDL, Gao *et al.* observed that vascular endothelial growth factor (VEGF) could significantly promote LDL transcytosis by upregulation of Cav-1 and Cav-2 expression while decrease LDL paracellular transport by downregulation of occludin and claudin-5 (the tight junction-associated proteins) in aortary ECs (41). Salvianolic acid B enabled to inhibit the effect of VEGF on LDL transcytosis. Cav-1-mediated cholesterol trafficking is important for HDL

stimulated cholesterol efflux, which functions as an intracellular cholesterol transporter (42). A recent study suggested that HIV or HIV envelope Nef would disrupt Cav-1-mediated cholesterol efflux from human aortic ECs on HDL stimulation (43). Recently, based on a newly established *in vitro* transcytosis model of LDL and confocal microscopic analysis, our studies investigated that C-reactive protein (22) and TNF- $\alpha$  (21) can increase caveolae-mediated LDL transcytosis across ECs and therefore promote LDL retention in human umbilical veins, as well as atherosclerotic lesion formation. Additionally, we also showed that ceramide largely promotes caveolae-mediated oxLDL transcytosis across ECs and accumulation under endothelium in vascular wall (23).

Of note, some literatures highlighted that Cav-1 would promote or inhibit the initial and progression of AS depending on Cav-1 expression in different cell types. Cav-1 in smooth muscle cell may suppress AS formation while Cav-1 in ECs may have a pro-atherogenic role by negatively regulating cell proliferation (44).

### 2.3.2. Caveolae-mediated transcytosis and diabetes

Diabetes is accompanied by enhanced transcytosis of reversibly glycosylated Alb and irreversibly glycosylated Alb (Alb modified by advanced glycation end products, AGE-Alb). In fact, glycosylated Albs are taken up by ECs at a much greater rate than their native forms. Compared to physiological condition, aortic ECs in diabetes present an increased number of caveolae and enhanced transcytosis of AGE-Alb (by ~20%) (45). In fact, after its secretion into the bloodstream by pancreatic  $\beta$  cells, insulin must first cross the endothelial barrier and then has access to its target tissues. A recent study found that NO can directly increase insulin transport across aortic ECs (46), thereby enhancing insulin signaling. Of note, it is well known that endothelial NO synthase (eNOS) activity is considered to be tightly controlled by caveolae. The IR and part of the downstream signaling mediators, like insulin receptor substrate 1 (IRS1), are localized in or recruited to caveolae. Moreover, the insulin-regulated glucose transporter-4 (GLUT4) appears to be endocytosed by caveolae under both basal and insulin-stimulated conditions, whereas the endocytosis of GLUT4 may also involve a clathrin-mediated process particularly during insulin stimulation (47). To date, data clearly confirmed a pivotal role of caveolae in insulin signaling and insulin resistance. However, more direct evidence should be explored that how caveolae related to the pathogenesis of insulin resistance in diabetes.

### 2.3.3. Caveolae-mediated transcytosis and Alzheimer's disease (AD)

A vast amount of fundamental and clinical data has confirmed that the development of AD is directly linked to the accumulation of beta-amyloid (A $\beta$ ), which

is formed by aberrant cleavage of the amyloid precursor protein (APP). Some evidence demonstrated that Cav-1 is upregulated in all parts of old rat brain, namely hippocampus, cerebral cortex and in elderly human cerebral cortex. Additionally, Cav-3 was not detected in caveolae in normal human brain, but it was strongly increased in AD brain (48). Cav-1 was also colocalized with APP in caveolae (49). Moreover, phospholipase D 1 was significantly accumulated in reactive astrocytes and colocalized with APP or Cav-3 in the brains of AD patients. It is suggested that the receptor for AGE seems to mediate apical-to-basolateral passage of A $\beta$  peptides across the BBB. Sakamoto *et al.* observed that the receptor for AGE-mediated A $\beta$ 40 and A $\beta$ 42 transport through brain capillary endothelial cells is caveolae-dependent transcytosis (50). Using Cav-1 specific shRNA lentiviral particles, Toborek *et al.* indicated that HIV-induced A $\beta$  accumulation in human brain microvascular endothelial cells is LRs and caveolae dependent and involves the caveolae-associated Ras signaling (51). Recently, it is reported that ginsenoside Rg3-triggered A $\beta$ 42 uptake via SRA involves clathrin- and caveolae-mediated endocytic mechanisms and further accelerates the degradation of A $\beta$  peptide through upregulating the levels of ADE, NEP and IDE, which may be beneficial to AD therapy (52). Lin *et al.* investigated that estrogen receptor  $\alpha$ -inhibited death domain-associated protein translocation and the communication between membrane estrogen receptor  $\alpha$  and caveolin in caveolae may protect against AD, which confirmed a higher AD incidence in women (53).

### 2.3.4. Caveolae-mediated transcytosis and lung diseases

It is reported that lungs show an exceptionally high Cav-1 level. The crucial role of caveolae-mediated Alb transcytosis across pulmonary endothelium is well described, which was demonstrated from initial gold-labeled Alb electron microscopy studies in wild type animals (54, 55) to similar investigations in Cav-1<sup>-/-</sup> mice (56, 57) as well as studies in isolated cultured lung ECs (15, 58). Using rat pulmonary endothelial cells, Zhang *et al.* found that Alb transport is not only caveolae-dependent but also clathrin- and macropinocytosis-mediated (15). Recently, studies in Cav-1<sup>-/-</sup> *Niemann Pick C1*<sup>-/-</sup> mice indicated the regulatory function of Cav-1 on pulmonary cholesterol metabolism (59). Human translationally controlled tumor protein (TCTP) contains a protein transduction domain (PTD) at its NH2-terminus, which we called TCTP-PTD. TCTP-PTD can facilitate the transduction of other protein into cells. Lee *et al.* found that TCTP-PTD is partly via LR/caveolae-dependent endocytosis and partly by macropinocytosis in a dynamin/actin/microtubule-dependent pathway across human lung carcinoma cells (60). Frameshift mutations in Cav-1 were identified in two patients with non bone morphogenetic protein type II receptors 2-associated hereditary pulmonary arterial hypertension (PAH). Recently, Mark P *et al.* demonstrated that heterozygous

null bone morphogenetic protein type II receptors 2 gene mutations induce caveolae trafficking defects in pulmonary endothelial cells and pulmonary endothelial barrier dysfunction in hereditary PAH patients (61). In addition, it was reported that nanoparticles (NPs) up to approximately 200 nm are primarily internalized by CME and those up to 500 nm are mainly taken up via caveolae-mediated endocytosis. Yusuke and co-workers found that carbon nanotube (204.5 nm-234.2 nm) is taken up into A549 human alveolar carcinoma epithelial cells via clathrin- and caveolae-mediated endocytosis, leading to inflammation-related carcinogenesis. What is more, inflammation would also stimulate paracellular and caveolae-mediated transcellular pathway in pulmonary vessels to promote protein-rich edema formation (62).

### 2.3.5. Caveolae-mediated transcytosis and tumors

Early work indicated that combination of low-frequency ultrasound and small-dose bradykinin can significantly increase caveolae-dependent transport of blood-tumor barrier (BTB) (63). Recently, Wang *et al.* investigated that low dose endothelial monocyte-activating polypeptide-II (a proinflammatory cytokine applied to antitumor therapy research) can increase BTB permeability via the paracellular pathway and caveolae-mediated transcytosis (64). Xue *et al.* reported that the association of the C-terminus of alpha subunit of human large-conductance calcium-activated potassium channels with Cav-1 triggering caveolae trafficking can enhance the permeability of BTB (65). In addition, VEGF and papaverine together can also upregulate BTB permeability via the paracellular pathway and caveolae-dependent transcellular pathway, which play a pivotal role in the induction of tumor angiogenesis and vascular permeability (66).

### 2.3.6. Caveolae-mediated transcytosis and inflammation

Leukocyte migration is a key step in inflammation, which is mediated by chemokines on the surface of ECs. Interferon gamma-inducible protein-10 (CXCL10), a chemokine secreted in many Th1 inflammatory diseases, exhibits important roles in trafficking of monocytes and activated T cells. It was suggested that CXCL10 is transferred from the basal membrane to the apical membrane in ECs by CME. Studies investigated by Lee showed that chemokines endocytosis occurs through a macropinocytosis like pathway in ECs and Cav-1 is not involved in CXCL10 internalization (67). However, Duffy antigen receptor for chemokines mediates chemokines (e.g. CC and CXC chemokines) transcytosis across the endothelium to different tissues and co-localizes with CCL2, CXCL8 and Cav-1 in caveolae (67). Additionally, David suggested that CCL3/MIP-1 $\alpha$  and CCL5/RANTES at the ECs surface are endocytosed primarily by caveolae (68). Of note, it was reported that dendritic cells (DCs) move paracellularly, whereas peripheral

blood lymphocytes traveled through the ECs via Cav-1-mediated pathway (69).

## 2.4. Transcytosis and drug delivery

Certain bioactive macromolecules, such as plasmid DNA, small-interfering RNA, anti-microRNA oligonucleotides, and antisense oligonucleotides, are commonly delivered to specific intracellular target to elicit therapeutic effect. Due to their large molecular weight and charged surfaces, these macromolecules are not able to permeate across the lipophilic cell membrane and therefore compact them into gene delivery vectors (viral or nonviral vectors) to deliver them into the cytosol, thus offering potential for site-specific gene delivery. It is now acknowledged that the initial binding steps of the vectors may determine its entire intracellular itinerary. So far, CME, caveolae-mediated endocytosis and macropinocytosis are major pathways that associate with the internalization of transfection agents (6). Early studies showed that 200 nm fluorescent latex beads rely on CME, whereas 500 nm beads are primarily taken up via caveolae-mediated endocytosis (70). By contrast, another study indicated that particles with diameters  $\leq 100$  nm are internalized by caveolae, while particles in the size range of 100 nm-200 nm are taken up by CME and large complexes with sizes of  $>200$  nm undergo the macropinocytosis process (71).

A recent report confirmed that polyethylenimine (PEI) and polyamidoamine (PAMAM) polyplexes are internalized by both CME and caveolae pathway in HeLa cells (72). PAMAM dendrimers are modified with angiopep through bifunctional PEG and then complexed with DNA, yielding PAMAM-PEG-Angiopep/DNA NPs. These modified NPs appear to be internalized via clathrin- and caveolae-mediated endocytosis pathway in brain capillary endothelial cells, as well as partly via macropinocytosis (73). Examining the endocytosis of two cationic liposomes, DMRIE-C and Lipofectamine LTX in chinese hamster ovary cells, Wong *et al.* observed that DMRIE-C-transfected DNA is taken up via caveolae-mediated endocytosis, while LTX-transfected DNA is internalized by CME (74). Interestingly, Rejman *et al.* showed that the uptake of lipoplex by DCs is preferentially mediated by macropinocytosis (75). Recently, comparing the different transfection mechanisms of polyplexes, lipoplexes, and stealth liposomes in DLD-1 human colorectal cancer cells, Kokkoli *et al.* found that 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) polyplexes are predominantly taken up by CME, branched polyethylenimine lipoplexes and branched polyethylenimine encapsulated in PEGylated nontargeted liposomes are primarily internalized using macropinocytosis pathway, and a combination of caveolae and macropinocytosis is the major form of endocytosis for PR<sub>b</sub> peptide functionalized liposomes (76). Cyclic



asparagines-glycine-arginine modified gene loaded poly (lactic acid)-poly (ethylene glycol) NPs, effectively condense DNA into small-sized complexes (<200 nm) with positive charge (~10 mv), could be taken up by human umbilical vein endothelial cells mainly through caveolae-mediated endocytosis, yet CME is involved to a lesser degree (77).

In addition, it was suggested that the injection of a fluorescent-labeled monoclonal antibody against aminopeptidase P2 (a marker uniquely enriched in lung endothelial caveolae) contributes to the rapid permeation of the antibodies to lung epithelial cells (78). Evidence suggested that Alb-bound paclitaxel (Abraxane) is transported across endothelial barrier via caveolae-mediated transcytosis in the treatment of metastatic breast cancer (79). The oral delivery of paclitaxel through encapsulation into lipid NPs is transferred by caveolae-mediated transcytosis across the intestinal epithelial cells barrier to deliver the drug to the general circulation (80). The above examples show that the exploitation of transcytosis for drug and gene delivery is a promising therapeutic approach, providing each specific physiopathological condition is carefully assessed.

### 3. LIPID RAFTS (LRS)

#### 3.1. Origin and development of the concept

Plasma membrane is a selectively permeable structure that separates the intracellular milieu of the cell from the outside environment. It is well known that plasma membrane is mainly composed of a phospholipid bilayer with embedded proteins that is held together via non-covalent interactions between the hydrophobic tails.

According to the fluid mosaic model first proposed by Seymour Jonathan Singer and Garth L. Nicolson in 1972, the glycerophospholipid bilayer is a two-dimensional fluid construct that allows the lateral movement of membrane components. In 1977, Jain and White introduced a "microdomain or lipid domain model" of membrane structures, which proposed cell membrane is made of dynamic membrane microdomains. Until 1997, Singer and Nicolson proposed the so called "Lipid raft model" for cell membrane structures functioning on membrane trafficking and signaling, which is much more ordered and tightly packed than the surrounding lipid bilayer and can move freely within the liquid-disordered cell membrane (81). At the 2006 Keystone Symposium of Lipid Rafts and Cell Function, a consensus replaced the term "Lipid rafts" with "membrane rafts" and clarified the definition of a membrane raft: "Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions." (82).

It is well known that LRs are involved in a great variety of cellular functions and biological events. However, LRs have been often a subject of controversy mainly due to (83):a) the difficulty to directly visualize such LR structures in living cells; and b) the use of detergents in LRs isolation that may promote the production of artefacts and misinterpretations.

#### 3.2. LRs on cell membranes

Based on the structure and components, LRs on cell membranes can be divided into two types, caveolar and noncaveolar rafts. Accordingly, caveolar and noncaveolar LRs are characterized by its cholesterol sensitivity and clathrin-independence. In fact, some cell types have only caveolar or noncaveolar rafts, while some other cell types may have both in their plasma membrane. Of note, caveolar and noncaveolar LRs induce different signaling pathways in different cells or even in the same cells in response to different stimuli. In the 1990s, it was suggested that caveolae, discovered in the early 1950s, were widely considered to be a distinct version of LRs that contain the specific protein caveolins and share numerous biochemical characteristics with LRs, including detergent insolubility and decreased density properties. Caveolins-mediated formation of caveolae in cells represents a form of LR clustering, which is present even under resting conditions. Nevertheless, noncaveolar LRs are not constitutively present, but capable of clustering in response to stimulations.

By modifying sucrose density gradient flotation centrifugation, Yao *et al.* observed that caveolar LRs are membrane vesicles about 50-100 nm in diameter and enriched with caveolins and flotillin-1, whereas noncaveolar LRs are amorphous membranes and contain much more cholesterol and sphingolipids than caveolae. Meanwhile, they also suggested that many membrane proteins, like IGF-1R and AQP-1, are specifically linked with noncaveolar LRs, but not with caveolae. They concluded that due to the difference in lipid and protein compositions, these two membrane microdomains exhibit different cellular functions. Interestingly, numerous studies indicated that many membrane proteins, like IGF-1R, AQP-1, eNOS, Na<sup>+</sup>/K<sup>+</sup>ATPase, can be detected in both caveolar and noncaveolar LRs. Westwood M *et al.* confirmed that although IGF-1R colocalizes within caveolae, caveolae may not be obligatory for IGF signaling. However, a recent study reported that there was a strong evidence for an association between Cav-1 and IGF-1R in cancer development (84). It was suggested that AQP-1 in mouse hearts is observed in ECs membranes and is localized with caveolae (85). The reduced levels of eNOS in caveolae are caused by a translocation of eNOS from the caveolar fractions to noncaveolar fractions, as studied by Gross and colleagues (86). In addition, some findings showed that caveolar and noncaveolar pools of the kidney Na<sup>+</sup>/K<sup>+</sup>-ATPase have similar catalytic properties,



but that Cav-1 oligomers, annexin-2 tetramers, and oligomers of the  $\alpha, \beta, \gamma$ -protomers of  $\text{Na}^+/\text{K}^+$ -ATPase are able to form a large multiprotein complex in the caveolar pool (87). Recently, some evidence provided new insights into the regulation of noncaveolar clathrin-independent endocytosis by specific caveolar proteins, like caveolins and cavin, which show multiple levels of crosstalks between these pathways (88).

### 3.3. Ceramide and LRs

The hydrophilic headgroups of glycosphingolipids and the hydrophilic parts of cholesterol tightly interact with each other. The ceramide moiety binds to cholesterol via hydrophobic van der Waal interactions. The tight hydrophilic and hydrophobic interactions as well as the high local concentration of sphingolipids and cholesterol contribute to a lateral association between the sphingolipids and the cholesterol, thereby spontaneously forming distinct microdomains. The cholesterol-sphingomyelin (SM)-enriched microdomains seem to exist in a more liquid-ordered or gel like phase that is the unique characteristic of LRs. Ceramide and LRs can co-exist with disordered fluid state membrane domains that rich in unsaturated lipids. Importantly, ceramide enables to stabilize and associate strongly with LRs. Meanwhile, ceramide can also promote unusually large raft domains or platforms formation in plasma membranes.

Ceramide is composed of fatty acid chains in varied length, saturated and hydroxylated, bound to an amino group of a sphingoid base via amide linkage. The fatty acid chain can vary from 2 to 28 carbons and C16- to C24-ceramide are expressed in abundance in mammalian cells. Of note, the biological functions are considered to be associated with ceramides' unique biophysical properties, which include the very small functional group, high melting temperature, very low polarity, strong hydrophobicity and the thermotropic properties (Figure 2). These unique molecular structures result in decreased miscibility with other lipids and are not able to exist in biological fluids or cytosol (89). Furthermore, ceramides mixed with phospholipid monolayers or bilayers mainly exhibit two effects: increasing the order of lipid chains and segregating laterally into rigid domains. Additional effects of ceramides in bilayers contains: increasing membrane permeability, inducing transbilayer motion of lipids and promoting non-lamellar phase formation (90).

It is well known that ceramide molecules can easily and spontaneously bind with each other resulting in the formation of small ceramide-enriched microdomains. Various stimuli are able to induce ceramide-enriched microdomains formation, such as ultraviolet irradiation, cytokines, endostatin, death receptors ligands, LDL and so on. Indeed, accumulating evidence suggested that ceramide-enriched membrane microdomains play an extremely critical role in LRs clustering or platform formations, where the ceramide is produced

by sphingomyelinase (SMases) catalyzed in individual LRs. Ceramide-enriched LRs are involved in a variety of biological processes and in diseases in different cells, including cell growth, differentiation and apoptosis, T-cell activation, tumor metastasis, and neutrophil and monocyte infiltration and so on (89).

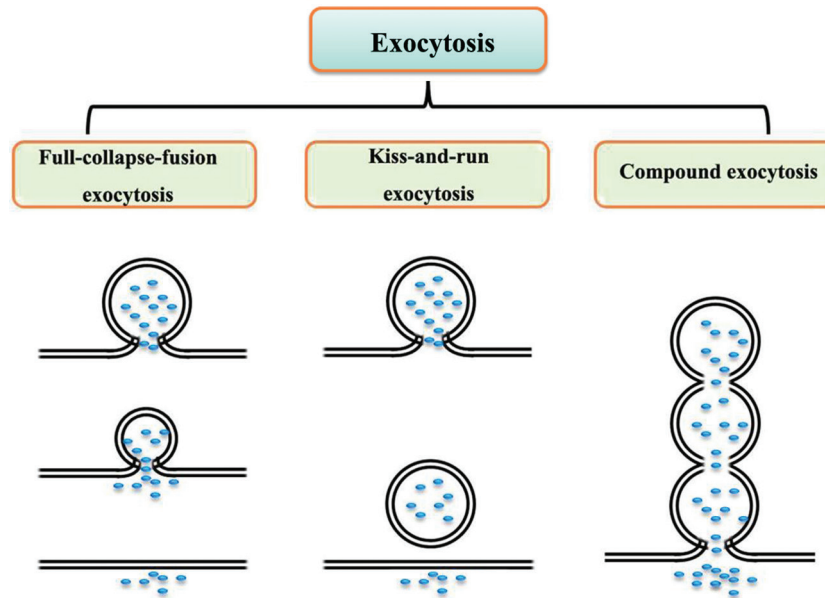
It should be noted that ceramide-enriched membrane platforms can be formed without the classically defined rafts-the small structures enriched in cholesterol, sphingolipids, and associated proteins. As described in Figure 3, ceramide-generation pathways primarily include (89, 91): 1) De novo synthesis pathway: this process is conducted within ER and begins with the condensation of serine and palmitoylCoA; 2) Sphingolipid recycling pathway: glucosylceramidase (GCase) enables to hydrolyze glucosylceramide to regenerate ceramide and SMase, acid or neutral SMase, which can hydrolysis SM to ceramide. 3) Salvage pathway: salvage of free sphingosine by N-acylation by ceramide synthase (CS). 4) Ceramide-1 phosphate pathway: ceramide-1 phosphate can be catalyzed by ceramide-1 phosphate phosphatase (C1PP) to generate ceramide. Furthermore, ceramide-metabolism pathways primarily include (89, 91): 5) SM synthase (SMS) catalyzes the transfer of choline phosphate groups from phosphatidylcholine to ceramide, generating both SM and diacylglycerol; 6) Glucosylceramide synthase (GCS) catalyzes ceramide to glucosylceramide; 7) Ceramidase (CDase) catalyzes ceramide to SM; 8) Ceramide kinase (CK) catalyzes ceramide to generate ceramide-1 phosphate.

## 4. TRANSCYTOSIS MOLECULES ASSOCIATED WITH LRS AND CERAMIDE

### 4.1. Molecular machinery for LR-mediated endocytosis

#### 4.1.1. Caveolae-mediated endocytosis

Caveolar transcytosis is a multi-step process that involves concentrating cargos within caveolae through specific molecular interactions (e.g. receptors), caveolae budding and fission from the plasma membrane and translocation across the cell, followed by docking and fusion with the target plasma membrane, and subsequently discharging cargos into the perivascular space. Our previous study established a proposed model of caveolae-mediated LDL transcytosis across ECs. We observed that C-reactive protein enables to promote LDL transcytosis across ECs (22). Mechanistically, C-reactive protein upregulates PKC and Src activity through increasing the levels of reactive oxygen species (ROS) to enhance the expression of endocytic proteins (Cav-1, Cavin-1, Dynamin 2), and further promote NSF binding with  $\alpha$ -SNAP, following by interaction with its receptor VAMP3, subsequently together with the target receptor Syntaxin-4, and thus fusion with the target plasma membrane, thereby discharging LDL particles from ECs.



**Figure 3.** Schematic modes of exocytosis. There are mainly three modes of exocytosis to regulate the release of cargos: 1) Full-collapse-fusion exocytosis: the vesicles are completely absorbed into the target plasma membrane; 2) Kiss-and-run exocytosis: the vesicles leave the target plasma membrane after the partial release of cargos; 3) Compound exocytosis: the very large and interconnected vesicles are formed via vesicle-vesicle fusion, which thus fuse with the target PM and discharge the cargos from cells.

A more general schematic model of caveolae-mediated transcytosis across cells was illustrated in Figure 4. The cargos are internalized via the receptors located in caveolae. Dynamin (Dyn) and intersectin (ITSN) around the neck region of caveolae induces the fission of caveolae to generate free transport vesicles, which transport cargos along actin-based myosin and microtubule-based motors. Meanwhile, NSF recruits its receptor SNAP and binds with v-SNARE, which further recognizes t-SNARE and triggers docking, thus fusing with the target plasma membrane and discharging cargos from cells. In addition, other important proteins are also involved in regulating membrane transport in eukaryotic cells including Rabs, Munc, Synaptotagmin (Synt) and Complexin (CPX).

#### 4.1.1.1. Receptor clusters in caveolae

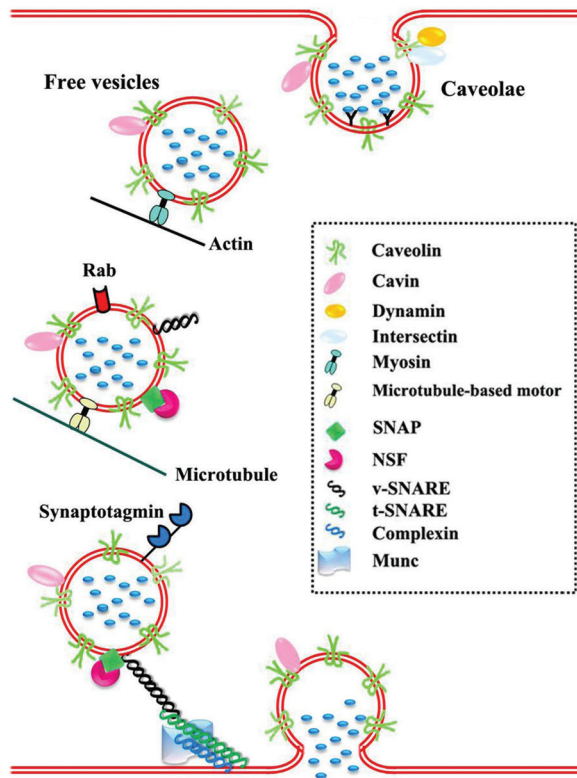
The first step of caveolae-mediated endocytosis is the binding of plasma molecules to specific sites or receptors localized on caveolae or their precursors (uncoated pits). Receptor-mediated transcytosis has been demonstrated for a variety of plasma molecules including LDL, HDL, Tf, CP, insulin, Alb and so on. It is reported that a lot of "life receptors", like LDL-R, HDL-R, Alb-R, Tf-R, CP-R, Ins-R, and "death receptors", such as IL-1-R, p75-R, are located within caveolae (4).

#### 4.1.1.2. Budding and fission mechanisms

Membrane budding requires first the formation of membrane invagination (caveolae) followed by the fission at the neck of the invagination to release discrete

vesicles. Accordingly, the formation of caveolae is primarily driven by three caveolin proteins. Cav-1 (also called vesicular integral membrane protein/(VIP)21) and Cav-2 are co-expressed in most terminally differentiated cells, whereas Cav-3 expression is restricted to smooth and striated muscle cells. Recently, accumulating evidence also suggests that cavinins, identified as caveolar proteins, may also play an important role in caveolae formation. The cavin family includes cavin-1 (poly-merase transcript release factor, PTRF), cavin-2 (serum deprivation protein response, SDPR), cavin-3 (srd-related gene product that binds to c-kinase, SRBC), and cavin-4 (muscle-restricted coiled-coil protein, MURC).

Vinten *et al.* first observed the direct link between cavin-1 and caveolae. They identified cavin-1 as a prominent caveolar protein (92) and found the correlation between the levels of cavin-1 and Cav-1 and the abundance of caveolae. Lack of cavin-1 resulted in the loss of morphologically distinct caveolae and decreased protein stability of all three caveolins. Recently, Regazzetti C and colleagues suggested that hypoxia-inhibited cavin-1 and cavin-2 expression results in the disappearance of caveolae in adipocytes, thereby leading to insulin resistance (93). In addition, cavin-1, localizing to caveolae in both muscle and non-muscle tissues displays a broader tissue distribution than that of either Cav-1 or Cav-3. Importantly, cavin-1 can be recruited by caveolins to plasma membrane caveolar domains and is necessary for caveolae formation (94).



**Figure 4.** A schematic model of caveolae-mediated transcytosis across cells. The cargos are internalized via the receptors located in caveolae. Dynamin (Dyn) and Intersectin (ITSN) around the neck region of caveolae induces the fission of caveolae to generate free transport vesicles, which transport cargos along actin-based myosin and microtubule-based motors. Meanwhile, NSF recruits its receptor SNAP and binds with v-SNARE, which further recognizes t-SNARE and triggers docking, thus fuse fusing with the target plasma membrane and discharging cargos from cells. Other important proteins are also involved in regulating membrane transport in eukaryotic cells including Rabs, Munc, Synaptotagmin (Syt) and Complexin (CPX).

Consequently, the recruitment of cavin-1 to caveolae is considered to stabilize the caveolae.

Cavin-2 was first purified as a phosphatidylserine-binding protein from human platelets, and was considered as a substrate for PKC isoforms (95). Mineo *et al.* found that cavin-2 localizes to caveolae (96). Cavin-2 was also identified as a protein with greater expression upon serum deprivation. Hansen *et al.* found that cavin-2 downregulation would lead to loss of cavin-1 and Cav-1 expression and thus limit caveolae formation, which suggests that cavin-1, cavin-2, and Cav-1 are functionally inter-dependent (97). Furthermore, they also revealed that cavin-2 can promote recruitment of cavin-1 to caveolae (97). Interestingly, cavin-2 would not increase caveolae number but induce elongated caveolar morphology and caveolae-associated tubule formation (97). Taken together, cavin-2 may be a necessary component for inducing membrane-curvature of caveolae.

Earlier studies demonstrated that cavin-3 is a phosphatidylserine-binding protein upon serum starvation and is also a substrate of PKC (98). Based on homology with cavin-1, cavin-3 is also considered to play a role in caveolae formation. Cavin-3 can associate with Cav-1 to form vesicles. Furthermore, lack of cavin-3 would significantly impair intracellular Cav-1 traffic along microtubules (99). However, a recent study indicated that cavin-3 is not absolutely required for making caveolae (100). Cavin-4 has been previously described as a muscle-restricted, coiledcoil protein and able to interact with cavin-2 (101). Cavin-4 expression is predominantly in muscle, and its distribution is impaired in human muscle disease associated with Cav-3 dysfunction, suggesting cavin-4 as a novel candidate caveolar protein (102). Recently, Ogata *et al.* observed that cavin-4 facilitates recruitment of ERK1/2 to caveolae in cardiomyocytes, whereas cavin-4 is not essential for caveolae formation (103).

It is well known that Dyn as the GTPase induces the fission of caveolae to generate free transport vesicles, which can quickly transfer cargos into or even across the cell. The large GTPase Dyn oligomerises to form a spring-shaped, structural collar around the neck of caveolae and binds to Cav-1. To date, three distinct Dyn genes have been identified in mammals and the different dynamin proteins are localized to distinct cytoplasmic or membrane compartments. Dyn I (Dyn 1) is considered as a conventional brain dynamin, Dyn II (Dyn 2) is ubiquitously expressed, and Dyn III (Dyn 3) is restricted to testis, brain, and lung. Of note, ITSN-1s plays a key role in Dyn recruitment to the caveolar necks creating a high local concentration of Dyn, since the GTPase activity of Dyn is allosterically dependent on its protein concentration (104). Similarly to Dyn, ITSN-1s is found to associate preferentially with the neck region of caveolae (104). Moreover, evidence supported that ITSN-1s is required for caveolae function and efficient transendothelial transport (104).

#### 4.1.2. Flotillin-mediated endocytosis

The mechanism of flotillin-dependent endocytosis is similar to that involving in caveolae-dependent endocytosis. However, lacking of caveolins, vesicles in this pathway are enriched in flotillin-1 (reggie-2) and flotillin-2 (reggie-1) transmembrane proteins (6), which are first found in retinal ganglion cells from fish. Notably, it is suggested that lack of caveolae and Cav-1, the coassembly of flotillin-1 and flotillin-2 is not sufficient to induce caveolae-like invaginations formation (105). Flotillins are reported as LR proteins in various cell types, like neurons, astrocytes. Flotillin-mediated endocytosis is regulated by Src family kinase Fyn phosphorylating flotillins. There exists some controversies that whether flotillin-dependent endocytosis is in a Dyn-dependent manner. Previously, evidence for flotillin-dependent endocytosis involved in



the uptake of the cholera toxin B and the GPI-anchored protein (GPI-AP) CD59 in COS-7 cells suggested that flotillin-mediated endocytosis is Dyn-independent (106). A recent study indicated that Dyn-2 activity is required for flotillin-dependent endocytosis upon epidermal growth factor (EGF) stimulation, and inhibition of Dyn-2 GTPase activity decreases flotillin-mediated endocytosis (107). In addition, overexpression of Dyn-2 mutants or lack of clathrin, flotillins are permanently trapped in endosomes (107).

### 4.1.3. GRAF1-regulated endocytosis

This endocytic pathway was first introduced by Sabharanjak and colleagues and indicated that GPI-APs are transported by a distinct cell division cycle 42 (Cdc42)-dependent, clathrin-independent pinocytic pathway. It was suggested that the GRAF1-dependent uptake pathway is responsible for the constitutive fluid uptake (>70%) in NIH 3T3 cells. Moreover, this endocytic pathway involves clathrin-independent carriers (CLICs) delivering cargos to GPI-APs enriched early endosomal compartments (GEECs), therefore it is also called CLICs/GEECs pathway (108). Vesicles in GRAF1-mediated endocytosis are termed GEECs, which is different from caveolae and CCVs and are not coated with caveolins or clathrin (6). Furthermore, Lundmark *et al.* proposed that GRAF1 would serve as a specific protein marker of this uptake route (109). Cholera toxin B is also a known marker for the CLICs/GEECs (6). Howes *et al.* reported that actin and GRAF1 promote the initial formation of the CLICs (110). In addition, during vesicle formation in Golgi, combination Cdc42 with Arf1 is able to drive actin polymerization, indicating that Arf1 may also be responsible for vesicle budding (111). Meantime, some reports supported the view that Dyn is involved in GRAF1-mediated endocytosis (110).

### 4.1.4. Arf6-regulated endocytosis

The uptake of class I major histocompatibility complex, CD1a,  $\beta$ 1-integrin E-cadherin, prion protein, coxsackievirus type B3 and the hERG (human ether-a-go-go related gene) potassium channel (Kv11.1.) is an Arf6-dependent, Dyn-independent pathway, as reviewed by Hideyoshi Harashima *et al.* (6). However, the uptake of Coxsackie virus A9 (112) and the green fluorescent protein fused with the herpes simplex virus protein VP22 (113) are shown to be an Arf6-mediated, dynamin-dependent pathway. Vesicles in Arf6-mediated endocytosis are considered to be coated with actin (114). Stefano Confalonieri and co-workers showed that the primate-specific protein TBC1D3 is required for optimal macropinocytosis in Arf6-mediated endocytosis (115). Recently, Tebar F and colleagues observed that small GTPase Rac1 and the main cellular  $\text{Ca}^{2+}$  sensor calmodulin interactions can regulate dynamics of Arf6-dependent endocytosis (116). Of note, they confirmed that Rac1 may be essential for the scission of vesicles (116).

### 4.1.5. RhoA-regulated endocytosis

It was suggested that RhoA-dependent endocytosis is of high specificity for a limited number of cargos. The uptake of IL-2R, A $\beta$ 42, dopamine, *Clostridium* toxins,  $\gamma$ c cytokine receptor and Alb was shown to follow clathrin-independent, dynamin-mediated, and RhoA-dependent endocytosis, as described in the review of Hideyoshi Harashima *et al.* (6). Some molecules are reported to function as regulators of this uptake pathway. This pathway is also sensitive to cholesterol depletion. Coractin, Rac1 and its downstream p21-activated kinases, PAK-1 and PAK-2 are also able to regulate this uptake pathway (117). Recently, DLC3, a functional Rho-specific GTPase-activating protein is also found to coordinate endocytic membrane transport by regulating Rho activity (118).

### 4.1.6. Other molecular mediators for LRs-dependent endocytosis

#### 4.1.6.1. Annexins (Anxs)

Anxs are a family of membrane-binding proteins that can interact preferentially with membrane phospholipids and intracellular protein partners in a  $\text{Ca}^{2+}$ -dependent manner, which are believed to be involved in membrane domain formation and membrane transport. Several members of Anxs family, in particular AnxA1, AnxA2, AnxA6 and AnxA8, have the ability to bind with their interacting partners in specific membrane microdomains resulting in specific signaling platforms formation during the endocytic pathway. AnxA1 was not required for multivesicular endosome/body formation and internal vesicle formation did not absolutely require AnxA1. However, under EGF stimulation, AnxA1 was necessary for internal vesicle formation (119). The data of Pollard HB *et al.* strongly suggested that AnxA1 is able to induce vesicle fusion *in vitro* and may play an important role in vesicle scission (120). Han DK and colleagues revealed that AnxA1 binding to phosphorylcholine in the plasma membrane of apoptotic cells can promote recognition and uptake of apoptotic cells by ECs (121). Consistent with Han DK, Divangahi *et al.* also reported that AnxA1, as an endogenous engulfment ligand, has the ability to enhance the phagocytosis of apoptotic cells (efferocytosis) by DCs during *Mycobacterium tuberculosis* infection (122).

It was reported that AnxA2 inducing LRs-dependent trafficking is associated with some proteins, including AQP2, transient receptor potential vanilloid type 5/6 channel, proprotein convertase subtilisin/kexin-type 9, EGFR, tetrodotoxin-resistant sodium channel Na(V)1.8 and furosemide-sensitive  $\text{Na}^{+}$ - $\text{K}^{+}$ -2Cl $^{-}$  cotransporter. In cells, AnxA2 is known as a monomer mainly locating in cytosol and on early endosomes (EEs) or as a heterotetramer (AnxA2-S100A10 heterotetramer; A2t) with S100A10 in the subplasmalemmal region. AnxA2 is a major component of LRs and of purified caveolae colocalizing with Cav-1. Indeed, AnxA2 can not



only bind membrane phospholipids in the presence of  $\text{Ca}^{2+}$  but also aggregate lipid vesicles (123). Furthermore, Gerke *et al.* also demonstrated that all AnxA2 derivatives have the ability of promoting LRs formation (123). Using giant unilamellar vesicles and Texas Red-labeled A2t, Gokhaleet and co-workers observed that A2t can promote 1-microm-sized pots on the giant unilamellar vesicles membrane (124). However, due to their strong affinity to phospholipids, in particular AnxA2 and AnxA6 are also found in non-raft domains such as clathrin-coated pits (125). Recently, AnxA2 are also been reported to play an important role in the mesothelial phagocytosis of crocidolite (126).

AnxA6 is involved in receptor-mediated endocytosis as well as LR/caveolae formation (127). Evidence indicated that different mechanisms are involved in the effect of AnxA2 and AnxA6 on the structural and functional changes at the plasma membrane. Cubells *et al.* described that overexpression of AnxA6 would induce cholesterol accumulation in late endosomes, resulting in cholesterol diminution of the plasma membrane and Golgi apparatus, thus decreasing the number of caveolae at the plasma membrane (128). Furthermore, AnxA6 seems to not only serve as a scaffold for several signaling proteins including  $\text{PKC}\alpha$  and p120GAP (129), but also interact with cytoskeleton proteins, like actin and spectrin (130). Kamal *et al.* previously reported that AnxA6 appears to regulate receptor-mediated endocytosis via spectrin for the internalization of LDL (130). Overexpression of AnxA6 increases the trafficking of LDL to the prelysosomal compartment. In addition, a recent study confirmed that cells expressing AnxA6 can decrease the level of plasma membrane cholesterol and can affect the actin cytoskeleton meshwork, which influences the membrane order (131). In summary, with the combination of cholesterol and actin-mediated events, AnxA6 is able to influence the membrane order, the recruitment of signaling proteins to the plasma membrane as well as modulating transient membrane-actin interactions during endocytic and exocytic transport. In contrast to the widely expressed AnxA1, AnxA2 and AnxA6 in human, AnxA8 is only found in lung, skin, liver and kidney in low amounts. So far, AnxA8 is yet poorly characterized. AnxA8 is reported to interact with phospholipid and F-actin to specifically regulate late endosomes organization and function (132).

### 4.1.6.2. Syndecan (Syn)

Early studies showed that chylomicron remnants binding to LDLR-related protein at the surface of hepatocytes may be stabilized by colocalizing with Syn-1, eventually followed by endocytosis. A study by Orlando *et al.* demonstrated that Syn-1 mediates the uptake of apoE-VLDL in transfected human fibroblasts via clathrin-independent, LDLR-related protein -independent pathway, which is dependent on LRs function (133). In addition, Fuki and co-workers revealed that Syn-1, Syn-2,

and Syn-4 all enhance lipoprotein lipase dependent LDL binding, internalization, and degradation in transfected Chinese hamster ovary cells (134). Durocher *et al.* observed that overexpression of Syn-1 could slightly increase PEI-mediated gene expression. Furthermore, by using LRs inhibitor M $\beta$ CD, they determined that integrity of LR is essential for the clustering of Syn-1 and Syn-2 (135). Notably, Kevin Jon Williams revealed the molecular mechanisms for Syn-1-mediated endocytosis, including (136): a) ligands stimulate rapid M $\text{KKK}$  (a conserved juxtamembrane motif in Syn-1)-dependent activation of ERK, which subsequently results in the dissociation of Syn-1 from  $\alpha$ -tubulin and the localization of Syn-1 into LRs; b) tyrosine phosphorylation of Syn-1 by Src family kinases triggers the robust recruitment of cortactin, which is proven to be an essential mediator for efficient actin-dependent endocytosis.

Evidence previously reported that Syn-4-mediated endocytosis is via a Cdc42-dependent macropinocytic pathway (137). Webb *et al.* confirmed that Syn-4 could induce LDL modification by group V secretory phospholipase  $\text{A}_2$  uptake by macrophages, possibly through macropinocytosis (138). Of note, they found that Syn-3 deletion is not able to inhibit the uptake of LDL modification by group V secretory phospholipase  $\text{A}_2$  (138). Recently, Ramji DP and colleagues indicated that eicosapentaenoic acid and docosahexaenoic acid decreased the uptake of acetylated LDL by human macrophages may involve the inhibition of macropinocytosis and Syn-4 expression (139).

## 4.2. Trafficking motors

Transport in the cell is achieved by molecular motors, which transport cargos along microtubules. Depending on the intrinsic polarity, microtubules drive both short- and long-range intracellular transport. Microtubules possess a dynamic plus end that grows towards the periphery in most cells, and a minus end located near the nucleus in non-dividing cells, which is anchored in the microtubule organizing centre. There are two main types of microtubule-based molecular motor, including kinesins and dyneins (140). Kinesins walk to the plus end, whilst dyneins move towards the minus end. It is suggested that kinesin-1 functions on the transport of secretory vesicles, while kinesin-3 is involved in the transport of secretory vesicles, EEs, peroxisomes, and ribonucleoprotein complexes (141-143). Accordingly, these two sets of molecular motors are both involved in caveolae- and clathrin-dependent endocytic shuttling (144).

In addition, actin-based myosin is another type of molecular motor, which drive endocytic or exocytic movement along actin filaments. Actin filaments also exhibit polarity and generally support short-range transport in cells. Actin contains the barbed and pointed end, which is similar to the plus and minus end of microtubules, respectively. It is reported that myosins I,

V and VI are associated with endocytic trafficking (145). Actin filaments are well known to be particularly required during phagocytosis and clathrin-dependent endocytosis (146). It is considered that actin generates a larger force for internalization to assist uptake. Of note, actin filaments are not always essential in mammalian cells. Swaan PW *et al.* emphasized that vesicular trafficking driven by kinesins and dyneins could regulate receptor-mediated internalization. Cortical actin may play an accessory role through its interaction with myosin in endocytic translocation, which cannot be dismissed (144). Additionally, microtubules, actin filaments and motor proteins are all considered to deform and assist in the scission of membranes, thereby promoting endosomal sorting and transport intermediates generation (147).

### 4.3. Molecular machinery for LR-mediated exocytosis

As illustrated in Figure 3, there are mainly three modes of exocytosis to regulate the release of cargos: a. Full-collapse-fusion exocytosis: the vesicles are completely absorbed into the target plasma membrane; b. Kiss-and-run exocytosis: the vesicles leave the target plasma membrane after the partial release of cargos; c. Compound exocytosis: the very large and interconnected vesicles are formed via vesicle-vesicle fusion, which thus fuse with the target plasma membrane and discharge the cargos from cells. No matter what modes of exocytosis, the molecular machinery for membrane fusion and docking are key steps in this process.

#### 4.3.1. The SNARE hypothesis

The “SNARE hypothesis” describes the mechanism by which the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) drive membrane docking and fusion through interactions with an ATPase, N-ethylmaleimide sensitive factor (NSF) and its adaptor proteins, soluble NSF attachment protein (SNAPs). Pamela L. Tuma and L. Hubbard previously reviewed that there are three families of proteins which play key roles in vesicle targeting and fusion (5), a) SNARE complex; b) the Sec1/Munc18 proteins; c) small GTP-binding proteins, the Rabs. Notably, NSF recruits its receptor SNAP and then binds with v-SNARE (vesicular SNARE), which in turn recognizes and docks with t-SNARE (target-membrane SNARE), eventually forming the functional NSF/SNAP/SNARE (20S) supercomplex for membrane fusion (148).

#### 4.3.2. SNARE complex

SNAREs are a superfamily of proteins which include 25 members in *Saccharomyces cerevisiae*, 36 in *Homo sapiens*, and 54 in *Arabidopsis thaliana*. Most SNAREs share a common SNARE motif that comprises 60-70 amino acid residues that contain  $\alpha$ -helix-forming heptad repeats with a central “0” layer, which assemble into a 4-helix bundle consisting of 16 layers of interacting

amino acids called the SNARE complex (*trans*-configuration) (149). Of note, SNAP-25 and its homologs (SNAP-23, -29, and -47) contain two SNARE motifs. The core SNARE complex in the 4-helix bundle is formed by the ionic interactions of amino acids from the coiled-coil SNARE motifs: 1 from Syntaxin, 1 from VAMP, and 2 from SNAP-25 or other SNAP proteins. The SNARE complex is found in two forms, the fully assembled complex-*cis*-SNARE complex and the loosely assembled form-*trans*-SNARE complex. According to the zipper hypothesis, membrane fusion is mediated by the *trans*-SNARE complex, which can bridge the vesicle and target membranes mediating vesicle docking and fusion (150). During SNARE complex assembly, the energy released provides the power overcoming the energy barriers to membrane fusion in that sense. SNARE proteins are considered to be the true fusion motors.

Based on the key residue in the central “0” layer, SNARE proteins are classified as Q-SNAREs and R-SNAREs. Furthermore, Q-SNAREs contain t-SNAREs—the Syntaxin subfamily and SNAP-25 subfamily as they contribute a glutamine (Q), while R-SNAREs are often referred to as v-SNAREs, the vesicle associated membrane proteins (VAMPs) or synaptobrevins as they contribute an arginine (R). Additionally, based on the amino acid sequence of the SNARE motifs, Q-SNAREs can also be classified as Qa, Qb or Qc SNAREs (151). In general, Qa SNAREs are often known as the Syntaxin subfamily. Qbc SNAREs include SNAP-25 or -23, -29 or -47 (Qb, N-terminal motif and Qc, C-terminal motif). v-SNAREs, communicating with its targeting receptors located on the vesicles, have a variety of N-terminal extensions, like a proline-rich flexible 35-residue region in VAMP2/synaptobrevin-2, and about 120-residue longin domain in VAMP7, Sec22b, and Ykt6 (152). t-SNAREs, interacting with the receptors on the target membrane, have an additional ~25-residue long N-terminal peptide, followed by the Habc domain with a three-helix bundle.

To date, the VAMP family is composed of isoforms 1, 2, 3 (cellubrevin), 4, 5, 7 (initially known as tetanus toxin insensitive TI-VAMP), 8 (endobrevin), Sec22b and ykt-6. In addition, at least 18 Syntaxin family members have been identified in mammals, of which five (STX 1A, 1B, 2-4) are plasma membrane-specific isoforms. More importantly, VAMPs together with Syntaxins are distinct from their functions and expressions according to different species and tissues. Neuronal SNARE complex including synaptobrevin-1/VAMP-1, Syntaxin, and SNAP-25 were first identified in studying fusion process in neuronal exocytosis. VAMP1 expressing in sensory neurons could modulate pain-peptide exocytosis (153), and mediate acetylcholine release in motor neurons (154). VAMP2 is able to regulate exocytosis of neurotransmitters from small vesicles in the brain (155). Kavalali ET *et al.* suggested that reelin, a glycoprotein being critical for proper layering of neocortex, can selectively augment

spontaneous neurotransmission via VAMP7 and SNAP-25 but not VAMP4 or VAMP2 (156). Furthermore, studies demonstrated that the SNARE complex consisting of the VAMP-2, SNAP-23 and Syntaxin-4 are involved in GLUT4 trafficking and vesicle fusion in adipocytes (157). VAMP4 is reported to be implicated in GLUT4 sorting but not exocytosis in adipocytes (158), and mediates insulin-induced CD36 translocation in cardiomyocytes (159). Rose and colleagues revealed that VAMP2, VAMP5 and VAMP7 play critical roles in docking and fusion of the GLUT4 transporters at the surface membrane in skeletal muscle (160). However, others indicated that VAMP3, VAMP8 or VAMP5 acting as the major v-SNARE, might also link to GLUT4 trafficking.

In a recent study, we also investigated that VAMP3 and Syntaxin-4 are involved in LDL transcytosis in ECs (22). Recently, studies by Williams and co-workers suggested that VAMP7, SNAP23 and Syntaxin-4 could mediate the transport of membrane type 1-matrix metalloproteinase into invadopodia (the subcellular degradative structures) to accelerate matrix degradation and cell invasion (161). VAMP-8 is enriched on the membrane of zymogen granules and is required for zymogen granules exocytosis together with Syntaxin-4 and SNAP23 in acinar cells (162). As a specific regulator of antigen crosspresentation, Sec22b and Syntaxin-4 are believed to participate in Ag export to the cytosol and phagosomal degradation in DCs (163). Ykt-6 is also involved in intracellular vesicular protein sorting and membrane fusion (164).

In pancreatic  $\beta$  cells, Syntaxin-1, Syntaxin-2 and Syntaxin-4 are mainly located on plasma membrane, while Syntaxin-3 is rich in secretory insulin-containing granules (SGs). The process of glucose stimulated insulin secretion (GSIS) began with a rapidly initiated and transient first phase (5-10 min), followed by a sustained second phase (several hours). Evidence indicated that Syntaxin-1A and Syntaxin-4 are essential for docking and fusion of insulin granules in the first phase. Recently, Li *et al.* confirmed that Syntaxin-4 mediates exocytosis of pre-docked and newcomer SGs during both the first and second phase in human pancreatic  $\beta$  cells (165). Early results revealed that Syntaxin-2 is probably involved in insulin exocytosis in pancreatic  $\beta$  cells (166). The overexpression of Syntaxin-3 would promote biphasic GSIS by increasing newcomer SGs exocytosis. By contrast, endogenous Syntaxin-3 depletion by RNA interference inhibited the recruitment and fusion of newcomer SGs during the first and second phases of GSIS, with no effect on pre-docked SGs (167). Additionally, early studies demonstrated that Syntaxin-2 and VAMP8 are implicated in cytokines exocytosis (168). Similarly, Syntaxin-3 is found to be essential for granule exocytosis and a variety of cytokines secretion, including IL-1 $\alpha$ , IL-1 $\beta$ , IL-12b, and CC chemokine 4 (169). Ye and colleagues found that the SNARE complex formed

by VAMP-8, Syntaxin-11 and SNAP-23 is required for platelet exocytosis (170). Recently, Poole AW *et al.* also indicated that Syntaxin-8 can regulate platelet granule secretion (171).

### 4.3.3. NSF and SNAP

NSF was the first protein found to play an essential role in eukaryotic trafficking. The well-documented role of NSF is to act as a chaperone to be strongly linked to the assembly-disassembly cycle of SNARE complex in vesicular transport, although additional roles of NSF have been investigated. NSF is a homo-hexamer with each promoter consisting of an N-terminal domain (NSF-N), and two adjacent AAA-domains (NSF-D1, NSF-D2). NSF-D1 is the catalytic ATPase domain, and NSF-N is responsible for SNAP-SNARE binding (148). As depicted above, the *trans*-SNARE complex triggers membrane fusion process. After fusion, once the *trans*-SNARE complex converting into the *cis*-SNARE complex, SNAPs binding to SNARE complex and recruiting NSF drive SNARE complex disassembly for another round of fusion in an ATP-dependent manner (150). Accordingly, NSF ATPase activity can be inhibited by alkylating (e.g. N-ethylmaleimide), S-nitrosylation and phosphorylation.

In mammals, the proteins in SNAP family are composed of  $\alpha$ -SNAP,  $\beta$ -SNAP and  $\gamma$ -SNAP. Both  $\alpha$ -SNAP and  $\gamma$ -SNAP have a much broader tissue distribution while  $\beta$ -SNAP is a brain-specific isoform. In human,  $\alpha$ -SNAP and  $\beta$ -SNAP share ~ 85% sequence identity;  $\gamma$ -SNAP only shares 20-25% sequence identity.  $\alpha$ -SNAP is proven to be associated with the formation of the *cis*-SNARE complex, which binds to SNARE complexes in an antiparallel manner. Our study and others suggested that NSF and its adaptor protein  $\alpha$ -SNAP are associated with caveolae and exert an important function on transcytosis across ECs (5, 22).  $\beta$ -SNAP is known to be involved in regulating neurotransmitter release and constitutive vesicular transport. Yang *et al.* revealed that the expression of  $\beta$ -SNAP in brain is significantly downregulated in the mouse model of AD (172). As a matter of fact, the intrinsic ATPase activity of NSF is extremely low. Once interacting with SNAP, the ATPase activity is upregulated. Moreover, the maximal ATPase activity is only achieved following the formation of NSF/SNAP/SNARE complex (173).

### 4.3.4. Other regulators for vesicle exocytosis

A large and diverse array of proteins is involved in regulating membrane transport in eukaryotic cells including Rabs, Munc-18, Munc-13, Syt and CPX.

#### 4.3.4.1. The Rabs

The Rabs belong to the largest family of small molecular mass (21-25kDa) GTP binding proteins, which appear to continuously cycle between the membranes (in an active GTP bound state) and the cytosol (in an



inactive GDP-bound state). 11 Rabs have been identified in *Saccharomyces cerevisiae*, 90 in *Entamoeba histolytica*, and 29 in *Caenorhabditis elegans*, 57 in *Arabidopsis thaliana*, 33 in *Drosophila melanogaster* as well as 70 in *Homo sapiens*. Moreover, most of the Rabs are ubiquitously expressed proteins and are implicated in various cellular functions, such as growth, transmembrane signal transduction, protein trafficking etc. Given that Rabs are efficient membrane microdomain organizers, Rabs are considered to be one of the best markers for different intracellular compartments in the steady state. Rabs have a central function on vesicle formation (endocytosis), vesicle motility (transport) and fusion (exocytosis) at target plasma membrane, as summarized in Table 1. Of note, Rabs primarily regulate membrane transport through the recruitment of cytosolic effector proteins. The cyclical activation, inactivation and translocation of Rabs are mainly modulated by three types of upstream regulators: Guanine Nucleotide Exchange Factors (GEFs), GAPs, and GDP-Dissociation Inhibitors (GDIs). Furthermore, GEFs serve as positive regulators whereas GAPs act as negative regulators. GDIs recycle back Rabs as Rab-GDP complex at donor membrane. In addition, current understanding about downstream effector proteins of Rabs has also been outlined in Table 1.

It is reported that nearly three-quarters of the 70 human Rab GTPases are involved in endocytic trafficking. Rab5 and Rab7 are the first mammalian Rabs found to be important regulators in endocytic pathway (174). Accordingly, Rab4, Rab5, Rab11 and Rab35 function on the early endocytic/recycling pathway, whereas Rab7, Rab9 and Rab22b/Rab31 are considered to regulate the late endocytic pathway. For example, Rab5 is located on the plasma membrane, participating in the formation of CCVs, the trafficking to the EEs and homotypic fusion between EEs (175, 176), while Rab7 and Rab9 mainly regulate transport from early to late endosomes and lysosomes. Rab4 and Rab11 could regulate the transport along the recycling pathway, from early and recycling endosomes to the cell surface (177).

Furthermore, Rabs have the capacity of modulating motors-driven transport and subsequent fusion of intracellular structures in a spatiotemporal manner. More importantly, Rabs communicate with motors by the intermediary protein. It was first hypothesized that Rabs might be directly associated with the cytoskeleton by observing the colocalization of Rab8 and actin and actin-driven transport of Rab8-positive vesicles. Recent studies indicated that myosin Va as an effector of Rab8A is involved in Rab8A-regulated GLUT4-containing vesicles exocytosis to the muscle cell surface (178). Rab6a is reported to regulate the recruitment of the dynactin (a protein that binds dynein directly) complex to Golgi membranes and is essential for activation of idling dynein (179). Rab4 and Rab5 are shown to regulate

GLUT4 exocytosis and endocytosis via interaction with kinesin and dynein motors (180). Additionally, Mathieu Kurowska *et al.* observed that an effector of Rab27a in cytotoxic T lymphocytes, synaptotagmin-like protein 3 is able to interact with kinesin-1 via the tetratricopeptide repeat of the kinesin-1 light chain (181).

Given that SNARE interacting proteins are effectors of Rabs, the function of Rabs are essential for fusion to the target membrane. Early studies suggested that SNARE function regulated by Rabs is exemplified by interaction of Rab5 effector endosome associated antigen (EEA1) with Syntaxin-13, which is essential for homotypic EEs fusion (182). What is more, NSF is required for Rab effector-SNARE interaction and effector complex formation. It was also suggested that modulating the Rab3A interacting molecule at the presynaptic active zone is able to regulate synaptic vesicle exocytosis (183). However, the exact functions of these interactions are yet poorly understood.

### 4.3.4.2. Munc18

SNARE-associated protein Munc18/Sec1p is believed to be essential for SNARE complex formation. It is well known that serving as the molecular chaperone of Syntaxins, Munc18 proteins at the plasma membrane are key regulators in the localization and expression of Syntaxins. In mammals, plasma membrane-associated Munc18 proteins contain Munc18-1, Munc18-2, and Munc18-3 (also known as Munc18a/STXBP1, Munc18b/STXBP2 and Munc18c/STXBP3, respectively); nonplasma membrane-associated mammalian proteins include mVps45 and mSly1. Endogenous Munc18-1 and Munc18-2 are able to interact with Syntaxin 1-3, whereas Munc18-3 binds only to Syntaxin-4 with high affinity.

The precise regulatory roles of Munc18 in membrane fusion are still highly debated. Some literatures about neuronal Munc18 proteins suggested that Munc18-1 negatively regulates membrane fusion by binding to the closed Syntaxin-1a and subsequently inhibiting the formation of SNARE complex (184). However, some confirmed that Munc18-1 has extremely important functions on priming/stimulating SNARE-mediated membrane fusion exocytosis through direct interaction with the SNARE complex (185). Accordingly, insulin secretion is linked to various Munc18-Syntaxin isoform pairs, while insulin action in the peripheral tissues appears to use only the Munc18-3-Syntaxin 4 pair. Recently, Shuzo Sugita *et al.* demonstrated that Munc18-1 and Munc18-2 (Munc18-1/2 DKD) in mast cells significantly reduce degranulation and the levels of Syntaxin-3 through interaction with the “closed” form of Syntaxin-3. Moreover, knockdown of Syntaxin-3 largely impaired degranulation (186). Nonetheless, binding of Munc18-1 to the “closed” Syntaxin-1a seems to drive the transport of Syntaxin-1a from the ER to the plasma membrane. Furthermore, after Syntaxin-1a arrives at the



**Table 1.** The Rabs involved in transcytosis

Rab	Localization	Function	Main effectors
Rab1	ER-Golgi intermediate	ER-Golgi trafficking	SIDD/M, LEPB, LIDA
Rab2	ER-Golgi intermediate	ER-Golgi trafficking	RICA, ICA69, CCCP-1, GRASP-GOLGIN
Rab3	TGN-apical-lateral membranes	Exocytosis of secretory granules and vesicles from TGN to apical-lateral membranes	NOC2, RIM1/2, SYNAPSIN, DENN/MADDw
Rab4	Early and recycling endosomes, CCV	Endocytic recycling to plasma membrane	AKAP10, CD2AP, FUFY1, GRIPAPA, RAB11FIP1, RABEP1, Rabenosyn-5, ZFYVE20-VPS45
Rab5	CCV, early endosomes, phagosomes	Endocytic internalization and early endosome fusion	ANKFY1, APPL1/2, EEA1, F8A1, OCRL, INPP4A/5B, PIK3CB/3R4, VPS8, RABEP1/2, ZFYVE20-VPS45
Rab6	Golgi	Golgi to plasma membrane transport, Golgi to endosome transport, Intra-Golgi transport and ER-Golgi transport	APBA3, BICD1, CCDC64, DENND5A, GOLGB1, GCC2, OCRL, MYH9, KIF20A, VPS52
Rab7	Late endosomes, lysosomes, autophagosome	Early to late endosome transport, late endosome to Golgi transport	FYCO1, KIAA0226, OSBPLIA, PIK3R4, PSMA7, RILP, RNF115, VSP35/41
Rab8	Golgi, TGN, recycling endosomes, GLUT4 vesicles	Basolateral protein transport from Golgi and TGN, GLUT4 vesicle translocation	MAP4K2, MICALL1/2, MYO5B, OCRL1, ODF2, PXR1, SYTL1
Rab9	Late endosomes	Late endosomes to TGN transport	TIP47, P40, GCC185
Rab10	Golgi, TGN, GLUT4 vesicles	Basolateral protein transport from Golgi and TGN, TGN to plasma membrane trafficking, GLUT4 vesicle trafficking	MICAL1, RIMS1, MYO5A/B/C
Rab11	Golgi, TGN, recycling endosomes	Transport from Golgi, apical and basolateral, endocytic recycling	DEEND5A, EXOC6, MYO5B, PI4KB, WDR44, RAB4IP/L1, TBC1D14, RAB11FIP1/2/3, SH3TC2
Rab12	recycling endosomes, lysosomes	Transport from peripheral region of cell to perinuclear centrosomes, transport to lysosomes	No data
Rab13	Early endosomes and tight junctions	Recycling endosome to plasma membrane transport, GLUT4 trafficking	LEPR/OBR, LEPROT, MICALL1/2
Rab14	Early endosomes and Golgi	Transport between early endosomes and Golgi	KIR16B, RUFY1, ZFYVE20
Rab15	Early and recycling endosomes	Inhibitor of endocytic internalization	RABIF, REP15
Rab17	Recycling endosomes and melanosomes	Transport through apical recycling endosomes and melanosome trafficking	No data
Rab18	ER-Golgi intermediate	ER-Golgi trafficking	No data
Rab20	Apical dense tubules, endocytic structures	Apical endocytosis/recycling	No data
Rab21	Early endosomes	Endocytic internalization	APPL, ITGA2/11
Rab22	Early endosomes, TGN	Transport between TGN and early endosomes	EEA1, RABGEF1, TBC1D2B, OCRL
Rab23	Plasma membrane and early endocytic vesicles	Transport between plasma membrane and early endocytic vesicles, phagosome-lysosome fusion	No data
Rab24	Autophagosomes and mitotic spindle	Autophagy-related process	GABARAP
Rab25	Apical recycling endosome	Transport through apical recycling endosomes, ER to plasma membrane transport	MYO5B, RAB3IP, RAB11FIP1/2/3/4/5
Rab26	Secretory granules and TGN-apical-lateral membranes	Exocytosis of secretory granules and vesicles	RIMS1

(Cond..)

**Table 1.** (Continued....)

Rab	Localization	Function	Main effectors
Rab 27A	Melanosomes, secretory granules	Dynamics of lysosome-related organelles and secretory granules	CORO1C, MLPH, MYO5A, MYRIP, SYTL1-5, ANKRD7
Rab31	Early endosomes, TGN	Transport between TGN and early endosomes	OCRL1, EEA1, GAPEX5
Rab32	Melanosomes, autophagosome, Tyrp1-containing organules	Trafficking of melanogenic enzymes to melanosomes, autophagy	ANKRD7, PRKAR2A
Rab33	Golgi, autophagosome	ER-Golgi and intra-Golgi transport and autophagy	ATG16L1, GOLGA2, RABEP1
Rab35	Plasma membrane, CCV, early recycling endosomes	Apical endocytic recycling	ACAP2, FSCN1, OCRL, MICALL1
Rab37	Golgi and secretory granules	Mast cell degranulation, TNF- $\alpha$ secretion	RIMS1, UNC13B
Rab38	Tyrp1-containing organelles, Melanosomes	Transport of tyrosinase to immature melanosomes	ANKRD7
Rab39	Golgi, endosomes and phagosomes	IL-1 and myocilin secretion	CASP1, MYO5A, UACA
Rab43	ER-Golgi intermediate	ER-Golgi trafficking	TBC1D20, TNTRE

target site, Munc18-1 is thought to promote the SNARE-complex assembly and thus facilitate membrane fusion. Recently, Brochetta C *et al.* revealed that Munc18-2 could directly interact with tubulin to promote secretory granules' translocation and Syntaxin-4 could enhance membrane fusion in mast cells (187).

So far, four proposed interaction modes have been used (188): 1) Munc18 binds to a "closed" form of Syntaxins; 2) Munc18 binds to an "open" form of Syntaxins; 3) Munc18 binds to the N-peptide of Syntaxins; 4) Munc18 binds to the assembled SNARE complex. Thus, due to different experimental design and proteins construct *in vitro*, conflicting roles of Munc18 are probably reported in different laboratories.

#### 4.3.4.3. Munc13

In mammals, the family of Munc13 proteins is composed of Munc13-1, Munc13-2, Munc13-3 and Munc13-4. Munc13-2 isoform exists in two splice variants, bMunc13-2 and ubMunc13-2. Munc13-1, bMunc13-2 and Munc13-3 are primarily expressed in neurons and neuroendocrine cells. Furthermore, all these isoforms contain a calmodulin-binding domain and a diacylglycerol-binding C1 domain that function to prime synaptic vesicles for the  $\text{Ca}^{2+}$ -dependent exocytosis. Munc13-4 exhibits strong expression in lung and spleen and lacks regulatory domains. Jens Rettig and colleagues indicated that Munc13-1 and Munc13-4 are functionally rich in cytotoxic T lymphocytes and are required for lytic granules secretion (189). Compared with brain slices from wild-type mice, slices from Munc13-1 KO and Munc13-1/2 KO mice are observed to significantly inhibit phorbol esters-stimulated APP release, whereas this effect is not found in Munc13-2 KO mice. Josep Rizo *et al.* proposed a mode in neurotransmitter release and revealed that Munc13 has ability of opening Syntaxin-1

and initiating *trans*-SNARE complex assembly together with Munc18-1, and the resulting Munc18-1-Munc13-SNARE assembly enables fast membrane fusion through the action of Synaptotagmin-1 and  $\text{Ca}^{2+}$  (190).

#### 4.3.4.4. Syt and CPX

It has been shown that CPXs enable to interact with assembled heterotrimeric SNARE complexes. CPX1 can bind to Syntaxin/SNAP25 binary complexes. Furthermore, it is found that Munc18-1 and CPX1 are able to simultaneously bind to the SNARE complex. Structurally, CPX is a small soluble protein of 134 residues and contains four domains: N-terminal domain (residues 1-27) for activating synaptic vesicle exocytosis and synaptic vesicle priming, C-terminal domain (residues 71-134) for the clamping and priming but not for the Syts-activating, an accessory helix domain (residues 28-48) for clamping synaptic vesicle exocytosis and a central  $\alpha$ -helical SNARE binding domain (residues 49-70) for all physiological functions. In neurons, it was reported that N-terminal domain of CPX1 stimulates fast synchronous neurotransmitter release whereas C-terminal domain and the accessory helix domain function on suppressing spontaneous release (191). The C-terminal domain is also essential for evoking neurotransmitter release (191).

Evidence revealed that CPXs play an important role in  $\text{Ca}^{2+}$ -triggered exocytosis of synaptic and neuroendocrine vesicles, which are regulated by the other family of SNARE-binding proteins including Syt1, Syt2, Syt7, Syt9 and Syt10 (192). Increasing CPX levels are also reported to facilitate the ability of Syt in stimulating synaptic vesicle fusion, suggesting a critical role for CPX as a regulator of Syt function (193). Syt consists of an N-terminal synaptic vesicle transmembrane anchor and two  $\text{Ca}^{2+}$ -binding C2 domains in cytoplasm. Studies of Syts in *Drosophila* suggested that  $\text{Ca}^{2+}$ -binding

C2B domain stimulates synchronous fusion, whereas  $\text{Ca}^{2+}$ -binding C2A domain is essential for inhibiting asynchronous release and facilitating synchronous fusion. It was found that SNARE binding by Syt triggers a conformational change of Syt into a more fusogenic form, which can increase spontaneous fusion in the absence of CPX (194). Whether both Syt and CPX bind the SNARE complex at the same time is still unclear. It was suggested that when SYT and CPX compete for SNARE complex binding, the binding affinities are probably to be regulated by intracellular  $\text{Ca}^{2+}$  concentration in the case of Syt (193).

### 4.4. Ceramid mediated transcytosis

Ceramide is composed of fatty acid chain of varied length, saturation and hydroxylation, bound to an amino group of a sphingoid base via amide linkage. The fatty acid chain can vary from 2 to 28 carbons and C16- to C24-ceramide are expressed in abundance in mammalian cells. C2-, C6-, and C18-ceramide were observed to significantly promote exocytosis in PC12 cells. In addition, C18-ceramide can increase exocytosis of glutamate from damaged neurons, thus aggravating neuronal injury. Tetsuma MURASE *et al.* previously found that C2-ceramide could enhance sperm acrosomal exocytosis during fertilization in boar spermatozoa (195). Using the *Drosophila* neuromuscular junction model, studies by Kendal Broaie *et al.* indicated that ceramide is involved in synaptic vesicles exocytosis and trafficking (196). Charles J. Lowenstein and co-workers suggested that endogenous ceramide could trigger Weibel-Palade body exocytosis to induce vascular inflammation and thrombosis in ECs and endogenous NO can downregulate ceramide-mediated exocytosis (197). Dae Kyong Kim and colleagues revealed that ceramide functions as an important mediator of dopamine, serotonin or arachidonic acid exocytosis in concert with  $\text{Ca}^{2+}$  (198). The structure of the ceramide domains in ganglioside GM1 was also reported to be essential for GM1-mediated cholera toxin transcytosis (199). Amira Klip and colleagues recently observed that C2-ceramide could trigger insulin resistance by preventing intracellular GLUT4 sorting and re-exocytosis (200). In an established *in vitro* transcytosis model, recent studies in our laboratory also found that oxLDL can transport across vascular ECs and endogenous ceramide enables to significantly promote the transcytosis of oxLDL across ECs (23). Ceramide inhibitors, ASM inhibitor desipramine and de novo ceramide synthesis inhibitor myriocin, significantly decreased the transcytosis of ox-LDL across ECs and the subendothelial retention in vesicular wall. However, ceramidase inhibitor N-oleylethanolamine and SMS inhibitor O-Tricyclo(5.2.1.0.2.,6)dec-9-yl dithiocarbonate potassium salt, which upregulated the endogenous ceramide production, greatly increased the transcytosis of ox-LDL across ECs and the subendothelial retention in vascular wall. We further confirmed that ceramide could upregulate the expression of Cav-1 and cavin-1 in

LRs, which is associated with ceramide-triggered ox-LDL transcytosis across ECs (23).

Ceramide has been shown to promote pore formation in model phospholipids bilayers. Of note, ceramide can also affect the movement of proteins into or from rafts or trigger conformational changes in membrane associated proteins (201). It is reported that SNARE complex can be clustered in LR, and alteration in ceramide resulting in conformation changes in the complex could probably influence exocytosis. To conclude, ceramide has particular roles in the process of vesicle trafficking and transcytosis. Further studies are required for elucidating the precise mechanisms of various ceramide species on the function of membrane raft-related proteins, and how they could affect transcytosis.

## 5. CONCLUSIONS AND PERSPECTIVES

Transcytosis is a widespread transport process of macromolecules and is extremely important for multicellular organisms to maintain cellular and body homeostasis. To date, there is no doubt that various transcytosis molecules are tightly linked to LR and ceramide, which form different functional membrane macrodomains or platforms upon stimulations. In particular, ceramide-enriched membrane microdomains play an extremely critical role in LR clustering or platform formations. Transcytosis is a complex multi-step process including endocytosis, intracellular trafficking and exocytosis. Endocytic routes taking place in LR include caveolae-mediated endocytosis, flotillin-mediated endocytosis and GTPases-regulated endocytosis (GRAF1-, RhoA-, Arf6-regulated endocytosis). Trafficking motors primarily consist of microtubule-based and actin-based molecular motors. Furthermore, molecular machinery for LR-mediated exocytosis mainly contains the SNARE proteins, NSF, SNAPs *etc.* Better and direct understanding of the mechanisms is imperative and essential: the mechanisms for cargo selection in the absence of coats and adaptors; the mechanisms for vesicle fission in the absence of dynamin as well as the precise mechanisms of various ceramide species on the function of LR-related proteins, and how they could affect transcytosis *etc.* Additional LR/ceramide-associated transcytosis mechanisms remain to be fully elucidated. It would be especially important to illustrate how the various transcytosis mechanisms are regulated and crosstalk with each other, as well as more attention should be paid on *in-vivo* transcytosis research strategies. The last, but not the least, more studies are especially critical for translating basic findings associated with transcytosis and LR/ceramide to clinical uses.

## 6. ACKNOWLEDGEMENTS

Fang Bian and Bin Xiong contributed equally

to this work. The authors' work cited in this review was supported by grants from the National Natural Science Foundation of China (81373413, 81072634, 81573432, 81503072) and a grant from Ministry of Education of China (NCET-2010-0409).

## 6. REFERENCES

1. PALADEGE.: The fine structure of blood capillaries. *J Appl Physiol*, 24, 1424 (1953)
2. S. N.: The microvascular endothelium: segmental differentiations; transcytosis, selective distribution of anionic sites. *Advances in Inflammation Research*, 61-70 (1979)
3. K. E. Mostov, J. P. Kraehenbuhl and G. Blobel: Receptor-mediated transcellular transport of immunoglobulin: synthesis of secretory component as multiple and larger transmembrane forms. *Proc Natl Acad Sci U S A*, 77(12), 7257-61 (1980)  
DOI: 10.1073/pnas.77.12.7257
4. M. Simionescu, D. Popov and A. Sima: Endothelial transcytosis in health and disease. *Cell Tissue Res*, 335(1), 27-40 (2009)  
DOI: 10.1007/s00441-008-0688-3
5. P. Tuma and A. L. Hubbard: Transcytosis: crossing cellular barriers. *Physiol Rev*, 83(3), 871-932 (2003)  
DOI: 10.1152/physrev.00001.2003
6. A. El-Sayed and H. Harashima: Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis. *Mol Ther*, 21(6), 1118-30 (2013)  
DOI: 10.1038/mt.2013.54
7. T. F. Roth and K. R. Porter: Yolk Protein Uptake in the Oocyte of the Mosquito *Aedes Aegypti*. *J Cell Biol*, 20, 313-32 (1964)  
DOI: 10.1083/jcb.20.2.313
8. T. Kirchhausen, D. Owen and S. C. Harrison: Molecular structure, function, and dynamics of clathrin-mediated membrane traffic. *Cold Spring Harb Perspect Biol*, 6(5), a016725 (2014)  
DOI: 10.1101/cshperspect.a016725
9. J. E. Preston, N. Joan Abbott and D. J. Begley: Transcytosis of macromolecules at the blood-brain barrier. *Adv Pharmacol*, 71, 147-63 (2014)  
DOI: 10.1016/bs.apha.2014.06.001
10. N. Ariotti and R. G. Parton: SnapShot: caveolae, caveolins, and caveolins. *Cell*, 154(3), 704-704 e1 (2013)
11. S. Engel, T. Heger, R. Mancini, F. Herzog, J. Kartenbeck, A. Hayer and A. Helenius: Role of endosomes in simian virus 40 entry and infection. *J Virol*, 85(9), 4198-211 (2011)  
DOI: 10.1128/JVI.02179-10
12. G. E. Palade, M. Simionescu and N. Simionescu: Structural aspects of the permeability of the microvascular endothelium. *Acta Physiol Scand Suppl*, 463, 11-32 (1979)
13. B. Razani, J. A. Engelman, X. B. Wang, W. Schubert, X. L. Zhang, C. B. Marks, F. Macaluso, R. G. Russell, M. Li, R. G. Pestell, D. Di Vizio, H. Hou, Jr., B. Kneitz, G. Lagaud, G. J. Christ, W. Edelmann and M. P. Lisanti: Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem*, 276(41), 38121-38 (2001)
14. E. Dobrinskikh, K. Okamura, J. B. Kopp, R. B. Doctor and J. Blaine: Human podocytes perform polarized, caveolae-dependent albumin endocytosis. *Am J Physiol Renal Physiol*, 306(9), F941-51 (2014)  
DOI: 10.1152/ajprenal.00532.2013
15. H. H. Li, J. Li, K. J. Wasserloos, C. Wallace, M. G. Sullivan, P. M. Bauer, D. B. Stolz, J. S. Lee, S. C. Watkins, C. M. St Croix, B. R. Pitt and L. M. Zhang: Caveolae-dependent and -independent uptake of albumin in cultured rodent pulmonary endothelial cells. *PLoS One*, 8(11), e81903 (2013)  
DOI: 10.1371/journal.pone.0081903
16. Z. J. Cheng, R. D. Singh, D. K. Sharma, E. L. Holicky, K. Hanada, D. L. Marks and R. E. Pagano: Distinct mechanisms of clathrin-independent endocytosis have unique sphingolipid requirements. *Mol Biol Cell*, 17(7), 3197-210 (2006)  
DOI: 10.1091/mbc.E05-12-1101
17. S. M. Armstrong, V. Khajoe, C. Wang, T. Wang, J. Tigdi, J. Yin, W. M. Kuebler, M. Gillrie, S. P. Davis, M. Ho and W. L. Lee: Co-regulation of transcellular and paracellular leak across microvascular endothelium by dynamin and Rac. *Am J Pathol*, 180(3), 1308-23 (2012)  
DOI: 10.1016/j.ajpath.2011.12.002
18. P. G. Frank, S. Pavlides, M. W. Cheung, K. Daumer and M. P. Lisanti: Role of caveolin-1



- in the regulation of lipoprotein metabolism. *Am J Physiol Cell Physiol*, 295(1), C242-8 (2008)  
DOI: 10.1152/ajpcell.00185.2008
19. P. G. Frank, A. Pedraza, D. E. Cohen and M. P. Lisanti: Adenovirus-mediated expression of caveolin-1 in mouse liver increases plasma high-density lipoprotein levels. *Biochemistry*, 40(36), 10892-900 (2001)  
DOI: 10.1021/bi0106437
20. S. Matveev, A. Uittenbogaard, D. van Der Westhuyzen and E. J. Smart: Caveolin-1 negatively regulates SR-BI mediated selective uptake of high-density lipoprotein-derived cholesteryl ester. *Eur J Biochem*, 268(21), 5609-16 (2001)  
DOI: 10.1046/j.1432-1033.2001.02496.x
21. Y. Zhang, X. Yang, F. Bian, P. Wu, S. Xing, G. Xu, W. Li, J. Chi, C. Ouyang, T. Zheng, D. Wu, Y. Li and S. Jin: TNF- $\alpha$  promotes early atherosclerosis by increasing transcytosis of LDL across endothelial cells: crosstalk between NF- $\kappa$ B and PPAR- $\gamma$ . *J Mol Cell Cardiol*, 72, 85-94 (2014)  
DOI: 10.1016/j.yjmcc.2014.02.012
22. F. Bian, X. Yang, F. Zhou, P. H. Wu, S. Xing, G. Xu, W. Li, J. Chi, C. Ouyang, Y. Zhang, B. Xiong, Y. Li, T. Zheng, D. Wu, X. Chen and S. Jin: C-reactive protein promotes atherosclerosis by increasing LDL transcytosis across endothelial cells. *Br J Pharmacol*, 171(10), 2671-84 (2014)  
DOI: 10.1111/bph.12616
23. W. Li, X. Yang, S. Xing, F. Bian, W. Yao, X. Bai, T. Zheng, G. Wu and S. Jin: Endogenous ceramide contributes to the transcytosis of oxLDL across endothelial cells and promotes its subendothelial retention in vascular wall. *Oxid Med Cell Longev*, 2014, 823071 (2014)  
DOI: 10.1155/2014/823071
24. D. Jelinek, R. A. Heidenreich, R. A. Orlando and W. S. Garver: The Niemann-Pick C1 and caveolin-1 proteins interact to modulate efflux of low density lipoprotein-derived cholesterol from late endocytic compartments. *J Mol Biochem*, 3(1), 14-26 (2014)
25. A. Nistor and M. Simionescu: Uptake of low density lipoproteins by the hamster lung. Interactions with capillary endothelium. *Am Rev Respir Dis*, 134(6), 1266-72 (1986)
26. S. Kim and D. P. Giddens: Mass transport of low density lipoprotein in reconstructed hemodynamic environments of human carotid arteries: the role of volume and solute flux through the endothelium. *J Biomech Eng*, 137(4), 041007 (2015)  
DOI: 10.1115/1.4028969
27. C. C. Michel, M. N. Nanjee, W. L. Olszewski and N. E. Miller: LDL and HDL transfer rates across peripheral microvascular endothelium agree with those predicted for passive ultrafiltration in humans. *J Lipid Res*, 56(1), 122-8 (2015)  
DOI: 10.1194/jlr.M055053
28. P. M. Azizi, R. E. Zyla, S. Guan, C. Wang, J. Liu, S. S. Bolz, B. Heit, A. Klip and W. L. Lee: Clathrin-dependent entry and vesicle-mediated exocytosis define insulin transcytosis across microvascular endothelial cells. *Mol Biol Cell*, 26(4), 740-50 (2015)  
DOI: 10.1091/mbc.E14-08-1307
29. H. Wang, A. X. Wang, K. Aylor and E. J. Barrett: Caveolin-1 phosphorylation regulates vascular endothelial insulin uptake and is impaired by insulin resistance in rats. *Diabetologia*, 58(6), 1344-53 (2015)  
DOI: 10.1007/s00125-015-3546-3
30. K. J. Kim and A. B. Malik: Protein transport across the lung epithelial barrier. *Am J Physiol Lung Cell Mol Physiol*, 284(2), L247-59 (2003)  
DOI: 10.1152/ajplung.00235.2002
31. R. Bahhady, K. J. Kim, Z. Borok, E. D. Crandall and W. C. Shen: Characterization of protein factor(s) in rat bronchoalveolar lavage fluid that enhance insulin transport via transcytosis across primary rat alveolar epithelial cell monolayers. *Eur J Pharm Biopharm*, 69(3), 808-16 (2008)  
DOI: 10.1016/j.ejpb.2008.01.028
32. B. Schroeder and M. A. McNiven: Importance of endocytic pathways in liver function and disease. *Compr Physiol*, 4(4), 1403-17 (2014)  
DOI: 10.1002/cphy.c140001
33. J. Chang, A. Paillard, C. Passirani, M. Morille, J. P. Benoit, D. Betbeder and E. Garcion: Transferrin adsorption onto PLGA nanoparticles governs their interaction with biological systems from blood circulation to brain cancer cells. *Pharm Res*, 29(6), 1495-505 (2012)  
DOI: 10.1007/s11095-011-0624-1

34. B. Ji, J. Maeda, M. Higuchi, K. Inoue, H. Akita, H. Harashima and T. Suhara: Pharmacokinetics and brain uptake of lactoferrin in rats. *Life Sci*, 78(8), 851-5 (2006)  
DOI: 10.1016/j.lfs.2005.05.085
35. R. L. Roberts and A. Sandra: Transport of transferrin across the blood-thymus barrier in young rats. *Tissue Cell*, 26(5), 757-66 (1994)  
DOI: 10.1016/0040-8166(94)90058-2
36. C. Puri: Loss of myosin VI no insert isoform (Nol) induces a defect in clathrin-mediated endocytosis and leads to caveolar endocytosis of transferrin receptor. *J Biol Chem*, 284(50), 34998-5014 (2009)  
DOI: 10.1074/jbc.M109.012328
37. I. Tabas, K. J. Williams and J. Boren: Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. *Circulation*, 116(16), 1832-44 (2007)  
DOI: 10.1161/CIRCULATIONAHA.106.676890
38. P. G. Frank, H. Lee, D. S. Park, N. N. Tandon, P. E. Scherer and M. P. Lisanti: Genetic ablation of caveolin-1 confers protection against atherosclerosis. *Arterioscler Thromb Vasc Biol*, 24(1), 98-105 (2004)  
DOI: 10.1161/01.ATV.0000101182.89118.E5
39. P. F. Nievelstein, A. M. Fogelman, G. Mottino and J. S. Frank: Lipid accumulation in rabbit aortic intima 2 hours after bolus infusion of low density lipoprotein. A deep-etch and immunolocalization study of ultrarapidly frozen tissue. *Arterioscler Thromb*, 11(6), 1795-805 (1991)  
DOI: 10.1161/01.ATV.11.6.1795
40. E. Vasile, M. Simionescu and N. Simionescu: Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium *in situ*. *J Cell Biol*, 96(6), 1677-89 (1983)  
DOI: 10.1083/jcb.96.6.1677
41. J. Ba, H. Peng, Y. Chen and Y. Gao: Effects and mechanism analysis of vascular endothelial growth factor and salvianolic acid B on 125I-low density lipoprotein permeability of the rabbit aortary endothelial cells. *Cell Biochem Biophys*, 70(3), 1533-8 (2014)  
DOI: 10.1007/s12013-014-0089-z
42. W. T. Chao, S. S. Fan, J. K. Chen and V. C. Yang: Visualizing caveolin-1 and HDL in cholesterol-loaded aortic endothelial cells. *J Lipid Res*, 44(6), 1094-9 (2003)  
DOI: 10.1194/jlr.M300033-JLR200
43. C. Mineo, H. Deguchi, J. H. Griffin and P. W. Shaul: Endothelial and antithrombotic actions of HDL. *Circ Res*, 98(11), 1352-64 (2006)  
DOI: 10.1161/01.RES.0000225982.01988.93
44. Q. Hu, X. J. Zhang, C. X. Liu, X. P. Wang and Y. Zhang: PPARgamma1-induced caveolin-1 enhances cholesterol efflux and attenuates atherosclerosis in apolipoprotein E-deficient mice. *J Vasc Res*, 47(1), 69-79 (2010)  
DOI: 10.1159/000235927
45. D. Popov and M. Simionescu: Cellular mechanisms and signalling pathways activated by high glucose and AGE-albumin in the aortic endothelium. *Arch Physiol Biochem*, 112(4-5), 265-73 (2006)  
DOI: 10.1080/13813450601094573
46. H. Wang, A. X. Wang, K. Aylor and E. J. Barrett: Nitric oxide directly promotes vascular endothelial insulin transport. *Diabetes*, 62(12), 4030-42 (2013)  
DOI: 10.2337/db13-0627
47. P. Stralfors: Caveolins and caveolae, roles in insulin signalling and diabetes. *Adv Exp Med Biol*, 729, 111-26 (2012)  
DOI: 10.1007/978-1-4614-1222-9\_8
48. J. K. Jin, B. H. Ahn, Y. J. Na, J. I. Kim, Y. S. Kim, E. K. Choi, Y. G. Ko, K. C. Chung, P. B. Kozlowski and S. Min do: Phospholipase D1 is associated with amyloid precursor protein in Alzheimer's disease. *Neurobiol Aging*, 28(7), 1015-27 (2007)  
DOI: 10.1016/j.neurobiolaging.2006.05.022
49. M. J. Kang, Y. H. Chung, C. I. Hwang, M. Murata, T. Fujimoto, I. H. Mook-Jung, C. I. Cha and W. Y. Park: Caveolin-1 upregulation in senescent neurons alters amyloid precursor protein processing. *Exp Mol Med*, 38(2), 126-33 (2006)  
DOI: 10.1038/emmm.2006.16
50. P. Candela, F. Gosselet, J. Saint-Pol, E. Sevin, M. C. Boucau, E. Boulanger, R. Cecchelli and L. Fenart: Apical-to-basolateral transport of amyloid-beta peptides through blood-brain barrier cells is mediated by the receptor for advanced glycation end-products and is restricted by P-glycoprotein. *J Alzheimers Dis*, 22(3), 849-59 (2010)

51. I. E. Andras, S. Y. Eum and M. Toborek: Lipid rafts and functional caveolae regulate HIV-induced amyloid beta accumulation in brain endothelial cells. *Biochem Biophys Res Commun*, 421(2), 177-83 (2012)  
DOI: 10.1016/j.bbrc.2012.03.128
52. S. K. Jang, J. M. Yu, S. T. Kim, G. H. Kim, W. Park da, I. Lee do and S. S. Joo: An Abeta42 uptake and degradation via Rg3 requires an activation of caveolin, clathrin and Abeta-degrading enzymes in microglia. *Eur J Pharmacol*, 758, 1-10 (2015)  
DOI: 10.1016/j.ejphar.2015.03.071
53. Y. L. Lan, J. Zhao and S. Li: Update on the neuroprotective effect of estrogen receptor alpha against Alzheimer's disease. *J Alzheimers Dis*, 43(4), 1137-48 (2015)
54. L. Ghitescu, A. Fixman, M. Simionescu and N. Simionescu: Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor-mediated transcytosis. *J Cell Biol*, 102(4), 1304-11 (1986)  
DOI: 10.1083/jcb.102.4.1304
55. M. Simionescu, A. Gafencu and F. Antohe: Transcytosis of plasma macromolecules in endothelial cells: a cell biological survey. *Microsc Res Tech*, 57(5), 269-88 (2002)  
DOI: 10.1002/jemt.10086
56. M. Drab, P. Verkade, M. Elger, M. Kasper, M. Lohn, B. Lauterbach, J. Menne, C. Lindschau, F. Mende, F. C. Luft, A. Schedl, H. Haller and T. V. Kurzchalia: Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science*, 293(5539), 2449-52 (2001)  
DOI: 10.1126/science.1062688
57. W. Schubert, P. G. Frank, B. Razani, D. S. Park, C. W. Chow and M. P. Lisanti: Caveolae-deficient endothelial cells show defects in the uptake and transport of albumin *in vivo*. *J Biol Chem*, 276(52), 48619-22 (2001)  
DOI: 10.1074/jbc.C100613200
58. M. R. Siddiqui, Y. A. Komarova, S. M. Vogel, X. Gao, M. G. Bonini, J. Rajasingh, Y. Y. Zhao, V. Brovkovich and A. B. Malik: Caveolin-1-eNOS signaling promotes p190RhoGAP-A nitration and endothelial permeability. *J Cell Biol*, 193(5), 841-50 (2011)  
DOI: 10.1083/jcb.201012129
59. D. I. Mundy, A. M. Lopez, K. S. Posey, J. C. Chuang, C. M. Ramirez, P. E. Scherer and S. D. Turley: Impact of the loss of caveolin-1 on lung mass and cholesterol metabolism in mice with and without the lysosomal cholesterol transporter, Niemann-Pick type C1. *Biochim Biophys Acta*, 1841(7), 995-1002 (2014)  
DOI: 10.1016/j.bbalip.2014.04.002
60. H. Y. Kim, S. Kim, H. J. Pyun, J. Maeng and K. Lee: Cellular uptake mechanism of TCTP-PTD in human lung carcinoma cells. *Mol Pharm*, 12(1), 194-203 (2015)  
DOI: 10.1021/mp500547f
61. A. R. Prewitt, S. Ghose, A. L. Frump, A. Datta, E. D. Austin, A. K. Kenworthy and M. P. de Caestecker: Heterozygous null bone morphogenetic protein receptor type 2 mutations promote SRC kinase-dependent caveolar trafficking defects and endothelial dysfunction in pulmonary arterial hypertension. *J Biol Chem*, 290(2), 960-71 (2015)  
DOI: 10.1074/jbc.M114.591057
62. Y. Sun, G. Hu, X. Zhang and R. D. Minshall: Phosphorylation of caveolin-1 regulates oxidant-induced pulmonary vascular permeability via paracellular and transcellular pathways. *Circ Res*, 105(7), 676-85, 15 p following 685 (2009)
63. C. Y. Xia, Z. Zhang, Y. X. Xue, P. Wang and Y. H. Liu: Mechanisms of the increase in the permeability of the blood-tumor barrier obtained by combining low-frequency ultrasound irradiation with small-dose bradykinin. *J Neurooncol*, 94(1), 41-50 (2009)  
DOI: 10.1007/s11060-009-9812-9
64. Z. Li, Y. H. Liu, Y. X. Xue, L. B. Liu and P. Wang: Low-dose endothelial monocyte-activating polypeptide-ii increases permeability of blood-tumor barrier by caveolae-mediated transcellular pathway. *J Mol Neurosci*, 52(3), 313-22 (2014)  
DOI: 10.1007/s12031-013-0148-8
65. Y. Song, P. Wang, J. Ma and Y. Xue: C-terminus of human BKca channel alpha subunit enhances the permeability of the brain endothelial cells by interacting with caveolin-1 and triggering caveolin-1 intracellular trafficking. *Neuromolecular Med*, 16(2), 499-509 (2014)
66. Z. H. Yang, L. B. Liu, L. N. Zhao, Y. H. Liu and Y. X. Xue: Permeability of the blood-tumor barrier is enhanced by combining vascular

- endothelial growth factor with papaverine. *J Neurosci Res*, 92(6), 703-13 (2014)  
DOI: 10.1002/jnr.23348
67. Y. Zhao, N. S. Mangalmurti, Z. Xiong, B. Prakash, F. Guo, D. B. Stolz and J. S. Lee: Duffy antigen receptor for chemokines mediates chemokine endocytosis through a macropinocytosis-like process in endothelial cells. *PLoS One*, 6(12), e29624 (2011)  
DOI: 10.1371/journal.pone.0029624
  68. P. Hillyer and D. Male: Expression of chemokines on the surface of different human endothelia. *Immunol Cell Biol*, 83(4), 375-82 (2005)  
DOI: 10.1111/j.1440-1711.2005.01345.x
  69. D. Sagar, A. Lamontagne, C. A. Foss, Z. K. Khan, M. G. Pomper and P. Jain: Dendritic cell CNS recruitment correlates with disease severity in EAE via CCL2 chemotaxis at the blood-brain barrier through paracellular transmigration and ERK activation. *J Neuroinflammation*, 9, 245 (2012)  
DOI: 10.1186/1742-2094-9-245
  70. J. Rejman, V. Oberle, I. S. Zuhorn and D. Hoekstra: Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J*, 377(Pt 1), 159-69 (2004)  
DOI: 10.1042/bj20031253
  71. S. Grosse, Y. Aron, G. Thevenot, D. Francois, M. Monsigny and I. Fajac: Potocytosis and cellular exit of complexes as cellular pathways for gene delivery by polycations. *J Gene Med*, 7(10), 1275-86 (2005)  
DOI: 10.1002/jgm.772
  72. M. E. Hwang, R. K. Keswani and D. W. Pack: Dependence of PEI and PAMAM Gene Delivery on Clathrin- and Caveolin-Dependent Trafficking Pathways. *Pharm Res*, 32(6), 2051-9 (2015)  
DOI: 10.1007/s11095-014-1598-6
  73. W. Ke, K. Shao, R. Huang, L. Han, Y. Liu, J. Li, Y. Kuang, L. Ye, J. Lou and C. Jiang: Gene delivery targeted to the brain using an Angiopep-conjugated polyethyleneglycol-modified polyamidoamine dendrimer. *Biomaterials*, 30(36), 6976-85 (2009)  
DOI: 10.1016/j.biomaterials.2009.08.049
  74. A. W. Wong, S. J. Scales and D. E. Reilly: DNA internalized via caveolae requires microtubule-dependent, Rab7-independent transport to the late endocytic pathway for delivery to the nucleus. *J Biol Chem*, 282(31), 22953-63 (2007)  
DOI: 10.1074/jbc.M611015200
  75. W. De Haes, G. Van Mol, C. Merlin, S. C. De Smedt, G. Vanham and J. Rejman: Internalization of mRNA lipoplexes by dendritic cells. *Mol Pharm*, 9(10), 2942-9 (2012)  
DOI: 10.1021/mp3003336
  76. M. M. Adil, Z. S. Erdman and E. Kokkoli: Transfection mechanisms of polyplexes, lipoplexes, and stealth liposomes in alpha(5)beta(1) integrin bearing DLD-1 colorectal cancer cells. *Langmuir*, 30(13), 3802-10 (2014)  
DOI: 10.1021/la5001396
  77. C. Liu, W. Yu, Z. Chen, J. Zhang and N. Zhang: cNGR conjugated poly(lactic acid)-poly(ethylene glycol) nanoparticles for targeted gene delivery. *J Control Release*, 152 Suppl 1, e155-7 (2011)  
DOI: 10.1016/j.jconrel.2011.08.059
  78. A. Chrastina, K. A. Massey and J. E. Schnitzer: Overcoming *in vivo* barriers to targeted nanodelivery. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, 3(4), 421-37 (2011)  
DOI: 10.1002/wnan.143
  79. F. Di Costanzo, S. Gasperoni and V. Rotella: Targeted delivery of albumin bound paclitaxel in the treatment of advanced breast cancer. *Onco Targets Ther*, 2, 179-88 (2009)  
DOI: 10.2147/OTT.S3863
  80. E. Roger, F. Lagarce, E. Garcion and J. P. Benoit: Lipid nanocarriers improve paclitaxel transport throughout human intestinal epithelial cells by using vesicle-mediated transcytosis. *J Control Release*, 140(2), 174-81 (2009)  
DOI: 10.1016/j.jconrel.2009.08.010
  81. K. Simons and E. Ikonen: Functional rafts in cell membranes. *Nature*, 387(6633), 569-72 (1997)  
DOI: 10.1038/42408
  82. L. J. Pike: Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res*, 47(7), 1597-8  
DOI: 10.1194/jlr.E600002-JLR200
  83. F. Mollinedo and C. Gajate: Lipid rafts as major platforms for signaling regulation in cancer. *Adv Biol Regul*, 57, 130-46 (2015)



- DOI: 10.1016/j.jbior.2014.10.003
84. M. Hamoudane, S. Maffioli, R. Cordera, D. Maggi and B. Salani: Caveolin-1 and polymerase I and transcript release factor: new players in insulin-like growth factor-I receptor signaling. *J Endocrinol Invest*, 36(3), 204-8 (2013)
  85. A. Rutkovskiy, M. Bliksoen, V. Hillestad, M. Amin, G. Czibik, G. Valen, J. Vaage, M. Amiry-Moghaddam and K. O. Stenslokken: Aquaporin-1 in cardiac endothelial cells is downregulated in ischemia, hypoxia and cardioplegia. *J Mol Cell Cardiol*, 56, 22-33 (2013)  
DOI: 10.1016/j.yjmcc.2012.12.002
  86. C. Meye, J. Schumann, A. Wagner and P. Gross: Effects of homocysteine on the levels of caveolin-1 and eNOS in caveolae of human coronary artery endothelial cells. *Atherosclerosis*, 190(2), 256-63 (2007)  
DOI: 10.1016/j.atherosclerosis.2006.03.009
  87. L. Liu, A. V. Ivanov, M. E. Gable, F. Jolivel, G. A. Morrill and A. Askari: Comparative properties of caveolar and noncaveolar preparations of kidney Na<sup>+</sup>/K<sup>+</sup>-ATPase. *Biochemistry*, 50(40), 8664-73 (2011)  
DOI: 10.1021/bi2009008
  88. N. Chaudhary, G. A. Gomez, M. T. Howes, H. P. Lo, K. A. McMahon, J. A. Rae, N. L. Schieber, M. M. Hill, K. Gaus, A. S. Yap and R. G. Parton: Endocytic crosstalk: cavins, caveolins, and caveolae regulate clathrin-independent endocytosis. *PLoS Biol*, 12(4), e1001832 (2014)  
DOI: 10.1371/journal.pbio.1001832
  89. B. M. Castro, M. Prieto and L. C. Silva: Ceramide: a simple sphingolipid with unique biophysical properties. *Prog Lipid Res*, 54, 53-67 (2014)  
DOI: 10.1016/j.plipres.2014.01.004
  90. F. M. Goni, J. Sot and A. Alonso: Biophysical properties of sphingosine, ceramides and other simple sphingolipids. *Biochem Soc Trans*, 42(5), 1401-8 (2014)  
DOI: 10.1042/BST20140159
  91. T. H. Beckham, J. C. Cheng, S. T. Marrison, J. S. Norris and X. Liu: Interdiction of sphingolipid metabolism to improve standard cancer therapies. *Adv Cancer Res*, 117, 1-36 (2013)  
DOI: 10.1016/B978-0-12-394274-6.00001-7
  92. J. Vinten, A. H. Johnsen, P. Roepstorff, J. Harpoth and J. Tranum-Jensen: Identification of a major protein on the cytosolic face of caveolae. *Biochim Biophys Acta*, 1717(1), 34-40 (2005)  
DOI: 10.1016/j.bbamem.2005.09.013
  93. C. Regazzetti, K. Dumas, S. Lacas-Gervais, F. Pastor, P. Peraldi, S. Bonnafeous, I. Dugail, S. Le Lay, P. Valet, Y. Le Marchand-Brustel, A. Tran, P. Gual, J. F. Tanti, M. Cormont and S. Giorgetti-Peraldi: Hypoxia inhibits Cavin-1 and Cavin-2 expression and down-regulates caveolae in adipocytes. *Endocrinology*, 156(3), 789-801 (2015)  
DOI: 10.1210/en.2014-1656
  94. Z. D. Nassar, M. M. Hill, R. G. Parton and M. O. Parat: Caveola-forming proteins caveolin-1 and PTRF in prostate cancer. *Nat Rev Urol*, 10(9), 529-36 (2013)  
DOI: 10.1038/nrurol.2013.168
  95. R. Burgener, M. Wolf, T. Ganz and M. Baggiolini: Purification and characterization of a major phosphatidylserine-binding phosphoprotein from human platelets. *Biochem J*, 269(3), 729-34 (1990)  
DOI: 10.1042/bj2690729
  96. C. Mineo, Y. S. Ying, C. Chapline, S. Jaken and R. G. Anderson: Targeting of protein kinase Calpha to caveolae. *J Cell Biol*, 141(3), 601-10 (1998)  
DOI: 10.1083/jcb.141.3.601
  97. C. G. Hansen, N. A. Bright, G. Howard and B. J. Nichols: SDPR induces membrane curvature and functions in the formation of caveolae. *Nat Cell Biol*, 11(7), 807-14 (2009)  
DOI: 10.1038/ncb1887
  98. S. Gustincich, P. Vatta, S. Goruppi, M. Wolf, S. Saccone, G. Della Valle, M. Baggiolini and C. Schneider: The human serum deprivation response gene (SDPR) maps to 2q32-q33 and codes for a phosphatidylserine-binding protein. *Genomics*, 57(1), 120-9 (1999)  
DOI: 10.1006/geno.1998.5733
  99. K. A. McMahon, H. Zajicek, W. P. Li, M. J. Peyton, J. D. Minna, V. J. Hernandez, K. Luby-Phelps and R. G. Anderson: SRBC/cavin-3 is a caveolin adapter protein that regulates caveolae function. *EMBO J*, 28(8), 1001-15 (2009)  
DOI: 10.1038/emboj.2009.46
  100. L. Liu, C. G. Hansen, B. J. Honeyman, B.

- J. Nichols and P. F. Pilch: Cavin-3 knockout mice show that cavin-3 is not essential for caveolae formation, for maintenance of body composition, or for glucose tolerance. *PLoS One*, 9(7), e102935 (2014)  
DOI: 10.1371/journal.pone.0102935
101. T. Ogata, T. Ueyama, K. Isodono, M. Tagawa, N. Takehara, T. Kawashima, K. Harada, T. Takahashi, T. Shioi, H. Matsubara and H. Oh: MURC, a muscle-restricted coiled-coil protein that modulates the Rho/ROCK pathway, induces cardiac dysfunction and conduction disturbance. *Mol Cell Biol*, 28(10), 3424-36 (2008)  
DOI: 10.1128/MCB.02186-07
102. M. Bastiani, L. Liu, M. M. Hill, M. P. Jedrychowski, S. J. Nixon, H. P. Lo, D. Abankwa, R. Luetterforst, M. Fernandez-Rojo, M. R. Breen, S. P. Gygi, J. Vinten, P. J. Walser, K. N. North, J. F. Hancock, P. F. Pilch and R. G. Parton: MURC/Cavin-4 and cavin family members form tissue-specific caveolar complexes. *J Cell Biol*, 185(7), 1259-73 (2009)  
DOI: 10.1083/jcb.200903053
103. T. Ogata, D. Naito, N. Nakanishi, Y. K. Hayashi, T. Taniguchi, K. Miyagawa, T. Hamaoka, N. Maruyama, S. Matoba, K. Ikeda, H. Yamada, H. Oh and T. Ueyama: MURC/Cavin-4 facilitates recruitment of ERK to caveolae and concentric cardiac hypertrophy induced by alpha1-adrenergic receptors. *Proc Natl Acad Sci U S A*, 111(10), 3811-6 (2014)  
DOI: 10.1073/pnas.1315359111
104. D. N. Predescu, R. Neamu, C. Bardita, M. Wang and S. A. Predescu: Impaired caveolae function and upregulation of alternative endocytic pathways induced by experimental modulation of intersectin-1s expression in mouse lung endothelium. *Biochem Res Int*, 2012, 672705 (2012)  
DOI: 10.1155/2012/672705
105. M. Kirkham, S. J. Nixon, M. T. Howes, L. Abi-Rached, D. E. Wakeham, M. Hanzal-Bayer, C. Ferguson, M. M. Hill, M. Fernandez-Rojo, D. A. Brown, J. F. Hancock, F. M. Brodsky and R. G. Parton: Evolutionary analysis and molecular dissection of caveola biogenesis. *J Cell Sci*, 121(Pt 12), 2075-86 (2008)  
DOI: 10.1242/jcs.024588
106. O. O. Glebov, N. A. Bright and B. J. Nichols: Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat Cell Biol*, 8(1), 46-54 (2006)  
DOI: 10.1038/ncb1342
107. M. Meister, A. Zuk and R. Tikkanen: Role of dynamin and clathrin in the cellular trafficking of flotillins. *FEBS J*, 281(13), 2956-76 (2014)  
DOI: 10.1111/febs.12834
108. G. J. Doherty and R. Lundmark: GRAF1-dependent endocytosis. *Biochem Soc Trans*, 37(Pt 5), 1061-5 (2009)  
DOI: 10.1042/BST0371061
109. R. Lundmark, G. J. Doherty, M. T. Howes, K. Cortese, Y. Vallis, R. G. Parton and H. T. McMahon: The GTPase-activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway. *Curr Biol*, 18(22), 1802-8 (2008)  
DOI: 10.1016/j.cub.2008.10.044
110. M. T. Howes, M. Kirkham, J. Riches, K. Cortese, P. J. Walser, F. Simpson, M. M. Hill, A. Jones, R. Lundmark, M. R. Lindsay, D. J. Hernandez-Deviez, G. Hadzic, A. McCluskey, R. Bashir, L. Liu, P. Pilch, H. McMahon, P. J. Robinson, J. F. Hancock, S. Mayor and R. G. Parton: Clathrin-independent carriers form a high capacity endocytic sorting system at the leading edge of migrating cells. *J Cell Biol*, 190(4), 675-91 (2010)  
DOI: 10.1083/jcb.201002119
111. M. Krauss, J. Y. Jia, A. Roux, R. Beck, F. T. Wieland, P. De Camilli and V. Haucke: Arf1-GTP-induced tubule formation suggests a function of Arf family proteins in curvature acquisition at sites of vesicle budding. *J Biol Chem*, 283(41), 27717-23 (2008)  
DOI: 10.1074/jbc.M804528200
112. O. Heikkila, P. Susi, T. Tevaluoto, H. Harma, V. Marjomaki, T. Hyypia and S. Kiljunen: Internalization of coxsackievirus A9 is mediated by {beta}2-microglobulin, dynamin, and Arf6 but not by caveolin-1 or clathrin. *J Virol*, 84(7), 3666-81 (2010)  
DOI: 10.1128/JVI.01340-09
113. K. Nishi and K. Saigo: Cellular internalization of green fluorescent protein fused with herpes simplex virus protein VP22 via a lipid raft-mediated endocytic pathway independent of caveolae and Rho family GTPases but dependent on dynamin and Arf6. *J Biol Chem*, 282(37), 27503-17 (2007)  
DOI: 10.1074/jbc.M703810200

114. F. D. Brown, A. L. Rozelle, H. L. Yin, T. Balla and J. G. Donaldson: Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J Cell Biol*, 154(5), 1007-17 (2001)  
DOI: 10.1083/jcb.200103107
115. E. Frittoli, A. Palamidessi, A. Pizzigoni, L. Lanzetti, M. Garre, F. Troglio, A. Troilo, M. Fukuda, P. P. Di Fiore, G. Scita and S. Confalonieri: The primate-specific protein TBC1D3 is required for optimal macropinocytosis in a novel ARF6-dependent pathway. *Mol Biol Cell*, 19(4), 1304-16 (2008)  
DOI: 10.1091/mbc.E07-06-0594
116. M. Vidal-Quadras, M. Gelabert-Baldrich, D. Soriano-Castell, A. Llado, C. Rentero, M. Calvo, A. Pol, C. Enrich and F. Tebar: Rac1 and calmodulin interactions modulate dynamics of ARF6-dependent endocytosis. *Traffic*, 12(12), 1879-96 (2011)  
DOI: 10.1111/j.1600-0854.2011.01274.x
117. A. Grassart, A. Dujeancourt, P. B. Lazarow, A. Dautry-Varsat and N. Sauvonnet: Clathrin-independent endocytosis used by the IL-2 receptor is regulated by Rac1, Pak1 and Pak2. *EMBO Rep*, 9(4), 356-62 (2008)  
DOI: 10.1038/embor.2008.28
118. A. C. Braun, J. Hendrick, S. A. Eisler, S. Schmid, A. Hausser and M. A. Olayioye: The Rho-specific GAP protein DLC3 coordinates endocytic membrane trafficking. *J Cell Sci*, 128(7), 1386-99 (2015)  
DOI: 10.1242/jcs.163857
119. F. Tebar, M. Gelabert-Baldrich, M. Hoque, R. Cairns, C. Rentero, A. Pol, T. Grewal and C. Enrich: Annexins and endosomal signaling. *Methods Enzymol*, 535, 55-74 (2014)  
DOI: 10.1016/B978-0-12-397925-4.00004-3
120. P. Raynal and H. B. Pollard: Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim Biophys Acta*, 1197(1), 63-93 (1994)  
DOI: 10.1016/0304-4157(94)90019-1
121. S. Arur, U. E. Uche, K. Rezaul, M. Fong, V. Scranton, A. E. Cowan, W. Mohler and D. K. Han: Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev Cell*, 4(4), 587-98 (2003)  
DOI: 10.1016/S1534-5807(03)00090-X
122. F. Tzelepis, M. Verway, J. Daoud, J. Gillard, K. Hassani-Ardakani, J. Dunn, J. Downey, M. E. Gentile, J. Jaworska, A. M. Sanchez, Y. Nedelec, H. Vali, M. Tabrizian, A. S. Kristof, I. L. King, L. B. Barreiro and M. Divangahi: Annexin1 regulates DC efferocytosis and cross-presentation during Mycobacterium tuberculosis infection. *J Clin Invest*, 125(2), 752-68 (2015)  
DOI: 10.1172/JCI77014
123. P. Drucker, M. Pejic, H. J. Galla and V. Gerke: Lipid segregation and membrane budding induced by the peripheral membrane binding protein annexin A2. *J Biol Chem*, 288(34), 24764-76 (2013)  
DOI: 10.1074/jbc.M113.474023
124. N. A. Gokhale, A. Abraham, M. A. Digman, E. Gratton and W. Cho: Phosphoinositide specificity of and mechanism of lipid domain formation by annexin A2-p11 heterotetramer. *J Biol Chem*, 280(52), 42831-40 (2005)  
DOI: 10.1074/jbc.M508129200
125. N. Zobiack, U. Rescher, C. Ludwig, D. Zeuschner and V. Gerke: The annexin 2/S100A10 complex controls the distribution of transferrin receptor-containing recycling endosomes. *Mol Biol Cell*, 14(12), 4896-908 (2003)  
DOI: 10.1091/mbc.E03-06-0387
126. K. Yamashita, H. Nagai and S. Toyokuni: Receptor role of the annexin A2 in the mesothelial endocytosis of crocidolite fibers. *Lab Invest*, 95(7), 749-64 (2015)  
DOI: 10.1038/labinvest.2015.28
127. R. Cornely, C. Rentero, C. Enrich, T. Grewal and K. Gaus: Annexin A6 is an organizer of membrane microdomains to regulate receptor localization and signalling. *IUBMB Life*, 63(11), 1009-17 (2011)  
DOI: 10.1002/iub.540
128. L. Cubells, S. Vila de Muga, F. Tebar, P. Wood, R. Evans, M. Ingelmo-Torres, M. Calvo, K. Gaus, A. Pol, T. Grewal and C. Enrich: Annexin A6-induced alterations in cholesterol transport and caveolin export from the Golgi complex. *Traffic*, 8(11), 1568-89 (2007)  
DOI: 10.1111/j.1600-0854.2007.00640.x
129. M. Koese, C. Rentero, B. P. Kota, M. Hoque, R. Cairns, P. Wood, S. Vila de Muga, M. Reverter, A. Alvarez-Guaita, K. Monastyrskaya, W. E. Hughes, A. Swarbrick, F. Tebar, R. J. Daly, C. Enrich and T. Grewal: Annexin A6 is a scaffold

- for PKC $\alpha$  to promote EGFR inactivation. *Oncogene*, 32(23), 2858-72 (2013)  
DOI: 10.1038/onc.2012.303
130. A. Kamal, Y. Ying and R. G. Anderson: Annexin VI-mediated loss of spectrin during coated pit budding is coupled to delivery of LDL to lysosomes. *J Cell Biol*, 142(4), 937-47 (1998)  
DOI: 10.1083/jcb.142.4.937
131. J. Wang, Y. Wang, H. Wang, X. Hao, Y. Wu and J. Guo: Selection of Reference Genes for Gene Expression Studies in Porcine Whole Blood and Peripheral Blood Mononuclear Cells under Polyinosinic:Polycytidylic Acid Stimulation. *Asian-Australas J Anim Sci*, 27(4), 471-8 (2014)  
DOI: 10.5713/ajas.2013.13471
132. V. Goebeler, D. Ruhe, V. Gerke and U. Rescher: Annexin A8 displays unique phospholipid and F-actin binding properties. *FEBS Lett*, 580(10), 2430-4 (2006)  
DOI: 10.1016/j.febslet.2006.03.076
133. L. C. Wilsie, A. M. Gonzales and R. A. Orlando: Syndecan-1 mediates internalization of apoE-VLDL through a low density lipoprotein receptor-related protein (LRP)-independent, non-clathrin-mediated pathway. *Lipids Health Dis*, 5, 23 (2006)  
DOI: 10.1186/1476-511X-5-23
134. I. V. Fuki, K. M. Kuhn, I. R. Lomazov, V. L. Rothman, G. P. Tuszyński, R. V. Iozzo, T. L. Swenson, E. A. Fisher and K. J. Williams: The syndecan family of proteoglycans. Novel receptors mediating internalization of atherogenic lipoproteins *in vitro*. *J Clin Invest*, 100(6), 1611-22 (1997)  
DOI: 10.1172/JCI119685
135. S. Paris, A. Burlacu and Y. Durocher: Opposing roles of syndecan-1 and syndecan-2 in polyethyleneimine-mediated gene delivery. *J Biol Chem*, 283(12), 7697-704 (2008)  
DOI: 10.1074/jbc.M705424200
136. K. Chen and K. J. Williams: Molecular mediators for raft-dependent endocytosis of syndecan-1, a highly conserved, multifunctional receptor. *J Biol Chem*, 288(20), 13988-99 (2013)  
DOI: 10.1074/jbc.M112.444737
137. E. Tkachenko, E. Lutgens, R. V. Stan and M. Simons: Fibroblast growth factor 2 endocytosis in endothelial cells proceed via syndecan-4-dependent activation of Rac1 and a Cdc42-dependent macropinocytic pathway. *J Cell Sci*, 117(Pt 15), 3189-99 (2004)  
DOI: 10.1242/jcs.01190
138. B. B. Boyanovsky, P. Shridas, M. Simons, D. R. van der Westhuyzen and N. R. Webb: Syndecan-4 mediates macrophage uptake of group V secretory phospholipase A2-modified LDL. *J Lipid Res*, 50(4), 641-50 (2009)  
DOI: 10.1194/jlr.M800450-JLR200
139. J. E. McLaren, D. R. Michael, I. A. Guschina, J. L. Harwood and D. P. Ramji: Eicosapentaenoic acid and docosahexaenoic acid regulate modified LDL uptake and macropinocytosis in human macrophages. *Lipids*, 46(11), 1053-61 (2011)  
DOI: 10.1007/s11745-011-3598-1
140. A. J. Roberts, B. S. Goodman and S. L. Reck-Peterson: Reconstitution of dynein transport to the microtubule plus end by kinesin. *Elife*, 3, e02641 (2014)  
DOI: 10.7554/eLife.02641
141. M. Schuster, S. Kilaru, G. Fink, J. Collemare, Y. Roger and G. Steinberg: Kinesin-3 and dynein cooperate in long-range retrograde endosome motility along a nonuniform microtubule array. *Mol Biol Cell*, 22(19), 3645-57 (2011)  
DOI: 10.1091/mbc.E11-03-0217
142. S. Baumann, T. Pohlmann, M. Jungbluth, A. Brachmann and M. Feldbrugge: Kinesin-3 and dynein mediate microtubule-dependent co-transport of mRNPs and endosomes. *J Cell Sci*, 125(Pt 11), 2740-52 (2012)  
DOI: 10.1242/jcs.101212
143. J. Zhang, L. Zhuang, Y. Lee, J. F. Abenza, M. A. Penalva and X. Xiang: The microtubule plus-end localization of Aspergillus dynein is important for dynein-early-endosome interaction but not for dynein ATPase activation. *J Cell Sci*, 123(Pt 20), 3596-604 (2010)  
DOI: 10.1242/jcs.075259
144. V. M. D'Souza, L. M. Bareford, A. Ray and P. W. Swaan: Cytoskeletal scaffolds regulate riboflavin endocytosis and recycling in placental trophoblasts. *J Nutr Biochem*, 17(12), 821-9 (2006)  
DOI: 10.1016/j.jnutbio.2006.01.008
145. F. J. Kull and S. A. Endow: Force generation by kinesin and myosin cytoskeletal motor proteins. *J Cell Sci*, 126(Pt 1), 9-19 (2013)  
DOI: 10.1242/jcs.103911
146. O. L. Mooren, B. J. Galletta and J. A. Cooper:



- Roles for actin assembly in endocytosis. *Annu Rev Biochem*, 81, 661-86 (2012)  
DOI: 10.1146/annurev-biochem-060910-094416
147. E. Granger, G. McNee, V. Allan and P. Woodman: The role of the cytoskeleton and molecular motors in endosomal dynamics. *Semin Cell Dev Biol*, 31, 20-9 (2014)  
DOI: 10.1016/j.semcdb.2014.04.011
148. M. Zhao, S. Wu, Q. Zhou, S. Vivona, D. J. Cipriano, Y. Cheng and A. T. Brunger: Mechanistic insights into the recycling machine of the SNARE complex. *Nature*, 518(7537), 61-7 (2015)  
DOI: 10.1038/nature14148
149. P. Kumar, S. Guha and U. Diederichsen: SNARE protein analog-mediated membrane fusion. *J Pept Sci* (2015)  
DOI: 10.1002/psc.2773
150. Y. Park, W. Vennekate, H. Yavuz, J. Preobraschenski, J. M. Hernandez, D. Riedel, P. J. Walla and R. Jahn: alpha-SNAP interferes with the zippering of the SNARE protein membrane fusion machinery. *J Biol Chem*, 289(23), 16326-35 (2014)  
DOI: 10.1074/jbc.M114.556803
151. J. B. Bock, H. T. Matern, A. A. Peden and R. H. Scheller: A genomic perspective on membrane compartment organization. *Nature*, 409(6822), 839-41 (2001)  
DOI: 10.1038/35057024
152. F. Filippini, V. Rossi, T. Galli, A. Budillon, M. D'Urso and M. D'Esposito: Longins: a new evolutionary conserved VAMP family sharing a novel SNARE domain. *Trends Biochem Sci*, 26(7), 407-9 (2001)  
DOI: 10.1016/S0968-0004(01)01861-8
153. J. Meng, J. Wang, G. Lawrence and J. O. Dolly: Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their antinociceptive potential. *J Cell Sci*, 120(Pt 16), 2864-74 (2007)  
DOI: 10.1242/jcs.012211
154. L. Peng, M. Adler, A. Demogines, A. Borrell, H. Liu, L. Tao, W. H. Tepp, S. C. Zhang, E. A. Johnson, S. L. Sawyer and M. Dong: Widespread sequence variations in VAMP1 across vertebrates suggest a potential selective pressure from botulinum neurotoxins. *PLoS Pathog*, 10(7), e1004177 (2014)  
DOI: 10.1371/journal.ppat.1004177
155. S. Schoch, F. Deak, A. Konigstorfer, M. Mozhayeva, Y. Sara, T. C. Sudhof and E. T. Kavalali: SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science*, 294(5544), 1117-22 (2001)  
DOI: 10.1126/science.1064335
156. M. Bal, J. Leitz, A. L. Reese, D. M. Ramirez, M. Durakoglugil, J. Herz, L. M. Monteggia and E. T. Kavalali: Reelin mobilizes a VAMP7-dependent synaptic vesicle pool and selectively augments spontaneous neurotransmission. *Neuron*, 80(4), 934-46 (2013)  
DOI: 10.1016/j.neuron.2013.08.024
157. A. Rezaei Farimani, M. Saidijam, M. T. Goodarzi, R. Yadegar Azari, S. Asadi, S. Zarei and N. Shabab: Effect of Resveratrol Supplementation on the SNARE Proteins Expression in Adipose Tissue of Streptozotocin-Nicotinamide Induced Type 2 Diabetic Rats. *Iran J Med Sci*, 40(3), 248-55 (2015)
158. J. B. Sadler, N. J. Bryant and G. W. Gould: Characterization of VAMP isoforms in 3T3-L1 adipocytes: implications for GLUT4 trafficking. *Mol Biol Cell*, 26(3), 530-6 (2015)  
DOI: 10.1091/mbc.E14-09-1368
159. R. W. Schwenk, E. Dirkx, W. A. Coumans, A. Bonen, A. Klip, J. F. Glatz and J. J. Luiken: Requirement for distinct vesicle-associated membrane proteins in insulin- and AMP-activated protein kinase (AMPK)-induced translocation of GLUT4 and CD36 in cultured cardiomyocytes. *Diabetologia*, 53(10), 2209-19 (2010)  
DOI: 10.1007/s00125-010-1832-7
160. A. J. Rose, J. Jeppesen, B. Kiens and E. A. Richter: Effects of contraction on localization of GLUT4 and v-SNARE isoforms in rat skeletal muscle. *Am J Physiol Regul Integr Comp Physiol*, 297(5), R1228-37 (2009)  
DOI: 10.1152/ajpregu.00258.2009
161. K. C. Williams, R. E. McNeilly and M. G. Coppelino: SNAP23, Syntaxin4, and vesicle-associated membrane protein 7 (VAMP7) mediate trafficking of membrane type 1-matrix metalloproteinase (MT1-MMP) during invadopodium formation and tumor cell invasion. *Mol Biol Cell*, 25(13), 2061-70 (2014)  
DOI: 10.1091/mbc.E13-10-0582

162. S. W. Messenger, M. A. Falkowski, D. D. Thomas, E. K. Jones, W. Hong, H. Y. Gaisano, N. M. Boulis and G. E. Groblewski: Vesicle associated membrane protein 8 (VAMP8)-mediated zymogen granule exocytosis is dependent on endosomal trafficking via the constitutive-like secretory pathway. *J Biol Chem*, 289(40), 28040-53 (2014)  
DOI: 10.1074/jbc.M114.593913
163. B. May-Mederake: Early intervention and assessment of speech and language development in young children with cochlear implants. *Int J Pediatr Otorhinolaryngol*, 76(7), 939-46 (2012)  
DOI: 10.1016/j.ijporl.2012.02.051
164. N. Thayanidhi, Y. Liang, H. Hasegawa, D. C. Nycz, V. Oorschot, J. Klumperman and J. C. Hay: R-SNARE ykt6 resides in membrane-associated protease-resistant protein particles and modulates cell cycle progression when over-expressed. *Biol Cell*, 104(7), 397-417 (2012)  
DOI: 10.1111/boc.201100048
165. L. Xie, D. Zhu, S. Dolai, T. Liang, T. Qin, Y. Kang, H. Xie, Y. C. Huang and H. Y. Gaisano: Syntaxin-4 mediates exocytosis of pre-docked and newcomer insulin granules underlying biphasic glucose-stimulated insulin secretion in human pancreatic beta cells. *Diabetologia*, 58(6), 1250-9 (2015)  
DOI: 10.1007/s00125-015-3545-4
166. S. Nagamatsu, H. Sawa, Y. Nakamichi, Y. Kondo, S. Matsushima and T. Watanabe: Non-functional role of syntaxin 2 in insulin exocytosis by pancreatic beta cells. *Cell Biochem Funct*, 15(4), 237-42 (1997)  
DOI: 10.1002/(SICI)1099-0844(199712)15:4<237:AID-CBF746>3.0.CO;2-U
167. D. Zhu, E. Koo, E. Kwan, Y. Kang, S. Park, H. Xie, S. Sugita and H. Y. Gaisano: Syntaxin-3 regulates newcomer insulin granule exocytosis and compound fusion in pancreatic beta cells. *Diabetologia*, 56(2), 359-69 (2013)  
DOI: 10.1007/s00125-012-2757-0
168. S. H. Low, X. Li, M. Miura, N. Kudo, B. Quinones and T. Weimbs: Syntaxin 2 and endobrevin are required for the terminal step of cytokinesis in mammalian cells. *Dev Cell*, 4(5), 753-9 (2003)  
DOI: 10.1016/S1534-5807(03)00122-9
169. I. Naegelen, S. Plancon, N. Nicot, T. Kaoma, A. Muller, L. Vallar, E. J. Tschirhart and S. Brechard: An essential role of syntaxin 3 protein for granule exocytosis and secretion of IL-1alpha, IL-1beta, IL-12b, and CCL4 from differentiated HL-60 cells. *J Leukoc Biol*, 97(3), 557-71 (2015)  
DOI: 10.1189/jlb.3A0514-254RR
170. S. Ye, Z. A. Karim, R. Al Hawas, J. E. Pessin, A. H. Filipovich and S. W. Whiteheart: Syntaxin-11, but not syntaxin-2 or syntaxin-4, is required for platelet secretion. *Blood*, 120(12), 2484-92 (2012)  
DOI: 10.1182/blood-2012-05-430603
171. E. M. Golebiewska, M. T. Harper, C. M. Williams, J. S. Savage, R. Goggs, G. Fischer von Mollard and A. W. Poole: Syntaxin 8 regulates platelet dense granule secretion, aggregation, and thrombus stability. *J Biol Chem*, 290(3), 1536-45 (2015)  
DOI: 10.1074/jbc.M114.602615
172. Z. Lin, D. Zhao, Y. Wang, W. Zhao, X. Yin, X. Zhou, Z. Zhang and L. Yang: Downregulation of beta-Soluble N-Ethylmaleimide-Sensitive Factor Attachment Protein: Proteomics-Based Identification in Early-Stage Prion Disease. *Neurodegener Dis* (2015)
173. E. Matveeva and S. W. Whiteheart: The effects of SNAP/SNARE complexes on the ATPase of NSF. *FEBS Lett*, 435(2-3), 211-4 (1998)  
DOI: 10.1016/S0014-5793(98)01071-0
174. P. Chavrier, R. G. Parton, H. P. Hauri, K. Simons and M. Zerial: Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell*, 62(2), 317-29 (1990)  
DOI: 10.1016/0092-8674(90)90369-P
175. H. McLauchlan, J. Newell, N. Morrice, A. Osborne, M. West and E. Smythe: A novel role for Rab5-GDI in ligand sequestration into clathrin-coated pits. *Curr Biol*, 8(1), 34-45 (1998)  
DOI: 10.1016/S0960-9822(98)70018-1
176. M. C. Gimenez, J. F. Rodriguez Aguirre, M. I. Colombo and L. R. Delgui: Infectious bursal disease virus uptake involves macropinocytosis and trafficking to early endosomes in a Rab5-dependent manner. *Cell Microbiol*, 17(7), 988-1007 (2015)  
DOI: 10.1111/cmi.12415
177. A. Wandinger-Ness and M. Zerial: Rab proteins and the compartmentalization of

- the endosomal system. *Cold Spring Harb Perspect Biol*, 6(11), a022616 (2014)  
DOI: 10.1101/cshperspect.a022616
178. Y. Sun, T. T. Chiu, K. P. Foley, P. J. Bilan and A. Klip: Myosin Va mediates Rab8A-regulated GLUT4 vesicle exocytosis in insulin-stimulated muscle cells. *Mol Biol Cell*, 25(7), 1159-70 (2014)  
DOI: 10.1091/mbc.E13-08-0493
179. B. Short, C. Preisinger, J. Schaletzky, R. Kopajtich and F. A. Barr: The Rab6 GTPase regulates recruitment of the dynactin complex to Golgi membranes. *Curr Biol*, 12(20), 1792-5 (2002)  
DOI: 10.1016/S0960-9822(02)01221-6
180. I. Jordens, M. Marsman, C. Kuijl and J. Neefjes: Rab proteins, connecting transport and vesicle fusion. *Traffic*, 6(12), 1070-7 (2005)  
DOI: 10.1111/j.1600-0854.2005.00336.x
181. M. Kurowska, N. Goudin, N. T. Nehme, M. Court, J. Garin, A. Fischer, G. de Saint Basile and G. Menasche: Terminal transport of lytic granules to the immune synapse is mediated by the kinesin-1/Slp3/Rab27a complex. *Blood*, 119(17), 3879-89 (2012)  
DOI: 10.1182/blood-2011-09-382556
182. H. M. McBride, V. Rybin, C. Murphy, A. Giner, R. Teasdale and M. Zerial: Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. *Cell*, 98(3), 377-86 (1999)  
DOI: 10.1016/S0092-8674(00)81966-2
183. S. Schoch, P. E. Castillo, T. Jo, K. Mukherjee, M. Geppert, Y. Wang, F. Schmitz, R. C. Malenka and T. C. Sudhof: RIM1 $\alpha$  forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature*, 415(6869), 321-6 (2002)  
DOI: 10.1038/415321a
184. P. Burkhardt, C. M. Stegmann, B. Cooper, T. H. Kloepper, C. Imig, F. Varoqueaux, M. C. Wahl and D. Fasshauer: Primordial neurosecretory apparatus identified in the choanoflagellate *Monosiga brevicollis*. *Proc Natl Acad Sci U S A*, 108(37), 15264-9 (2011)  
DOI: 10.1073/pnas.1106189108
185. D. Parisotto, M. Pfau, A. Scheutzw, K. Wild, M. P. Mayer, J. Malsam, I. Sinning and T. H. Sollner: An extended helical conformation in domain 3a of Munc18-1 provides a template for SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex assembly. *J Biol Chem*, 289(14), 9639-50 (2014)  
DOI: 10.1074/jbc.M113.514273
186. N. R. Bin, C. H. Jung, B. Kim, P. Chandrasegram, E. Turlova, D. Zhu, H. Y. Gaisano, H. S. Sun and S. Sugita: Chaperoning of closed syntaxin-3 through Lys46 and Glu59 in domain 1 of Munc18 proteins is indispensable for mast cell exocytosis. *J Cell Sci*, 128(10), 1946-60 (2015)  
DOI: 10.1242/jcs.165662
187. C. Brochetta, R. Suzuki, F. Vita, M. R. Soranzo, J. Claver, L. C. Madjene, T. Attout, J. Vitte, N. Varin-Blank, G. Zabucchi, J. Rivera and U. Blank: Munc18-2 and syntaxin 3 control distinct essential steps in mast cell degranulation. *J Immunol*, 192(1), 41-51 (2014)  
DOI: 10.4049/jimmunol.1301277
188. A. Rehman, J. K. Archbold, S. H. Hu, S. J. Norwood, B. M. Collins and J. L. Martin: Reconciling the regulatory role of Munc18 proteins in SNARE-complex assembly. *IUCrJ*, 1(Pt 6), 505-13 (2014)  
DOI: 10.1107/S2052252514020727
189. M. Dudenhofer-Pfeifer, C. Schirra, V. Pattu, M. Halimani, M. Maier-Peuschel, M. R. Marshall, U. Matti, U. Becherer, J. Dirks, M. Jung, P. Lipp, M. Hoth, M. Sester, E. Krause and J. Rettig: Different Munc13 isoforms function as priming factors in lytic granule release from murine cytotoxic T lymphocytes. *Traffic*, 14(7), 798-809 (2013)  
DOI: 10.1111/tra.12074
190. C. Ma, L. Su, A. B. Seven, Y. Xu and J. Rizo: Reconstitution of the vital functions of Munc18 and Munc13 in neurotransmitter release. *Science*, 339(6118), 421-5 (2013)  
DOI: 10.1126/science.1230473
191. Y. J. Kaeser-Woo, X. Yang and T. C. Sudhof: C-terminal complexin sequence is selectively required for clamping and priming but not for Ca<sup>2+</sup> triggering of synaptic exocytosis. *J Neurosci*, 32(8), 2877-85 (2012)  
DOI: 10.1523/JNEUROSCI.3360-11.2012
192. P. Cao, X. Yang and T. C. Sudhof: Complexin activates exocytosis of distinct secretory vesicles controlled by different synaptotagmins. *J Neurosci*, 33(4), 1714-27 (2013)  
DOI: 10.1523/JNEUROSCI.4087-12.2013

193. R. A. Jorquera, S. Huntwork-Rodriguez, Y. Akbergenova, R. W. Cho and J. T. Littleton: Complexin controls spontaneous and evoked neurotransmitter release by regulating the timing and properties of synaptotagmin activity. *J Neurosci*, 32(50), 18234-45 (2012)  
DOI: 10.1523/JNEUROSCI.3212-12.2012
194. R. W. Cho, Y. Song and J. T. Littleton: Comparative analysis of Drosophila and mammalian complexins as fusion clamps and facilitators of neurotransmitter release. *Mol Cell Neurosci*, 45(4), 389-97 (2010)  
DOI: 10.1016/j.mcn.2010.07.012
195. T. Murase, N. Imaeda, N. Kondoh and T. Tsubota: Ceramide enhances acrosomal exocytosis triggered by calcium and the calcium ionophore A23187 in boar spermatozoa. *J Reprod Dev*, 50(6), 667-74 (2004)  
DOI: 10.1262/jrd.50.667
196. J. Rohrbough, E. Rushton, L. Palanker, E. Woodruff, H. J. Matthies, U. Acharya, J. K. Acharya and K. Brodie: Ceramidase regulates synaptic vesicle exocytosis and trafficking. *J Neurosci*, 24(36), 7789-803 (2004)  
DOI: 10.1523/JNEUROSCI.1146-04.2004
197. R. Bhatia, K. Matsushita, M. Yamakuchi, C. N. Morrell, W. Cao and C. J. Lowenstein: Ceramide triggers Weibel-Palade body exocytosis. *Circ Res*, 95(3), 319-24 (2004)  
DOI: 10.1161/01.RES.0000136519.84279.7a
198. H. J. Jeon, D. H. Lee, M. S. Kang, M. O. Lee, K. M. Jung, S. Y. Jung and D. K. Kim: Dopamine release in PC12 cells is mediated by Ca(2+)-dependent production of ceramide via sphingomyelin pathway. *J Neurochem*, 95(3), 811-20 (2005)  
DOI: 10.1111/j.1471-4159.2005.03403.x
199. D. E. Saslowsky, Y. M. te Welscher, D. J. Chinnapen, J. S. Wagner, J. Wan, E. Kern and W. I. Lencer: Ganglioside GM1-mediated transcytosis of cholera toxin bypasses the retrograde pathway and depends on the structure of the ceramide domain. *J Biol Chem*, 288(36), 25804-9 (2013)  
DOI: 10.1074/jbc.M113.474957
200. K. P. Foley and A. Klip: Dynamic GLUT4 sorting through a syntaxin-6 compartment in muscle cells is derailed by insulin resistance-causing ceramide. *Biol Open*, 3(5), 314-25 (2014)  
DOI: 10.1242/bio.20147898
201. A. E. Cremesti, F. M. Goni and R. Kolesnick: Role of sphingomyelinase and ceramide in modulating rafts: do biophysical properties determine biologic outcome? *FEBS Lett*, 531(1), 47-53 (2002)  
DOI: 10.1016/S0014-5793(02)03489-0

**Abbreviations:** ECs, endothelial cells; GRAF1, GTPase regulator associated with focal adhesion kinase; Arf6, adenosine diphosphate-ribosylation factor 6; LRs, lipid rafts; CME, clathrin-mediated endocytosis; CCVs, clathrin-coated vesicles; LDL-R, low density lipoprotein receptor; HDL-R, high density lipoprotein receptor; Alb-R, albumin receptor; Tf-R, transferrin receptor; Ins-R, insulin receptor; Cav-1, caveolin-1; SR-BI, scavenger receptor class B type I; VLDL, very-low-density lipoprotein; ox-LDL, oxidative LDL; TIRF, total internal reflection fluorescence; BBB, blood-brain barrier; AS, atherosclerosis; VEGF, vascular endothelial growth factor; AGE-Alb, Alb modified by advanced glycation end products; eNOS, endothelial NO synthase; IRS1, insulin receptor substrate 1; GLUT4, glucose transporter-4; A $\beta$ , beta-amyloid; AD, Alzheimer's disease; TCTP, T translational controlled tumor protein; PTD, protein transduction domain; PAH, pulmonary arterial hypertension; NPs, nanoparticles; BTB, blood-tumor barrier; CXCL10, interferon gamma-inducible protein-10; DCs, dendritic cells; PEI, polyethylenimine; PAMAM, polyamidoamine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; Abraxane, Alb-bound paclitaxel; SM, sphingomyelin; SMase, sphingomyelinase; GCase, glucosylceramidase; CS, ceramide synthase; C1PP, ceramide-1 phosphate phosphatase; SMS, SM synthase; GCS, glucosylceramide synthase; Cdase, ceramidase; CK, ceramide kinase; ROS, reactive oxygen species; Dyn, dynamin; Intersectin, ITSN; GPI-AP, GPI-anchored protein; EGF, epidermal growth factor; Cdc42, cell division cycle 42; CLICs, clathrin-independent carriers; GEECs, GPI-APs enriched early endosomal compartments; Anxs, Annexins; EEs early endosomes; Syn, Syndecan; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; NSF, N-ethylmaleimide sensitive factor; SNAPs, soluble NSF attachment protein; VAMPs, vesicle associated membrane proteins; SGs, secretory insulin-containing granules; GSIS, glucose stimulated insulin secretion; Syt, synaptotagmin; CPX, Complexion; GEFs, guanine nucleotide exchange



factors; GDIs, GDP-dissociation inhibitors; EEA, endosome associated antigen.

**Key Words:** Transcytosis, Lipid Raft, Ceramide, Atherosclerosis, Review

**Send correspondence to:** Si Jin, Department of endocrinology, Institute of geriatric medicine, Liyuan hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, 430077, China, Tel: 8627-8369-1182, Fax: 8627-8369-2608, E-mail: Jinsi@mail.hust.edu.cn