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Caveolae-Dependent Endocytosis in Viral Infection

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1. Introduction

1.1. Caveolae structure and morphogenesis

Morphologically described as small “caves” in the plasma membrane, caveolae are highly specialized membrane domains with important roles in cell endocytosis, lipid metabolism, and signaling. Since their discovery sixty years ago [1,2], the functional relevance of caveolae has challenged many scientists, raising numerous debates and controversies. The electron microscopy images of caveolae show a rather cell-type-dependent appearance. In endothelial cells, caveolae opening is more constricted [3], while in epithelial cells they appear open to the extracellular medium and smaller in size [4]. In muscle cells, multiple caveolae units cluster together forming T-like tubules invaginating from sarcolemma [5]. Regardless their shape, caveolae appear as immobile structures, in tight connection with the cortical actin cytoskeleton underlying the plasma membrane. Video microscopy and fluorescence recovery after photobleaching analysis have shown that caveolae detach from the membrane only upon ligand binding and specific signaling [6].

The discovery of caveolins (Cav), the structural proteins enveloping caveolae in a spike-like coat [7] marked a significant breakthrough in understanding the nature and importance of these organelles.

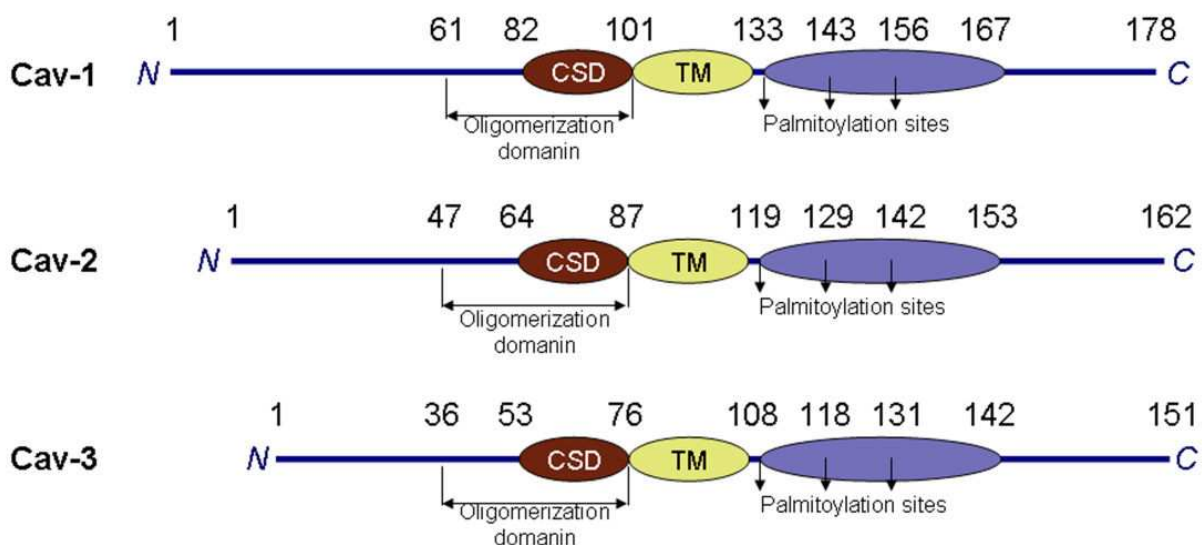
Three members of the Cav family have been described in mammalian cells, to date: Cav-1, -2, -3, which share a significant homology and are conserved throughout evolution [8]. Cav-1 and -2 are relatively ubiquitous, with highest distribution in fibroblasts, adipocytes, endothelial cells, and pneumocytes, being co-expressed in most cells types [9]. Cav-3 is expressed independently of Cav-1 and -2 and is limited to skeletal muscle fibers and cardiac myocytes [10-12]. Over-expression of Cav-1 in caveolae-deficient cells is necessary and ap-

parently sufficient to drive caveolae biogenesis [13]. Moreover, Cav-1 expression is required for the membrane localization and stability of Cav-2. Although unable to drive caveolae biogenesis on its own, Cav-2 may however influence it, at least in several polarized, epithelial cells [14,15]. The capacity to modulate caveolae assembly, shape and size has been shown to depend on Cav-2 phosphorylation status [16]. Similar to Cav-1, Cav-3 protein is sufficient to drive formation of caveolae in muscle cells [17].

Most of the molecular data available on caveolins refer to Cav-1; therefore the following discussion will focus on this protein, as a representative of the caveolin family, which is shown schematically in Fig.1.

Cav-1 is an integral membrane protein of 21kDa with an unusual topology. Both the N- and C-termini are cytoplasmically oriented and connected by a central hydrophobic domain, comprising approximately residues 102-134, inserted into, but not spanning the membrane bilayer, in a hairpin (or U bent, or horseshoe) configuration [18,19]. Interestingly, a peptide corresponding to the last 20 residues of the hydrophilic N-domain, (amino acids 82–101) enriched in aromatic residues, can also bind to membranes independently [20,21]. This so-called caveolin scaffolding domain (CSD) is a highly conserved region responsible for many functions associated with Cav-1.

In silico analysis of the conformation of this hydrophobic domain, showed that mutation of a single residue, Pro (110), changes the stable conformation to a straight hydrophobic helix that would span the membrane. Expression of the Cav-1 P110A mutant in HEK 293 cells followed by confocal immunofluorescence microscopy further confirmed the in silico data and the estimated topology [22].



CSD- scaffolding domain
 TM- transmembrane domain

Figure 1. Schematic presentation of the structural similarities of the caveolin family of proteins.

Very recently, circular dichroism and NMR spectroscopy analysis have shown that the transmembrane domain of Cav-1 is primarily α -helical (57-65%). In addition, the helix-break-helix structure was suggested to be critical for the formation of the intra-membrane horseshoe conformation predicted for the protein. Interestingly, mutations of Ile (109) and Pro (110) to Ala dramatically altered the helix-break-helix structure. Moreover, it was shown that substitution of Pro (110) with any other residue results in disruption of the helix-break-helix structure, confirming the importance of the residue in the stability of the hydrophobic domain [23].

An important structural feature of Cav-1 is represented by its arrangement in high molecular mass oligomers of about 350 kDa, composed of 14-16 individual molecules [24,25]. Oligomerization is initiated in the endoplasmic reticulum (ER), where the Cav-1 monomer is cotranslationally inserted into the membrane [19] and is rapidly assembled to form SDS-resistant, 8S complexes considered the building units of caveolae structure [24,25]. Intriguingly, blue native gel analysis evidenced only a few intermediate sized oligomers, suggesting that Cav-1 oligomerization is a highly cooperative process [26]. Oligomerization of the full-length protein requires the presence of the CSD and of the C-terminal domain [27] and appears to be stabilized by the palmitoylation of cysteine residues located at positions 133, 143 and 156 [18,28]. At this stage of their assembly, the complexes appear highly mobile in the ER membrane and rapidly concentrate at the ER exit sites, a process favored by the existence of a di-acidic export sequence located at the N-terminal domain. In the absence of this signal sequence, Cav-1 accumulates in lipid droplets [29,30]. This is an important observation, suggesting that ER exit and lipid droplets localization of caveolin complexes are competing processes, highlighting the role of the di-acidic motif in caveolin trafficking.

Interestingly, co-expression of Cav-1 and -2 results in assembly of mixed 8S complexes, where the Cav-1 to Cav-2 ratio may vary from 2:1 to 4:1 [31]; however, expression of Cav-2 alone does not result in oligomer formation.

The process continues in the trans-Golgi where the oligomers are exported in a COPII-dependent manner and self-associate into a large network of caveolin. However, formation of the 8S complex is not a prerequisite for Golgi transport, as expression of Cav-2 alone, as well as that of an oligomerization-incompetent variant of Cav-1 does not result in their retention within the ER [32,33].

The Golgi oligomerization step is sensitive to BFA, clearly indicating that formation of large oligomer complexes is dependent on caveolins trafficking to this compartment, probably requiring a specific lipid composition of the membrane. The assembly process continues in a cholesterol-dependent manner, resulting in formation of 70S stable complexes, also evidenced by using a panel of anti-Cav-1 conformational antibodies. These complexes were assumed to correspond to the intact protein scaffold of the caveolae structure [34]. It is interesting that the caveolin assemblies colocalized with medial, rather than trans-Golgi markers, suggesting that, unlike other cargos transported through the secretory pathway, caveolar carrier vesicles are formed in the medial cisternae, being further exported to the

plasma membrane in a dynamin-2 independent manner, similar to other raft-associated proteins [34]. This assembly process is schematically shown in Fig.2.

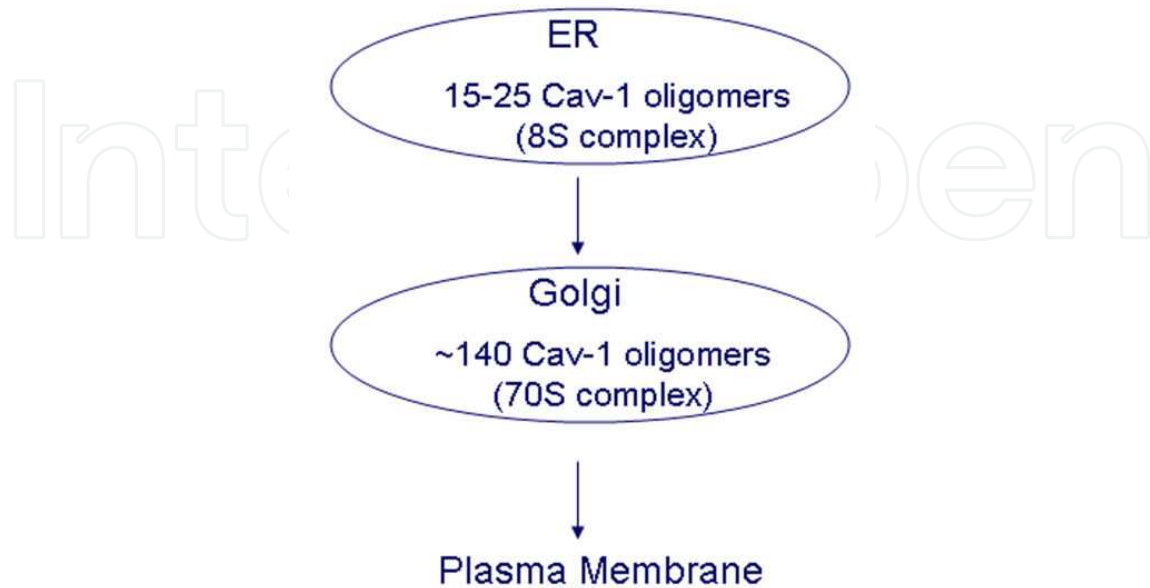


Figure 2. Caveolin oligomerization and assembly

It can be concluded from these observations that the tight regulation of Cav-1 trafficking along the secretory compartments (and the multiple check points) is totally justified by the complexity of the assembly process.

In addition to oligomerization, caveola formation involves association of the complexes with cholesterol-rich lipid-raft domains at plasma membrane. It is estimated that Cav-1 binds to 1–2 cholesterol molecules through the conserved basic and hydrophobic residues of the scaffolding domain [35]; thus, the relative amount of cholesterol concentrated in isolated caveolae can be as high as 20,000 molecules [36]. The relationship between cholesterol and caveolins is very complex. Treatment of cells with cholesterol binding or depleting agents results in caveolae with altered morphology and disrupted protein coat [7]. Moreover, cholesterol regulates Cav-1 expression at both, transcriptional and translational levels, through binding to either two steroid regulatory elements in the Cav-1 promoter, or the protein itself, thus modulating the level of Cav-1 mRNA or the protein stability [37,38].

Caveolae are enriched in glycosphingolipids (like GM1 and GM3) and sphingomyelin, the total lipid density being significantly higher than within the rest of the plasma membrane [36]. This is an important observation implying that certain lipids are recruited in caveolae, possibly to ensure their invagination-competent composition.

Recently, a crucial role in the last steps of caveolae biogenesis has been attributed to PTRF (Polymerase I and transcript release factor), originally described as an RNA Pol I transcription factor (also called Cav-P60 or cavin-1) [39,40]. Interestingly, cavin-1 is able to associate

with plasma membrane caveolae but not with caveolins with other intracellular distribution (such as Golgi caveolins) [34]. Cavin-1 is recruited by Cav-1 to plasma membrane caveolar domains, where the two proteins are found to be in close proximity and an approximate ratio of 1:1 [39]; however, whether or not they directly interact with each other is still a matter of debate. It was clearly demonstrated that cavins bind to phosphatidylserine and are phosphorylated at multiple sites, suggesting they may act as regulatory proteins of caveolae functions [41].

Based on sequence homology with cavin-1, three other proteins named cavin-2 to 4, sharing similar molecular organization, have been identified as part of the cavin family [42-44]. While cavin-1 expression is strictly associated to that of Cav-1 [39] contributing to the stability of the caveolae unit like a scaffolding protein, cavin-2 promotes recruitment of cavin-1 in caveolae and appears to have a role in the membrane-curvature [45].

The role of cavin-3 and -4 in caveolae biogenesis is less well understood. Cavin-3 was shown to regulate caveolae budding and Cav-1 trafficking, suggesting a function in coupling caveolae to the intracellular transport network [44]. Cavin-4 is co-expressed with Cav-3 in cardiac and muscle tissues where their function appears to be tightly correlated [42].

All members of the cavin family interact in a multimeric complex of about 60-80 cavins, in a Cav-1 independent manner. These complexes were detected both in the cytosol and plasma membrane fractions, suggesting they are the result of a succession of events, starting with cavin association into the cytosol and ending with the recruitment of the multimeric complexes to caveolae, during the final step of their biogenesis [42].

In contrast to caveolins, cavins are peripheral membrane proteins, and bind molecular components of the caveolar domain facing the cytosol. Given the high affinity of cavins for phosphatidylserine and the rapid dissociation from caveolae in the presence of nonionic detergents, it was suggested that binding to the lipid membrane, rather than to the protein scaffold, was highly probable.

The identification of cavins in caveolae opened new perspectives in understanding the complexity of caveolar structure. Although our knowledge on caveolae architecture and molecular composition has improved since their discovery, the main structural pillars defined at the time have not dramatically changed. Thus, today, caveolae are referred to as invaginations of the plasma membrane lipid bilayer, enriched in cholesterol and sphingolipids, embedding an integral membrane scaffold formed by caveolin oligomers assembled in a stable network, peripherally covered by a protein layer of cavin complexes. Once formed, this structure appears to remain stable also during endocytosis [46].

2. Caveolae signaling

The protein composition of caveolae has been addressed in a more systematic manner within the last years, using proteomic approaches [47-49]. A variety of signal transduction pro-

teins were found to localize in caveolae, in tight connection with either the CSD or the lipid domains.

According to the caveolin signaling hypothesis, the role of caveolae is to trigger specific signal transduction by concentrating downstream effectors close to plasma membrane receptors, through direct interaction with the CSD [50,51]. Palmitoylation appears to play an important function in this process by facilitating the caveolar localization of proteins [52]. Of the signaling molecules identified, several have been more thoroughly investigated:

- a. G proteins were abundant in caveolae; their binding to caveolin has a role in maintaining the $G\alpha$ subunits in an inactive GDP-bound state [53]. Small GTP-binding proteins of the Ras superfamily also localized in caveolae, a process enhanced by palmitoylation of the C-terminal hypervariable region [54,55]. Binding of the H-Ras to the CSD results in inactivation of the protein, which is relevant in certain human cancers where H-Ras-caveolin interaction is prevented and the protein is maintained in an active state [56].
- b. Src family kinases, such as c-Src, Fyn, Lyn [57] are nonreceptor tyrosine kinases, also enriched in caveolae. Their localization depends on the N-terminal myristoylation and subsequent interaction with the caveolins. Interestingly, Cav-1 palmitoylation is equally important for caveolae/c-Src interaction [52], which results in c-Src and Fyn inactivation [58]. In turn, the tyrosine phosphorylation of Cav-1 and -2 facilitates the recruitment of matrix metalloproteinases [58,59], and promotes caveolins localization to focal adhesions [60,61].
- c. Several steroid hormone receptors were localized in caveolae, a process depending on both, palmitoylation and association with Cav-1 [62] and facilitating their activation [63].
- d. Endothelial nitric oxide synthase (eNOS) is one of the most extensively studied Cav-1 interacting protein [64]. eNOS binds to the CSD of both Cav-1 and -3, which inhibit its enzymatic activity [65,66]. This observation led to a novel concept of eNOS regulation, whereby, the interaction with caveolins is necessary to keep the enzyme inactive under basal conditions, while its concentration in caveolae will allow a quick response upon stimulation [67].
- e. Many ion channels and pumps are targeted to caveolae and interact with caveolins, such as the calcium signaling molecules calmodulin, Ca^{2+} -ATPase, L-type Ca^{2+} channels [68,69]. Transient receptor potential (TRP) channels, and large-conductance Ca^{2+} -activated K^{+} channels also localize in cholesterol-rich membrane areas, suggesting an important role of these domains in Ca^{2+} homeostasis [70,71]. Other transporters, like the Na/K-ATPase, involved in maintaining the Na^{+} membrane gradient, are also found in caveolae, owing this localization to the existence of two caveolin-binding motifs in their amino acid sequence [72].
- f. Protein kinases of different families were found in caveolae, due to their direct interaction with the Cav-1 CSD. For PKA, this interaction results in inhibition of the enzymatic activity [73], with consequences on regulation of other proteins, such as ATP-dependent

K⁺ channels or eNOS in muscle and endothelial cells, respectively [74,75]. Different isoforms of the PKC family of enzymes are also caveolae resident and appear to participate in regulation of caveolar proteins [76]. Caveolae interaction with PKC is more complex, leading to either activation (via ceramide interaction) [77], or inactivation, following endosomal delivery [78]. Caveolae also recruit the phosphatidylinositol-3-kinase (PI3K) through direct binding to Cav-1 [79] and the protein kinase B (PKB). Integration of this signaling pathway by caveolae plays a significant role in managing the cellular physiological stress, and regulating cell survival and death [80].

- g. Phosphodiesterases (PDEs), involved in cyclic nucleotides (cAMP and cGMP) hydrolysis, have a preference for lipid rafts association (Abrahamsen H, 2004). For some isoforms, such as PDE3B, a direct interaction with Cav-1, with a stabilizing effect on PDE3B, has been clearly confirmed by co-immunoprecipitation [81]. Other PDEs (PDE5, PDE4A4) appear to be recruited by caveolae through indirect mechanisms, possibly involving adapter proteins; [82]. Nevertheless, this association influences the establishment of cAMP/cGMP gradients and the downstream events [83].

3. Caveolae internalization and trafficking

Electron microscopy data show that caveolae are tightly connected to submembranous actin filaments [84,85], suggesting a function of the cytoskeleton in caveolae-mediated endocytosis. However, the exact role of the actin cytoskeleton is not clearly defined, as its disruption inhibits uptake of the caveolae ligand, alkaline phosphatase, on one hand [86] and promotes clustering of caveolae and internalization of Cav-1-labeled vesicles, on the other hand [6,87]. These observations fit into a model whereby actin would play a dual role in caveolae internalization: one is to keep the organization of caveolae and maintain their immobility at the plasma membrane, and the other to promote vesicle budding and release from the membrane.

Caveolae endocytosis relies heavily on dynamin, a multi-domain GTPase [88,89], which was shown to interact directly with Cav-1 [90]. Ligand binding initially disrupts the local actin cytoskeleton and promotes dynamin II recruitment to the site of internalization [91,92]. Dynamin oligomerization and the GTP-dependent conformational changes result in a structural collar around the neck of caveolae, directly mediating formation of free transport vesicles, following vesicular fission from the plasma membrane. It was shown that the protein regulates the actin tail formation [93,94], possibly through binding to cortactin [95-97] or intersectin, which promotes actin polymerization [98].

Another player in this complex molecular game was recently suggested, following the initial observation that Cav-1 binds to actin cross-linking proteins, filamin A and B, both *in vitro* and *in vivo* [99]. The main intracellular function of these proteins is to organize the actin cytoskeleton. The Cav-1 filamin A interaction was further confirmed in different cell types [100,101] and it was implicated in activation of the actin-folding and chaperone protein T-complex protein-1, [100] and inhibition of calpain-mediated cleavage of filamin A [102].

Thus, by providing the missing link between Cav-1 and the actin cytoskeleton, filamin is an important regulator of caveolae-mediated endocytosis and trafficking [103].

It was proposed that following budding, caveolae can fuse with either preformed vesicles, called “caveosome” at the time of their discovery, or early endosome, the latter process being dependent on Rab5 expression [46].

The caveosome was initially described as an immobile structure which did not co-localize with fluid phase markers or ligands of the clathrin-dependent pathway [104]. Moreover, the compartment was characterized by neutral pH and was unable to accumulate a lysosomal dye (lysotracker), reinforcing the notion of an independent organelle, clearly distinct from other endocytic compartments, which delivers its cargo to other cellular locations, such as the ER [104,105]. However, in a recent investigation of Cav-1 trafficking using pH-sensitive fluorophores, the existence of a neutral pH compartment was seriously doubted [106]. Rather, it was suggested that the caveosomes would correspond to modified late endosomes, where Cav-1 accumulates when over-expressed, undergoing ubiquitination and being further targeted to degradation [106]. Conversely, under physiological conditions, caveolae would bud from the plasma membrane transporting their viral cargo to early endosomes and eventually to the ER, in a microtubule-dependent manner [107], following a series of maturation events, which will be detailed below.

Clearly, more work is necessary to have the correct picture of the highly atypical caveolar trafficking, which appears to allow access of its ligands to intracellular destinations that are not reachable from other endocytic pathways. Despite the remaining uncertainties, the continuous development of the field has considerably advanced our knowledge of virus infection of host cells using caveolae endocytosis.

The current view of caveolae internalization and trafficking is depicted in Fig. 3.

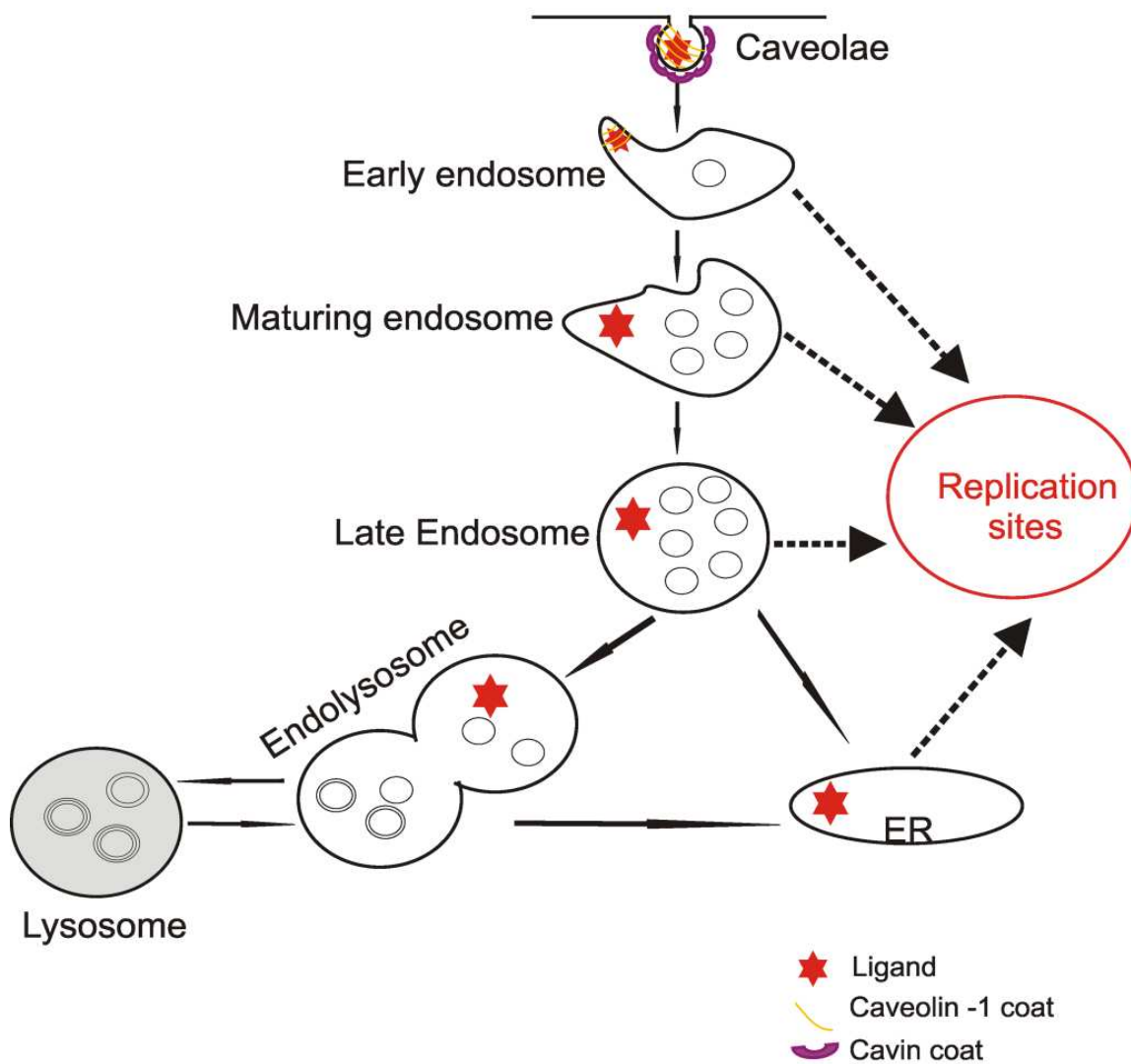


Figure 3. Ligand internalization and intracellular trafficking following caveolae-mediated endocytosis

4. Caveolae dependent viral infections

Owing to the vast amount of experimental data characterizing cell infection by the simian virus 40 (SV40), the pathogen has become the “star” of the caveolae-mediated entry pathway, being now extensively used in other studies as a marker of it [104,108]. Other well-characterized viruses using this entry pathway belong to the polyoma virus family, which has gained more interest recently, with the increasing number of human viruses identified. These include the KI polyoma virus, the WU polyoma virus and the Merkel cell polyoma virus [109-111], the latter being associated with an aggressive form of neuroendocrine skin cancer, the Merkel cell carcinoma. These are all non enveloped DNA viruses that replicate in the nucleus.

Viruses that use the same pathway to initiate a productive infection in target cells are listed in Table 1. Amongst them, Echovirus 1 (EV1), Human Hepatitis B virus (HBV) [112], Murine Leukemia Virus (MLV) [113], enteroviruses [114], have been more intensely investigated.

Virus	Reference
Simian virus 40	Stang, E., J. Kartenbeck, and R.G. Parton. 1997. Major histocompatibility complex class I molecules mediate association of SV40 with caveolae. <i>Mol Biol Cell.</i> 8:47-57. Pelkmans, L., J. Kartenbeck, and A. Helenius. 2001. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. <i>Nat Cell Biol.</i> 3:473-83.
K1 polyoma virus	Allander, T., K. Andreasson, S. Gupta, A. Bjerkner, G. Bogdanovic, M.A. Persson, T. Dalianis, T. Ramqvist, and B. Andersson. 2007. Identification of a third human polyomavirus. <i>J Virol.</i> 81:4130-6
WU polyoma virus	Gaynor, A.M., M.D. Nissen, D.M. Whiley, I.M. Mackay, S.B. Lambert, G. Wu, D.C. Brennan, G.A. Storch, T.P. Sloots, and D. Wang. 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. <i>PLoS Pathog.</i> 3:e64
Merkel cell polyoma virus	Feng, H., M. Shuda, Y. Chang, and P.S. Moore. 2008. Clonal integration of a polyomavirus in human Merkel cell carcinoma. <i>Science.</i> 319:1096-100
Echovirus 1	Marjomaki, V., V. Pietiainen, H. Matilainen, P. Upla, J. Ivaska, L. Nissinen, H. Reunanen, P. Huttunen, T. Hyypia, and J. Heino. 2002. Internalization of echovirus 1 in caveolae. <i>J Virol.</i> 76:1856-65. Stuart, A.D., H.E. Eustace, T.A. McKee, and T.D. Brown. 2002. A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. <i>J Virol.</i> 76:9307-22
Human Hepatitis B virus	Macovei, A., C. Radulescu, C. Lazar, S. Petrescu, D. Durantel, R.A. Dwek, N. Zitzmann, and N.B. Nichita. 2010. Hepatitis B virus requires intact caveolin-1 function for productive infection in HepaRG cells. <i>J Virol.</i> 84:243-53.
Murine Leukemia Virus	Beer, C., D.S. Andersen, A. Rojek, and L. Pedersen. 2005. Caveola-dependent endocytic entry of amphotropic murine leukemia virus. <i>J Virol.</i> 79:10776-87
Tiger Frog Virus	Guo, C.J., D. Liu, Y.Y. Wu, X.B. Yang, L.S. Yang, S. Mi, Y.X. Huang, Y.W. Luo, K.T. Jia, Z.Y. Liu, W.J. Chen, S.P. Weng, X.Q. Yu, and J.G. He. 2011. Entry of tiger frog virus (an Iridovirus) into HepG2 cells via a pH-dependent, atypical, caveola-mediated endocytosis pathway. <i>J Virol.</i> 85:6416-26

Infectious spleen and kidney necrosis virus	Guo, C.J., Y.Y. Wu, L.S. Yang, X.B. Yang, J. He, S. Mi, K.T. Jia, S.P. Weng, X.Q. Yu, and J.G. He. 2011. Infectious spleen and kidney necrosis virus (a fish iridovirus) enters Mandarin fish fry cells via caveola-dependent endocytosis. <i>J Virol.</i> 86:2621-31.
Influenza viruses	Nunes-Correia, I., A. Eulalio, S. Nir, and M.C. Pedrosa de Lima. 2004. Caveolae as an additional route for influenza virus endocytosis in MDCK cells. <i>Cell Mol Biol Lett.</i> 9:47-60.
Coronavirus 229E	Nomura, R., A. Kiyota, E. Suzaki, K. Kataoka, Y. Ohe, K. Miyamoto, T. Senda, and T. Fujimoto. 2004. Human coronavirus 229E binds to CD13 in rafts and enters the cell through caveolae. <i>J Virol.</i> 78:8701-8
Papillomavirus 31	Bousarghin, L., A. Touze, P.Y. Sizaret, and P. Coursaget. 2003. Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. <i>J Virol.</i> 77:3846-50
Respiratory syncytial virus	Brown G, Jeffree CE, McDonald T, Rixon HW, Aitken JD, et al. Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. <i>Virology</i> 2004;327(2) 175-185. Werling D, Hope JC, Chaplin P, Collins RA, Taylor G, et al. Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. <i>J Leukoc Biol</i> 1999;66(1) 50-58.
Newcastle disease virus	Cantin, C., J. Holguera, L. Ferreira, E. Villar, and I. Munoz-Barroso. 2007. Newcastle disease virus may enter cells by caveolae-mediated endocytosis. <i>J Gen Virol.</i> 88:559-69
Ebola virus	Empig, C.J., and M.A. Goldsmith. 2002. Association of the caveola vesicular system with cellular entry by filoviruses. <i>J Virol.</i> 76:5266-70.
BK polyoma virus	Eash, S., W. Querbes, and W.J. Atwood. 2004. Infection of vero cells by BK virus is dependent on caveolae. <i>J Virol.</i> 78:11583-90.
Marburg virus	Empig, C.J., and M.A. Goldsmith. 2002. Association of the caveola vesicular system with cellular entry by filoviruses. <i>J Virol.</i> 76:5266-70.

Table 1. Viruses that use caveolae-mediated endocytosis

After binding to the host cell, the virus particles are able to cluster the receptor molecules such as certain integrins ($\alpha 2\beta 1$ in the case of EV1) [118] or glycosphingolipids (GM1 or GD1a in the case of SV40 or polyoma viruses), within the lipid rafts [119]. Accumulation of viral particles in caveolae induces a cascade of tyrosine phosphorylation reactions followed by rearrangements of the cortical actin cytoskeleton, as described above [91].

Several models addressing virus capturing into the plasma membrane invaginations have been proposed, the most recent relying on the observation that Cav-1 polymer remains intact once formed in the Golgi complex, during transport to the plasma membrane. According to this model, caveolae result from the fusion of a pre-existing caveolar vesicle with the plasma membrane [46]. As a consequence of virus binding to an increasing number of sphingolipids and/or integrins, the affinity of the pathogen for caveolar domains increases, facilitating entrapment in these areas. Another possibility is that virus particles bind and release the plasma membrane gangliosides in a transient manner, thus screening the whole cell surface. When reaching a caveolar region where multiple gangliosides interactions can occur simultaneously, binding becomes permanent and the virus particles are sequestered [104].

The intracellular trafficking of caveolae cargos has been recently re-evaluated using SV40 as a model and a series of complementary, state-of-the art techniques, including live-cells and electron microscopy, video recordings, pharmacological inhibitors and inhibition of expression of trafficking regulators [120]. It was shown that productive SV40 infection depends on the virus transport through a series of classical endocytic vesicles. Initially, the virus is found in Rab5-, EEA1-positive early endosomes and subsequently becomes associated with Rab7-positive domains, during endosome maturation. As this process proceeds, SV40 co-localizes with LAMP1-, Rab9-, and Rab7-positive late endosomes resembling multivesicular bodies and possibly endolysosomes. Endosome maturation also involves acidification of the compartments, as a consequence of vacuolar ATPase (v-ATPase) recruitment and activity. At this stage, acidification is required for SV40 subsequent transport steps and the initiation of productive infection. Interestingly, BK and JC viruses were also shown to enter the endosomes and depend on acidification for infection [121-123]. In the case of the mouse polyoma virus, the recycling, as well as late endosomes have been involved in infection [124,125].

From the late compartments of the endocytic pathway, SV40 appears to be directly transported to the ER, although an indirect ER targeting, via the Golgi complex, has not been completely excluded. Similarly, other polyoma viruses are transported to the ER lumen, before reaching the nucleus [121,125].

However, there are some notable exceptions of virus trafficking diverting from this pathway, despite being internalized through caveolae. Thus, cellular penetration of the EV1, a positive-stranded RNA human pathogen, depends on caveolins, dynamin II, and signaling events but does not require actin filaments or microtubules. The virus uptake was much faster than that of SV40 and was followed by rapid co-localization with Cav-1. Beyond this step, the virus failed to enter the Golgi complex, the ER, or the lysosomes, as none of the markers used to label these organelles co-localized with viral proteins. This observation raised the hypothesis that the virus particles remain sequestered in the Cav-1 positive endocytic vesicles until replication is initiated, further using them for cytoplasmic penetration and uncoating.

The lack of transport to the ER or Golgi was also confirmed by the absence of any inhibition of infection in the presence of nocodazole. Although more experimental data is needed to substantiate it, it is tempting to speculate that the different sorting pathway of the two virus-

es is related to their replication mechanism; thus, a DNA virus such as SV40 needs the nucleus for replication, which might be more accessible through the ER, whereas for a positive-stranded RNA virus the release of the genome into the cytoplasm is sufficient to initiate replication.

Either way, the precise molecular details characterizing this segment of the trafficking pathway are still to be defined, but understanding the factors involved in these unusual trafficking pathways is crucially important as other, yet uninvestigated viruses, may well use them when accessing the host cell via caveolae.

Importantly, there is accumulating evidence suggesting that several viruses take advantage of cross talk between endocytosis routes. For instance, JCV, bovine papillomavirus type 1 and human papillomavirus type 16 have been shown to access cells using the clathrin-dependent endocytosis, but intriguingly, they require Cav-1-mediated trafficking to initiate productive infection [123,126,127]. The internalized virions were trafficked to early endosomes before being transported to the caveolar pathway. From the Cav-1-positive vesicles, the viral cargo is further moved to the ER in a COPI-mediated, BFA-sensitive manner [127].

The newly described trafficking routes taken by these viruses may have an explanation in their absolute requirement to reach the ER compartment, a target that is not on the clathrin-mediated route.

The intriguing question as to why these viruses travel to the lumen of the ER, instead of using the endosomes for genome release, have received several interesting answers lately. One possibility is that the pathogens take advantage of the ER machinery of folding enzymes and chaperones, for uncoating and membrane penetration, being activated by luminal thiol oxidoreductases before release into the cytosol/nucleoplasm [128].

Very recently, BiP and the ER-membrane protein BAP31 (both involved in ERAD) were shown to be essential factors for SV40 infection; thus incoming SV40 particles are structurally remodeled leading to exposure of the amino-terminal sequence of the minor viral protein VP2. These hydrophobic sequences anchor the virus to the ER membranes helping the particles release into the cytosol [129].

5. Investigation of the caveolae entry pathway

The molecular details of virus entry have been investigated through a variety of techniques, by perturbing endocytotic internalization with various inhibitors or interfering with the expression or function of key regulator proteins, using siRNA or dominant-negative mutants of the proteins, or by using transgenic animals. Because no single method to assess caveolae is perfect, the use of complementary techniques is crucial for such a task. These can employ cell fractionation, immunoprecipitation, protein and organelle labeling, immunofluorescence microscopy. Since caveolae are best characterized by their microscopic appearance, studies employing alteration of the intracellular level of caveolins, cholesterol, or different molecules enriched, but not exclusively present in caveolae, should ideally follow the im-

pact of such changes to other internalization and trafficking pathways, by electron microscopy

5.1. Pharmacological approaches

A pharmacologic approach involves the treatment of cells with agents that deplete membranes of cholesterol, or inhibit various structural or signaling molecules involved in controlling the pathway. Having the advantage of being readily available and convenient to use, chemical inhibitors have been extensively employed to characterize different endocytic pathway; however, stringent controls must be included and results should be interpreted with care, because of the pleiotropic effects these drugs may have within the treated cell.

The usefulness as well as the pitfalls associated with the use of these agents will be detailed below.

Methyl- β -cyclodextrin (M β CD) – Cyclodextrins are cyclic oligomers of glucose that have the property to bind and extract lipophiles, including cholesterol, from their hydrophobic core [130]. Based on the tight dependency of caveolae stability and function on the amount of cholesterol present in the lipid rafts, the compound has been widely used to define the caveolar-mediated entry of many pathogens [6,117,131-135].

A major issue of M β CD treatment is its cellular toxicity, which was initially associated with longer incubation times (for example, the cell viability decreases from 90% during a 30 minutes incubation, to as low as 64%, if the drug is used for 12 h) [117]. A thorough study performed on multiple cell lines showed, however, that M β CD significantly decreased cellular viability, even after short treatment and at concentrations routinely used to inhibit endocytosis, a phenomenon which was cell line dependent [136]. Moreover, a low level of plasma membrane cholesterol was shown to interfere with other endocytic pathways, such as the clathrin-mediated endocytosis [137,138], or even with cholesterol independent endocytosis [139], demonstrating a rather poor specificity of the drug in inhibiting a distinct pathway.

Statins – are a group of drugs that lower the intracellular cholesterol level by competitively inhibiting the 3 hydroxy 3 methylglutaryl coenzyme A reductase involved in its biosynthesis. As a consequence, efficient depletion of membrane cholesterol and decreased formation of caveolae are observed [140]. Despite showing good toxicity profiles, statins also exert pleiotropic effects through a variety of mechanisms, which appear to be unrelated to their cholesterol-lowering activity. Thus, several immunosuppressive effects have been involved; amongst them well-documented are the prevention of activation of the transcription factor NF-kappaB or up-regulation of the pro-inflammatory cytokine production [141,142].

Filipin - is a macrolide pentene polyene with antibiotic properties relying on sterol binding with high affinity [143]. Filipin III has been employed to block caveolae entry since it complexes with membrane cholesterol, thus interfering with cholesterol-sensitive processes [144]. Treatment with filipin dispersed the receptors found in caveolae and promoted disassembly of these structures [145]. Similar to M β CD, filipin treatment is toxic and at least for certain cell lines, its inhibitory effect on endocytosis was exclusively due to cytotoxicity

[146]. However, a narrow window of specific inhibitory function can be identified in most cell lines.

Genistein – inhibits several tyrosine kinases in mammalian cells and thus, caveolae internalization [86,147]. Genistein has been however shown to suppress the entry of several types of viruses that use different endocytic ways to gain access to the replication sites: SV40 [91], adenoviruses [148], human herpesvirus 8 (HHV-8) [149], HBV [112]. However, the general need of tyrosine kinases-mediated signaling of diverse families of viruses, both at early entry steps, or later in infection, makes it difficult to clearly ascertain the endocytic route used, only by using genistein.

U18666A - is an amphiphilic amine that arrests cholesterol transport and suppresses sterol biosynthesis. Treatment of cells with this inhibitor was shown to induce cholesterol accumulation in late endosomes/lysosomes and deplete cholesterol from the Golgi complex [150]. Interestingly, the mobility of Cav-1 significantly increased in the Golgi complex of U18666A-treated cells.

Phorbol 12-myristate 13-acetate (PMA) – is an activator of classical 2,3-diacylglycerol (DAG)-dependent protein kinase isoforms, owing this property to their high affinity for the DAG binding site. Interestingly, PMA treatment results in constitutive phosphorylation of caveolin [151] with significant inhibitory effects on caveola invagination from the intracellular face of the plasma membrane [86,152,153].

PMA has been shown to specifically inhibit the caveolae-mediated entry of Ebola and Marburg viruses, two negative-stranded RNA pathogens, members of the *Filoviridae* family [154]. PMA has a low cytotoxicity even when used for longer incubation times (82% of the cell are still viable following incubation for 24 h, at concentrations required to inhibit caveolae endocytosis [117].

It is important to note that PMA can stimulate endocytosis of other ligands and interferes with the endocytic trafficking by stimulating a factor required for endosome fusion after Rab5 activation [155]. PMA treatment may have opposite effects on internalization of certain ligands (such as FITC-dextran) in polarized cells, increasing for instance, its basolateral, but not apical uptake.

Okadaic acid – is an inhibitor of phosphatases 1 and 2A, which are important in caveolae function [156]. Treatment with okadaic acid has been shown to promote removal of caveolar structures from the cell surface and stimulate endocytosis via these structures [6,86]. Importantly, the drug also inhibits the clathrin-mediated endocytosis [86] making the time of addition to the cells an important experimental factor in drawing the right conclusions on viral entry. Thus, pretreatment of cells with okadaic acid interferes with both, caveolae and clathrin pathways, while addition after virus binding to the target cells enhances infection, as it was shown for MLV [113].

Cytoskeleton inhibitors - the actin cytoskeleton localized near the plasma membrane appear to be a critical regulator of caveolae endocytosis [157].

Depolymerization of microtubules with colchicine or disruption of actin microfilaments with cytochalasin D resulted in a significant reduction of the amount of Cav-3 in plasma membrane fractions isolated from cardiac myocytes. Treatment with either drug also led to the exclusion of Cav-1 and -2 from similar fractions and the decrease of tyrosine-phosphorylated Cav-1 [101].

Other drugs interfering with actin polymerization are latrunculin A (actin monomer-sequestering drug) and jasplakinolide (an actin polymer-stabilizing compound), both were shown to reduced SV40 internalization by more than 60% [91]. Since function of many cellular processes such as trafficking and organelle movement are regulated by microtubules and the actin cytoskeleton, the biochemical assays should be combined with microscopy analysis to clearly define the role of the cytoskeleton in viral infection mediated by caveolae. Also, it is important to keep in mind that viruses induce cytoskeletal reorganization and reconfiguration to initiate, maintain and spread the infection. Therefore, the impact of the cytoskeleton perturbation on the outcome of infection highly depends on the stage of the viral life cycle the drug is acting upon [158].

5.2. Interference with expression and function of caveolae regulating proteins

As threshold levels of Cav-1 regulate caveolae formation [155,159,160], modulating the expression and/or function of this protein is one the most reliable approach to investigate caveolae entry. Cav-1 down-regulation using anti-sense, small interfering (si) or short hairpin (sh) RNA results in a significant decrease in the number of caveolae. For instance, the siRNA-mediated knockdown of Cav-1 expression was sufficient to inhibit albumin uptake in endothelial cells; however, intriguingly enough, the caveolae localization of signaling proteins, including eNOS, Rac, tyrosine kinase Src and insulin receptor was not altered. Using this technique, several viruses were shown to depend on caveolae for productive infection such as the Avian Reovirus [161], BK polyomavirus [133].

Cavin proteins are also important targets to study caveolae-mediated entry, since absence of cavin-1 results in lower expression level of caveolins and eventually, the loss of caveole. Down-regulation of cavin-1, using specific shRNA, increases mobility of caveolin-1, which is released from the cell surface and rapidly internalized and degraded [39]. Interfering with cavin-2 expression is also a valuable tool when assessing the role of caveolae in viral infection, since its down-regulation induces loss of cavin-1 and caveolin expression and therefore, it limits caveolae formation [43]. Similarly, suppression of cavin-3 biosynthesis uncouples caveolae from the intracellular transport machinery [44].

Caveolae budding from the plasma membrane and subsequent internalization strictly depends on dynamin II [89], thus silencing its expression is also often used in combination with caveolin inhibition.

An elegant alternative to silencing the expression of the proteins involved in caveolae architecture and function is over-expression of their mutant counterparts, which compete with the wild-type proteins for the same function. An important advantage of this technique is that, at any time during the experiment, the wild-type protein is still expressed ensuring the

functioning of the pathway at a basal level and reducing toxicity. This approach was used to show the dependence of HBV internalization on functional Cav-1 and dynamin II (Fig. 4) [112].

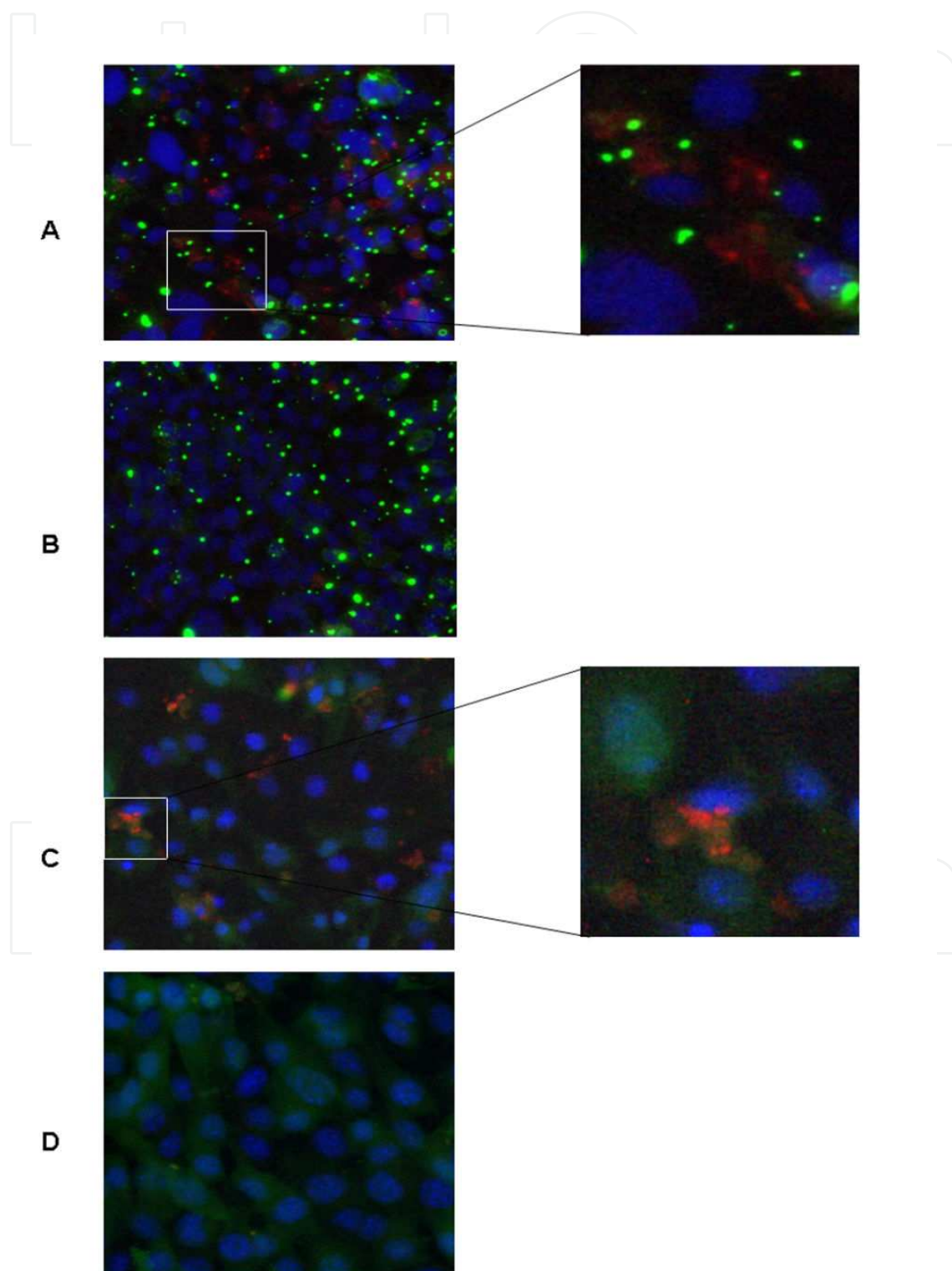


Figure 4. Hepatitis B Virus (HBV) infection of permissive HepaRG cells stably expressing dynamin II (A and B) and caveolin-1 (C and D) proteins with either wild-type (A and C) or dominant-negative (B and D) functions. HBV infection is evidenced by immunofluorescence microscopy using antibodies against the envelope proteins (in red). The dominant-negative dynamin II contains the K44A mutation which abolishes the GTP-ase activity (B). The dominant negative caveolin-1 contains a deletion of the 1-81 amino acid domain, at the N-terminal end (D). Expression of the wild-type and dominant-negative variants is evidenced through the Green Fluorescent Protein (GFP), which is either co-expressed from bicistronic GFP-caveolin DNA constructs (C and D) or expressed in fusion with dynamin II (A and B).

Cav -1 dominant negative proteins – disturb the formation of Cav-1-positive lipid rafts and cause the redistribution of endogenous caveolin to detergent soluble membrane fractions [162]. These are N-terminally truncated or N-terminally GFP-tagged caveolin constructs, which strongly inhibit SV40 entry [104,163] and were used to characterize the internalization pathway of many other viruses [134,164,165].

Other caveolin constructs containing the point mutations, Y14F and P132L were recently demonstrated to have dominant-negative activity [166]. Expression of the Cav-1 variant containing the P110A mutation was shown to determine a profound inhibition of caveolae endocytosis, cellular lipid accumulation and lipid droplet biogenesis. Moreover, this is a potent mutant to take into account when investigating the caveolae pathway, as it significantly reduces the Cav-1 localization into detergent-resistant domains of the plasma membrane and caveolae formation [22].

An interesting caveolin mutant is cav^{DGV} , a deleted Cav-3 form, which lacks the first 53 residues of the protein, but contains an intact scaffolding domain. The truncated protein acts as a dominant negative inhibitory mutant, causing the intracellular accumulation of free cholesterol in late endosomes, a reduction of surface cholesterol, efflux and synthesis [30].

Dynamin -II dominant negative proteins - as important regulators of clathrin, caveolae and other endocytic pathways [88,167], dynamin II inhibition is often used in combination with modulation of other, more specific proteins involved in caveolae function (listed above). By far, the most used mutant dynamin is the K44A variant, defective in GTP hydrolysis, which was clearly shown to inhibit release of caveolae from plasma membranes in an *in vitro* assay [89].

A long term expression of a dominant negative protein may be toxic for the cells, determining changes of morphology. Also it is important to keep in mind that down-regulation of a certain pathway may promote up-regulation of other, compensatory entry mechanisms, if cells express dominant negative proteins for a long time. A solution to overcome these potential problems is the use controlled/inducible expression systems (such as the TetOn/Tet Off switch).

Generation of knockout (KO) mice – is a powerful approach for the study of caveolae *in vivo*. Caveolin-KO mice (Cav-1, -2, -3 and Cav-1/-3 double KO mice have already been generated and characterized. They displayed different phenotypes, but interestingly, were viable and fertile [168]. While Cav-2 KO mice retain normal expression of caveolae, Cav-1 KO mice are devoid of Cav-2 expression and caveolae in certain cell lines, and develop many cardiac and pulmonary diseases. More work is needed to understand whether or not these pathologies are directly correlated with the loss of expression of caveolins and caveolae and

a good approach toward this aim would be to investigate each individual caveolins and the development of the corresponding phenotypes over a longer period of time.

6. Concluding remarks

The new experimental evidence emerged with the advance of the techniques used to investigate ligand internalization and intracellular trafficking, have consolidated the notion that endocytosis through caveolae is a true alternative to the clathrin-mediated pathway. By employing this route for entry into the target cell, viruses could benefit from the enormous advantage of being targeted directly to specific organelles that are essential for their replication; moreover, degradative compartments can be bypassed, which could enhance the efficiency of productive infection. Nevertheless, despite the tremendous development of the field in the last decade, many conceptual and mechanistic aspects are still to be clarified or reevaluated. Certainly, important issues regarding: a) the regulation of the crosstalk between different internalization pathways; b) the similarities between caveolae and other clathrin-independent entry routes; c) the exact mechanism of ligand sorting; d) the properties of the compartment(s) where it occurs; e) the preferential targeting of caveolae ligands to other intracellular compartments than the ER; are already under the scrutiny of many cell biologists and will find an answer in the near future.

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