Gulping rather than sipping: macropinocytosis as a way of virus entry
Jason Mercer and Ari Helenius

Macropinocytosis has emerged as a major endocytic mechanism in the cell entry of animal viruses. The process differs fundamentally from other endocytic mechanisms involved in virus internalization. By activating growth factor receptors or other signaling molecules, plasma membrane-bound viruses trigger the activation of a signaling pathway. When amplified, this causes a transient, global change in cell behavior. The consequences of this change include the actin-dependent formation of membrane protrusions, the elevation of non-specific uptake of fluid, and the internalization of membrane together with surface-bound ligands and particles including viruses. Recent studies show that this strategy is used by a variety of enveloped and non-enveloped viruses.

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Introduction
Most animal viruses take advantage of the host cell’s endocytic mechanisms for entry and infection [1]. Release of the viruses themselves or their capsids into the cytosol occurs through the penetration of membranes in endosomes or other downstream organelles such as the endoplasmic reticulum. While many viruses bind to receptors and follow these receptors into cells exploiting clathrin-coated vesicles for internalization, it is now increasingly evident that others depend on clathrin-independent endocytic mechanisms [2–4]. Macropinocytosis, a ligand-induced mechanism for the uptake of fluid and solutes, has in the last few years emerged as a major mechanism for virus infection. The viruses that use it belong to different families. They include enveloped and non-enveloped viruses, RNA and DNA viruses, large and small. For some, the macropinocytic entry pathway seems to be one of several possible pathways. Others seem to be using variants of the general theme with properties different from classical macropinocytosis.

In this review, we will describe the salient features of this rather unusual endocytic mechanism and the membrane trafficking pathways downstream of initial uptake. We will discuss the data collected for the various viruses, and attempt to fit these data into the larger cell biological picture.

What is macropinocytosis?
In contrast to phagocytosis, a cargo-triggered endocytosis mechanism involving the uptake of large particles through locally assembled machinery, macropinocytosis is primarily responsible for nonspecific uptake of fluid, solutes, membrane, ligands, and smaller particles attached to the plasma membrane. In most cell types, it is not a continuously ongoing process, but rather transiently triggered, and when activated, operational for a limited time. Physiological ligands such as growth factors, integrin substrates, and phosphatidylserine (PS)-containing cell remnants serve as specific triggers [5–7].

A complex signaling pathway is activated by these ligands. When amplified in the cell, the signal induces an increase of endocytosis and a transient global change in cell behavior. These changes may include alterations in cell shape, motility, plasma membrane dynamics, and so on [8]. Many of these events result from changes in actin dynamics. The activation of fluid phase endocytosis is the consequence of plasma membrane ruffling (see Box 1).

The collapse of these ruffles results in the formation of large uncoated, irregularly shaped, fluid-filled endocytic vacuoles called macropinosomes (reviewed in [18]). Uptake of fluid is transiently elevated (30–60 min) up to ten-fold above the basal rate, and as a rule it is not restricted to any specific region of the cell surface. It is also recognized that macropinocytosis can lead to uptake of particles as well as fluid. The uptake of particles is, in fact, physiologically important for the clearance of cell debris left behind after apoptosis of cells in tissues [7].

The non-specific aspect of the uptake process is probably an advantage for the viruses involved. Instead of having to bind to specific receptor molecules, as other viruses do, those that trigger macropinocytosis can initially bind to any surface component. Once bound these viruses somehow ‘tickle’ the appropriate receptors in the plasma membrane to turn on the macropinocytosis program.
Box 1 Macropinocytic protrusions

Depending on cell type, conditions, choice of receptors, activation mechanisms, and properties of the virus, the macropinocytic protrusions induced can take different forms [9,10*].

(a) Lamellopodial ruffles. Some viruses (Ebola and Adeno 35), as well as stimulation with epidermal growth factor [6] cause formation of large, motile, flattened lamellipodia.

(b) Circular ruffles. Stimulation of cells with platelet derived growth factor leads to the formation of thin crown-like projections called 'circular ruffles' on top of the cell [11]. The resulting macropinosome formation is dynamin-dependent. Macropinocytosis of HIV-1, HRV8, HRV14, and BTV-1 have been described as dynamin-dependent.

(c) Filopodial protrusions. In some instances, macropinocytosis involves the formation of thin finger-like filopodia. This has been described for VV MVs (strain IHD-J) and Nipah virus [9,10*,12].

(d) Blebs. Membrane blebbing was demonstrated for VV mature virions of the WR strain and extracellular virions [13,14†] as well as KSHV [15,16**]. Bleb formation relies on actin destabilization rather than stabilization.

The images presented have been reproduced with permission from [9] and [17].

When uptake begins, it is the consequence of a cell-wide change, and occurs in seemingly arbitrary locations. Thus any bound virus, whether associated with the activating receptors or not, has a chance for internalization. The non-specific nature of macropinocytosis also renders the pathway useful for delivery of other cargo: it is increasingly viewed as a promising gateway for the delivery of peptides, proteins, nanocarriers, therapeutics, and so on [18–21].

Criteria for macropinocytic virus entry

Macropinocytosis can be divided into five steps that depend on distinct sets of cellular factors; (1) Virus binding, (2) Activation of intracellular signaling, (3) Plasma membrane protrusion, (4) Vacuole closure and formation, and (5) Macropinosome trafficking. Some of the key players in these steps and their roles are illustrated schematically in Figure 1.

There is no single diagnostic test reliable enough to define whether a given virus uses macropinocytosis. However, reliance on actin dynamics and Na⁺/H⁺ exchange can provide a rapid and consistent first glance. To this end, disruption of actin with jasplakinolide or cytochalasin D, and Na⁺/H⁺ exchange using amiloride or
its derivatives can be employed to determine if macropinocytic virus entry is worth pursuing.

To define experimentally whether a virus uses macropinocytosis, several additional approaches are needed. Most important is to determine which cellular proteins the entry process depends on, and which not. This is typically done by determining whether infection – or preferably endocytosis of the virus – is affected by perturbing key players using inhibitors, siRNA-mediated or shRNA-mediated depletion, or expression of mutant constructs.

We have in a previous review recommended a set of experimental criteria to assess virus entry by macropinocytosis [17]. In addition to dependence on actin and Na\(^+\)/H\(^+\) exchangers, they include the Rho GTPases (Rac1 or Cdc42), p21-activated kinase 1 (Pak1), PI(3)K, and protein kinase C (PKC). Together with evidence for ruffling or blebbing and a transient elevation in fluid uptake, they provide positive evidence for a macropinocytic uptake process. The dependence on dynamin-2, receptor tyrosine kinases (RTKs), and myosin II are examples of supporting but less reliable discriminators. Exclusion of clathrin-mediated and caveolar/lipid raft-mediated endocytosis is also important. Electron microscopy is valuable to visualize the size of primary endocytic vesicles that contain viruses, and the presence or absence of cytoplasmic coats. Light-microscopy is especially useful for the characterization of ruffling/blebbing, as well as for the detection of viruses in fluid-containing vacuoles after endocytosis.

It is clear that any set of criteria chosen leaves open a degree of ambiguity that is amplified by cell type differences and the general complexity in the regulation of vesicular trafficking [22]. In the field of cell biology, the classification of endocytic mechanisms is still far from complete [23]. That many viruses use more than one entry mechanism [24] does not make definition of pathways easier. The limitations and side effects of drugs and other tools intro-

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**Figure 1**

Virus macropinocytosis: Stages and signaling. Macropinocytosis of viruses occurs in five stages: Binding, signaling, membrane protrusion, vacuole closure and formation, and trafficking. Each stage is governed by a unique set of cellular factors with some overlaps. Virus binding often occurs through rather non-specific attachment factors (such as glycosaminoglycans). These serve to concentrate the viruses on the plasma membrane or to bring them to specific sites that facilitate activation of internalization receptors. Engagement of a variety of cell-surface receptors (receptor tyrosine kinases (RTKs) such as EGFR, integrins, and in some cases PS receptors) is used to activate kinases (blue), GTPases (gray), adaptors (purple), and other factors such as Na\(^+\)/H\(^+\) exchangers, phospholipases, myosins and fission/fusion factors (orange). This signaling cascade triggers actin rearrangement and plasma membrane protrusion and ruffling (see Box 1). Circular (crown-like) ruffles, blebs, and lamellipodia are displayed here. To form an endocytic vacuole (a macropinosome) the protrusions collapse back on the plasma membrane forming an invagination. The membrane fission events that separate the macropinosomes from the extracellular space differ depending on the type of protrusion. Closing of circular ruffles is dynamin-dependent, lamellipodial ruffles close via the action of CIBP1/Bars, and the cellular factors that govern closure of bleb-derived macropinosomes are not known. After closure, the newly formed macropinosomes move into the cytoplasm where they rely on a further set of cellular factors for trafficking and maturation (see Figure 2). Solid arrows represent direct allosteric or catalytic activation and dashed arrows represent interactions that serve in signal amplification, macropinosome closure, and trafficking.
duce a further level of uncertainty. As the experimental read-out frequently relies on negative data, that is, lack of endocytosis and infection, it is useful to include control viruses for which the entry pathways are better known, such as vesicular stomatitis virus (VSV), simian virus 40 (SV40), and Semliki forest virus (SFV) that do not use macropinocytosis [25–27].

An alternative approach to defining the mechanisms of virus entry is provided by using targeted siRNA or shRNA screening libraries. The approach is based on depleting key factors in the various endocytic mechanisms, and comparing the effects of this on infection by different viruses. We are using a library of siRNAs called the ‘Usual Suspects’ in our lab jargon, and applying it to many different viruses. A pilot version was already published for SV40 [26]. In the future, it will be considerably easier to determine whether a virus enters by macropinocytosis using such diagnostic screens.

**Macropinocytic triggers used by viruses**

In the case of macropinocytosis, one must consider that the interaction between the virus and the cell surface is likely to involve multiple different types of contacts: some providing anchoring to the membrane, others needed for activation of receptors. Attachment can depend on direct contact with specific receptors or on less-specific adsorption to extracellular matrix components such a heparan sulphate proteoglycans (HSPGs) [28]. To trigger macropinocytosis, further contacts have to be established with RTKs, PS-receptors, integrins, or other surface molecules capable of activating the appropriate signaling pathways.

The interaction between the virus and the signaling receptors is not necessarily a direct one. The viruses simply need to activate the receptors, even if that occurs indirectly, for example through the assistance of bridging molecules. More specifically, it has been shown that vaccinia virus mature virions (VV MVs), vaccinia virus extracellular virions (VV EVs), influenza A virus (IAV), and possibly human papillomavirus-16 (HPV-16) bind to HSPGs and other glycans before activating classical growth factor receptors, such as epidermal growth factor receptor (EGFR) [10*,14*,29,30*]. Other viruses take advantage of integrins to which they bind directly: adenoviruses 3 and 35 to αv-integrins and CD46, echovirus 1 to α2β1-integrins, and Kaposi’s sarcoma-associated herpes virus (KSHV) to α3β1, αvβ3, and αvβ5 integrins [31–34]. The signaling pathways downstream of the different receptors are somewhat variable, but they all end up influencing actin dynamics and inducing endocytosis.

**Apoptotic mimicry**

Recent findings have demonstrated that ‘apoptotic mimicry’ is a mechanism used by some of these viruses to trigger macropinocytosis. This strategy relies on the presence of PS on the virus surface to mimic apoptotic debris. Thereby these viruses take advantage of the host cell’s PS-dependent apoptotic removal system for internalization [35]. First suggested for hepatitis B virus [36], and subsequently demonstrated experimentally for VV MVs [13], PS-mediated macropinocytosis has now been shown to be used by lentiviral vectors, pichinde virus (a model for Lassa fever virus), cytomegaloviruses, Ebola virus, Marburg virus, and possibly HIV-1 [37*,38–40]. VV MVs, HIV-1, and pichinde virus have been shown to contain PS in their envelope [38,40–42].

A connecting factor between virus PS and activating receptors was recently discovered: a serum protein called Gas6 [37**]. It belongs to a family of dual function molecules that recognize PS, and on the contrary activate the TAM receptors Tyro3, Axl, and Mer [43]. These RTKs serve as receptors for PS-dependent internalization of cell debris and apoptotic bodies [44]. Using lentivirus vectors, it was demonstrated that Gas6 could enhance PS-dependent infection of non-permissive cell lines by binding to Axl [37**].

It will be interesting to see how commonly Gas 6 and similar molecules, like Protein S, serve as bridging factors in virus entry. The macropinocytosis-like pathway used by IAV in A549 cells only appears functional in the presence of serum [45]. This raises the possibility that a serum factor acts as a bridging component to trigger endocytosis. Axl has also been shown to enhance macropinocytic entry of Ebola virus in some cell types [39,46], although no direct interaction between the Ebola virus glycoprotein and Axl was found [47].

Thanks to its non-specific nature, apoptotic mimicry may serve as a general strategy used by enveloped viruses to broaden their tissue and host tropisms. If so, targeting PS on these various pathogens may be an effective broad-range antiviral strategy [40]. Additionally, PS may provide a general passport for the internalization of nanoparticles and drugs into cells.

**Viruses that use macropinocytosis**

In Table 1, we have collected information about the macropinocytic and macropinocytosis-related entry of viruses as described in the current literature. It is important to note that for most of the viruses the experimental data are still incomplete. For many, the assays used depend on infection as a read-out rather than endocytosis, which leaves open the possibility of post-endocytosis effects. Nevertheless, it is apparent that seven of the viruses fit the criteria for macropinocytic uptake (see Table 1).

**Macropinocytosis-like mechanisms**

The various steps in the macropinocytic process depend on a large number of cell factors (shown in Figures 1 and 2) [17,18]. Here we focus on issues that involve some of these
proteins and protein families during macropinocytosis entry. In certain cases, the authors of papers have invoked ‘macropinocytosis-like’ mechanisms because the results are either incomplete or show deviations from classical macropinocytosis.

A clear case of macropinocytic-like virus entry was reported for HPV-16 [30*]. It was found that endocytosis and infection relies on many hallmarks of classical macropinocytosis (see Table 1), but does not depend on the Rho GTPases. Internalization is, moreover, inhibited by chlorpromazine, a reagent that inhibits clathrin-mediated endocytosis, but does not generally affect macropinocytosis [30*]. For HPV-16 there is no evidence of ruffling, and electron microscopy shows viruses in small, uncoated primary endocytic vesicles [30*].

Adeno-associated virus 2 (AAV2) has recently been shown to enter cells by a dynamin-independent pathway sensitive to EIPA, and dependent on Cdc42, Arf1, Graf1, and actin polymerization [56*]. While entry was associated with plasma membrane ruffling, there was no increase in fluid phase uptake after virus addition [56*]. Although the mechanism seems in many ways macropinocytosis-like, the authors concluded that the virus made use of the so-called CLIC/GEEC pathway, which has not previously been observed to carry viruses.

### Kinases, GTPases, and other factors

Macropinocytosis requires the coordinated activation of multiple kinases (Figure 1 and Table 1) [17]. The process is therefore sensitive to a variety of general and specific kinase inhibitors. Thus, it is surprising that infection by

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Table 1

<table>
<thead>
<tr>
<th>Cellular factors used by viruses for macropinocytic entry</th>
<th>Entry or Infection</th>
<th>Cell types</th>
<th>Ruffles</th>
<th>Fluid uptake</th>
<th>Fluid colocalization</th>
<th>Cytoskeleton</th>
<th>GTPases</th>
<th>Kinases</th>
<th>Other</th>
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<td>ENT &amp; INF</td>
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<td><strong>Genome</strong></td>
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<td>(+) ssRNA</td>
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<td>(+) ssRNA</td>
<td>dsRNA</td>
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<td><strong>Cells</strong></td>
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<td>Hela/HHK-2</td>
<td>Hela</td>
<td>HMEC-3</td>
<td>Hela/OCO</td>
<td>Hela</td>
<td>Vero</td>
<td>C15</td>
<td>HD Cells</td>
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<td>Blebbing</td>
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<tr>
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</table>

**REFS:** 39, 46, 47, 50, 51, 52, 53, 30
vaccinia virus MVs and EVs, as well as Nipah virus, is not blocked by the general tyrosine kinase inhibitor, genistein [10,12,14]. Since these viruses depend on RTKs for infection, this probably reflects a general insensitivity of certain tyrosine kinases to this inhibitor, which will be important to keep in mind when studying virus macropinocytosis.

PI(3) kinase has been implicated in several stages of macropinocytosis, from membrane protrusion, to macropinosome trafficking and fusion [18,57–62]. The presence of PI(3,4,5)P3 in the plasma membrane during macropinosome formation coincides with the binding of various SNX-PX-BAR family proteins [63–66]. Therefore it is unexpected that macropinocytosis of VV MVs and EVs of the IHD-J strain, as well as HIV-1 entry into macrophages, can occur in the presence of the PI(3)K inhibitor wortmannin [10,14*,67]. PI(3)K-independent macropinocytosis also occurs in neuronal cells [68]. Although the mechanism remains unclear, these results suggest that, under certain conditions other lipid kinases may regulate macropinocytosis.

A ‘macropinocytosis-like’ entry pathway has been invoked for IAV [45*]. This dynamin-independent mechanism is activated in A549 cells in the presence of serum. It has many of the properties of macropinocytosis (Table 1), but seems to be independent of Rac1 and Cdc42, which play a central role in actin dynamics during macropinocytosis [69,70]. Inconsistent with the observed Rho GTPase-independence, the amiloride derivative EIPA, which inhibits activation of Rac1 and Cdc42 by altering submembranous pH [71**], effectively blocked IAV infection [45*]. This suggests that EIPA has additional effects on the IAV lifecycle, or this virus relies on undefined EIPA-sensitive Rho GTPases.

The large, multi-functional GTPase dynamin-2 can be inhibited by several drugs including dynasore and Dyngo4a, or by overexpression of dominant negative forms of the protein [72,73]. Macropinocytosis of HIV-1 (in macrophages), human rhinovirus-14 (HRV-14), and bluetongue virus-1 (BTV-1) was inhibited by dynasore, and entry of human rhinovirus-8 (HRV-8) by dynasore and the dynamin-2 dominant negative [53–55,67]. Although not required for lamelipodia-associated and bleb-associated macropinocytosis [13,52,74], dynamin-2 is needed for closure of PDGF-stimulated circular ruffles [53,67,75,76]. This implies that the uptake of these viruses involves circular ruffling, but this needs to be confirmed using live cell microscopy. It is worth noting that owing to side effects on the actin cytoskeleton and lamellipodia formation, dynasore should not be used as the sole criteria for determining dynamin-dependence [77].

Taken together, these observations raise important questions. Are there endocytic mechanisms that have similarities to macropinocytosis but do not share the aspect of systemic activation and the formation of large fluid-filled vacuoles? How distinct is macropinocytosis from other clathrin-independent and caveolin-independent mechanisms? How far does the plasticity of the systems reach when extended to different cells types and tissue architectures?

**Macropinosome trafficking**

Viruses internalized by clathrin mediated-endocytosis and caveolar/lipid raft-endocytosis are delivered to early endosomes. This is a sorting compartment from which a portion of the incoming fluid and cargo is recycled back to the plasma membrane while some, including viruses, is targeted via multivesicular late endosomes to lysosomes for degradation. During movement into the perinuclear space and preparation for fusion with lysosomes, endosomes undergo dramatic maturation [78]. This includes changes in the phosphoinositide content, the cohort of proteins that cover the cytoplasmic surface, the Rab profile (Rab5 to Rab7 switch), further acidification, and formation of intralumenal vesicles (Figure 2 and reviewed in [23,24,78]).

Macropinosomes appear to undergo maturation as well (details in Figure 2). The main differences are that an initial sorting station comparable to early endosomes is apparently missing, the existence of a ‘maturing’ macropinosome that would contain both early and late macropinosome markers has not been described, and there is no evidence that macropinosome maturation involves the formation of intralumenal vesicles (ILVs).

After endocytosis, macropinosomes help to transport internalized viruses through the cortical cytoskeleton and cytoplasm while providing cues, such as low pH, to promote virus escape by membrane fusion. Many of the studies on macropinocytic entry of viruses have focused on the initial stages of uptake. Viruses are likely to serve as valuable tools for mapping later events in the pathway as well.

**Perspectives**

Interactions with cell surface factors and receptors define, to a large extent, the cell type and host species tropism of viruses, as well as the pathogenesis of viral diseases. It is therefore of utmost importance to analyze the initial interactions in detail and determine their consequences for the viruses and the cells. Here, we are dealing with a group of viruses that has evolved to exploit surface proteins that cells normally use as antennas for intercellular communication. By triggering their activation, the viruses turn on a complex, highly amplified downstream signaling cascade thus managing to induce dramatic global changes in cell behavior. The resultant reprogramming is transient, but sufficient to provide the viruses a window for endocytic uptake.
Macropinosome trafficking and maturation. The main organelles of the classic endocytic pathway are early endosomes (EEs), maturing endosomes (MEs), late endosomes (LEs), endolysosomes (ELs), and lysosomes (LYs). The maturation program of these organelles, their trafficking, and the fate of viruses that enter this network are relatively well defined (reviewed in [24]). Although macropinosome trafficking differs with cell type and the triggering ligand, largely, macropinosomes appear to mature in a parallel fashion to classical endosomes. They undergo changes in their phosphoinositide make up, Rab GTPase components, pH, and position within the cell. Upon detachment, PI(3)K generates PI(3,4,5)P3 on the newly formed early macropinosomes (EMs) [79]. Rab5 then accumulates together with its GEF, Rabex5, and the effector Rabankyrin 5 [79,80]. In some cell types macropinosomes acquire Rab34 and EEA1, which serve to mediate pinosome formation and homotypic fusion [81,82]. The action of sorting nexins (SNX) 1 and 5, allows macropinosome cargo to be recycled or trafficked to EEs [63-66]. As macropinosomes move deeper into the cytoplasm they become late macropinosomes (LMs), they undergo acidification, and acquire Rab7 and Rab21 [83,84]. While there is no consensus as to the final fate of LMs, at this stage they can fuse with each other, LEs, or LYs [83,85,86] by a process shown to rely on the lipid kinase activity of PIKfyve [87**].
Our present understanding of macropinocytosis leaves open many questions both at the conceptual and the molecular level. For example, it would seem likely that such reprogramming would have other consequences that favor productive infection. Growth factors turn on the cell cycle, they induce changes in the cytoskeleton, and so on [8]. Does this help us to understand how viruses penetrate tissue barriers, as suggested by the work on coxsackievirus B [88]? By activating macropinocytosis, this virus triggers a dramatic change in cell architecture in order to infect otherwise impermeable epithelial cells. The sudden internalization of large vacuoles and cargo induced by these viruses must have consequences for the entire endocytic network. Is this important for virus entry? The adenovirus 2 data suggest that it does. Although these viruses enter by clathrin-mediated endocytosis, they have to induce parallel macropinocytosis for subsequent penetration [6].

The answers to these and many other questions will require careful analysis of virus cell interactions using molecular, cell biological, physiological, and systems-based approaches. The results will hopefully increase our understanding of viral diseases and provide strategies to prevent infection by turning off cellular responses that are so helpful to the viruses. In addition, through these studies it may be possible to make cells more amenable to uptake of therapeutic nanoparticles and drug delivery vehicles.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


Different macropinocytic responses can be triggered in a single cell type using different vaccinia virus strains.

15. VACV EVs are shown to be internalized by macropinocytosis followed by low pH dependent rupture of the outer EV membrane in macrophages.
This paper characterizes serum-dependent macrocytosis as an alternative mechanism of host cell entry by influenza A virus.


Using a wide-range of endocytic perturbers and control viruses Schelhaas and co-workers demonstrate that HPV-16 uses a macropinocytic-like virus entry mechanism.


38. Paper demonstrates that lentiviral vectors use Gas6 to bridge the gap between virus phosphatidylserine and the cellular PS receptor Axl.


This paper characterizes serum-dependent macrocytosis as an alternative mechanism of host cell entry by influenza A virus.


51. Saeed MF, Kolokoltsov AA, Albrecht T, Davey RA: Cellular entry of ebola virus involves uptake by a macrocytosis-like mechanism and subsequent trafficking through early and late endosomes. PLoS Pathog 2010, 6:


AAV2 is reported to enter cells using the CLIC/GEEC pathway making it the first virus to use this endocytic mechanism.


Macropinocytosis as a way of virus entry Mercer and Helenius


This paper demonstrates that Na"/H" exchange inhibitors, such as amiloride, prevent macropinocytosis by altering submembranous pH, which in turn prevents activation of the GTPases Rac1 and Cdc42.


Using Salmonella as a model ligand, Kerr and co-workers demonstrate that macropinosome-late endosome/lysosome fusion depends on PI(3)Kfyre function.