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Current Opinion in Virology

How to get out: ssRNA enveloped viruses and membrane fission

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Enveloped viruses acquire their membrane from the host cell and accordingly need to separate their envelope from cellular membranes *via* membrane fission. Although some of the enveloped viruses recruit the endosomal sorting complex required for transport (ESCRT) to catalyze the final fission reaction, many enveloped viruses seem to bud in an ESCRT-independent manner. Here we describe the principles that govern membrane fission reactions in general and review progress in the understanding of ESCRT-mediated membrane fission. We relate ESCRT function to budding of single stranded RNA viruses and discuss alternative ways to mediate membrane fission that may govern ESCRT-independent budding.

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Current Opinion in Virology 2013, **3**:159–167

This review comes from a themed issue on **Virus structure and function**

Edited by **Joseph Marcotrigiano** and **Felix Rey**

For a complete overview see the Issue and the Editorial

Available online 11th April 2013

http://dx.doi.org/10.1016/j.coviro.2013.03.011

Introduction

Enveloped viruses assemble at cellular membranes, which provide the newly formed virus envelope. Viral proteins bend membranes into vesicular structures during assembly and use either their own or cellular host proteins to catalyze membrane fission, an important energetic barrier associated with virion release. The host cell may provide the blueprint for fission, since many membrane trafficking processes require a membrane fission step. Schematically, cellular vesicles have an extracellular, luminal or cytoplasmic content and come in two flavors. First, protein-coated vesicles, such as clathrin-coated endocytotic vesicles [1], COPI-coated or COPII-coated vesicles that bud off Golgi and ER membranes, respectively [2,3] or retromer-coated vesicles in the retrograde pathway [4]. Second, non-coated vesicles such as those retrieving for instance GPI-linked or glycolipid receptors from the plasma membrane [5] or vesicles that bud into the lumen of endosomes producing multivesicular bodies [6]. Clathrin-coated vesicles recruit helical dynamin polymers at the membrane neck connecting the vesicle and the donor membrane [7••,8••]. GTP hydrolysis then leads to polymer twisting and neck constriction [9], which sets the stage for membrane fission occurring at the frontier between the narrow dynamin coated membrane and the coexisting bare membrane with a larger diameter. Additionally, fission kinetics is affected by the mechanical properties of the membrane (tension and bending rigidity) [10••]. Dynamin has been also implicated in the budding of caveolae [11] and dynamin-like molecules catalyze mitochondrial fission [12]. An alternative model for vesicle fission stipulates that proteins containing amphipathic helices can induce fission by hydrophobic insertion; in the case of N-BAR domain proteins, this effect is modulated, by an antagonistic relationship between these helices and BAR-domain scaffolds [13••,14]. In contrast, vesicle budding into the lumen of endosomes is organized by endosomal sorting complexes for transport (ESCRT) [6,15]. Because endosomal vesicle budding is topologically similar to enveloped virus budding, some of the enveloped viruses hijack part of the ESCRT machinery to escape from host cells [16–18]. Here we review membrane fission release of enveloped ssRNA viruses catalyzed by the ESCRT machinery or by ESCRT-independent pathways. In order to place virus release and membrane fission into the context of membrane biophysics, we first summarize general physical principles of membranes favoring fission and relate these principles to ESCRT-dependent and ESCRT-independent budding processes.

Physical properties of membranes influencing fission: phase separation and line tension

In vitro experiments and theoretical approaches have shown that lipid domains in membranes can induce membrane bending and fission from quasi-flat membranes due to the constrictive line tension at the edge of the domains [19•,20]. Lateral tension in membranes limits this pinching-off effect, as it favors flat membranes [19•]. In the case of tubular geometries reminiscent of the neck of a budding virion, fission also occurs at the edge of domains and can be accelerated by membrane tension [21]. Proteins are in principle not required for this process and the membrane domains can be made of lipids only [22]. However, proteins including actin may contribute to induce phase separation in membranes [23,24] leading to membrane fission [25•] and forces generated by actin

polymerization pushing on the membrane can help fission [22]. Although this line-tension fission mechanism has been documented *in vitro* and in some cases *in vivo*, it is not clear to which extent it contributes to *in vivo* membrane fission reactions such as for example ESCRT-driven membrane fission.

Physical modeling of virus budding

Mechanisms for the entry and exit of enveloped virus from cells have been proposed by physicists, in the framework of colloid–membrane interactions. The virus is modeled as a solid particle with a spherical or elongated shape that can be wrapped by the interacting membrane. Theoretical models have been calculated describing the conditions under which particles can spontaneously bud off independently of fission proteins as a function of adhesion energy, particle size and shape or membrane tension. Membrane destabilization leading to fission is expected to occur when wrapping is total. An optimal particle size for budding depending on particle concentration and on the relative densities of interacting molecules on the particle has been derived from these models [26–28]. However, to date numerical simulations are limited to the effect of a single particle and to membranes of small size [29,30].

The ESCRT machinery and budding

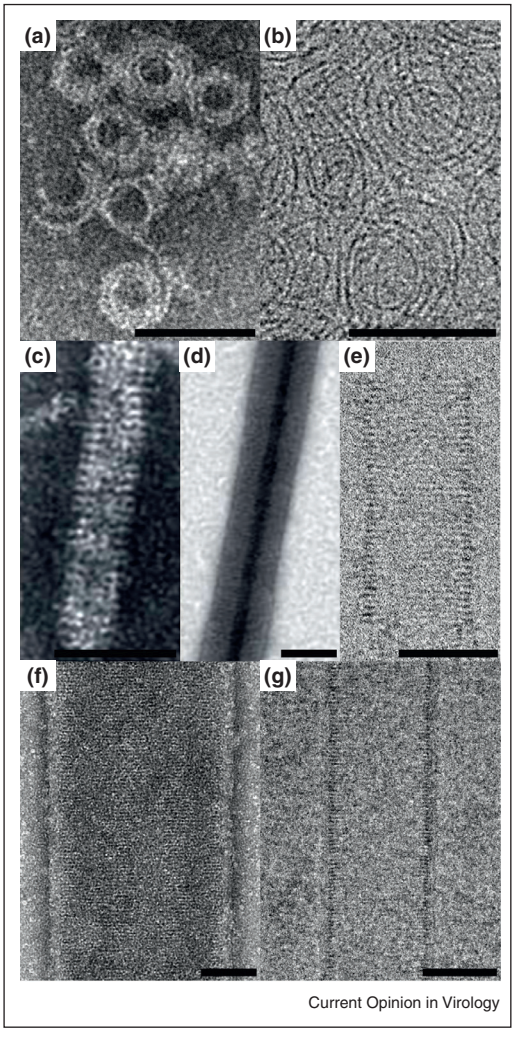
The ESCRT machinery is composed of five different complexes, ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and VPS4, which assemble sequentially on the endosomal membrane to concentrate cargo, sort it into vesicles, pinch off the vesicles and recycle ESCRTs [6,15,31,32]. Because ESCRT-III constitutes the membrane fission machinery [33•,34••], together with VPS4 [6,35••] they are also recruited to topologically similar processes such enveloped virus budding [36] and cytokinesis [37,38].

ESCRT-III and membrane fission

Higher eukaryotes express 12 ESCRT-III proteins named CHMP (*CH*arged *M*ultivesicular body *P*rotein) and IST1 (*I*ncreased *S*odium *T*olerance *1* gene product) [39–42]. CHMP6 (Vps20), CHMP4 (A, B or C) (snf7), CHMP3 (Vps24) and CHMP2 (A or B) (Vps2) are recruited in this order and may form a core complex in yeast [31], while the remaining ESCRT-III (CHMP1A, B, CHMP5, CHMP7 and IST1) exerts regulatory functions. All ESCRT-III proteins are required for MVB biogenesis [6] and during cell division [43]. In contrast, HIV-1 release requires only one CHMP4 and one CHMP2 family member that act sequentially and may thus constitute the minimal fission machinery [44••] together with VPS4 [35,45••]. However, CHMP3 exerts a significant synergistic effect on CHMP2A [46] and CHMP1B is recruited last [45••,47•].

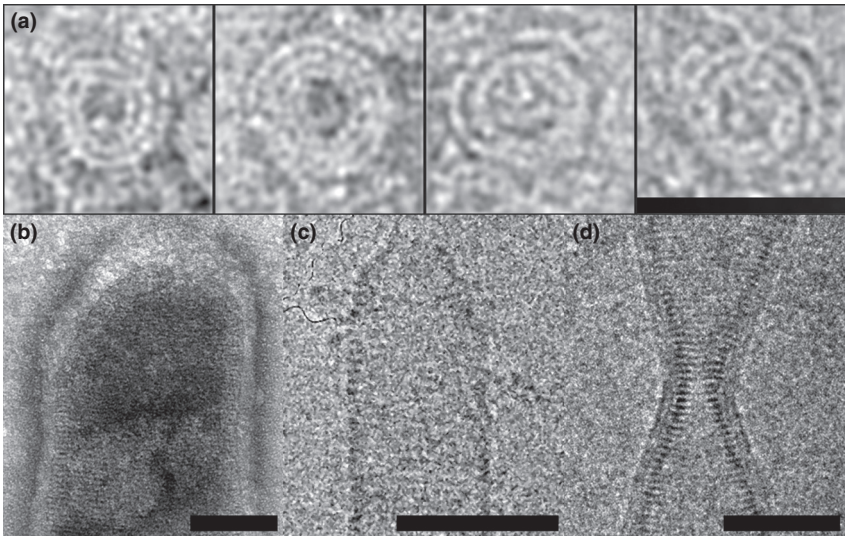
ESCRT-III proteins are small helical assemblies [48–50] and their autoinhibited conformation is controlled by the C-terminal region [49,51–53]. Displacement or artificial removal of the C-terminus leads to polymerization *in vitro* and on cellular membranes. Direct ESCRT-III-driven membrane deformation *in vivo* has been only observed twice. Expression of truncated CHMP4 together with dominant negative VPS4 produced membrane tubes that contained helical arrays of presumably CHMP4 [54] and expression of CHMP2B wild type led to the formation of long membrane tubes emanating from the plasma membrane. CHMP2B tube formation required CHMP4 at the base, depended on VPS4 and produced a tight helical layer of CHMP2B filaments spaced by 30 Å [55] similar to the CHMP2A-CHMP3 helical tubes [46] (Figure 1). *In*

Figure 1



Gallery of ESCRT-III polymers observed *in vitro* and *in vivo*. **(a)** CHMP2A negative stain. **(b)** CHMP4 cryo-EM. **(c)** CHMP1B negative stain. **(d)** IST1 negative stain. **(e)** CHMP2A-3 cryo-EM. **(f)** CHMP2B (with membrane) negative stain. **(g)** CHMP2B (with membrane) cryo-EM. Scale bars are all 50 nm.
Images shown in c and d are reproduced with permission from Bajorek et al. [49].

Figure 2



Gallery of closed coil-like structures, dome-like structures and constricted polymers. **(a)** Gallery of 4 CHMP2A spirals (negative stain). **(b)** CHMP2B dome (with membrane) (negative stain). **(c)** CHMP2A-CHMP3 dome (cryo-EM). **(d)** CHMP2B membranous bottleneck (cryo-EM). Scale bars are all 50 nm.

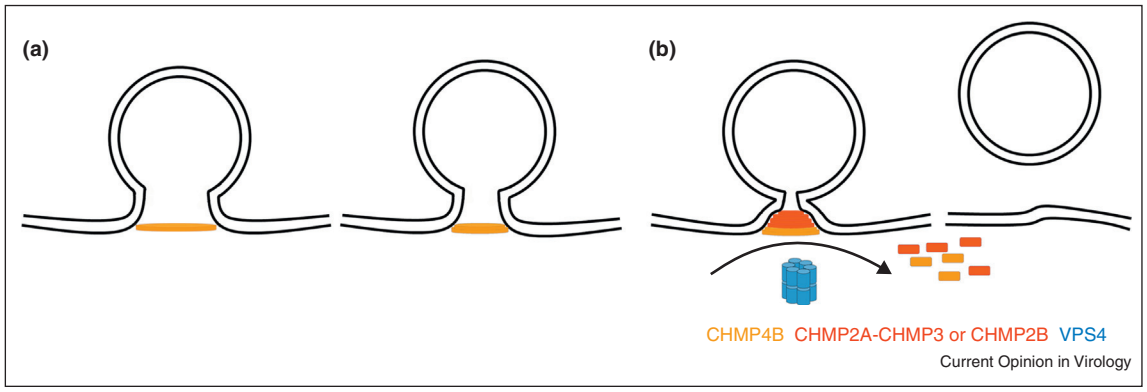
vitro, CHMP4B forms loose ~30 Å thick helical arrays or ring-like structures [56,57] (Figure 1) and CHMP2A polymerizes into circular ~30 Å thick coils [46] (Figure 1). In addition, IST1 and CHMP1B, tubes have large diameters and yeast Snf7-induced Vps24-Vps2 (CHMP3–CHMP2) polymers resemble their human counterparts [46,49,58••,59•] (Figure 1). However, it should be noted that yeast Vps24 (CHMP3) assembles into two stranded filaments [57] and the spiral ESCRT-III structures imaged at the midbody are much thicker [60] than those shown in Figure 1. *In vivo*, ESCRT-III and VPS4 have short transient residency times at budding sites [35,45••,61].

Although circular and tubular structures of ESCRT-III polymers observed *in vitro* and *in vivo* can assemble inside a bud neck, their large diameters varying from 50 to >200 nm impede spontaneous membrane fission. This led to the proposal of two main models. One model suggests that VPS4 induces constriction by filament sliding leading to fission [59•], while the second model proposes that ESCRT-III polymers forming a dome-like structure permit neck constriction and membrane fission [18,58••,62]. Albeit none of the models have been proven experimentally, the dome model is appealing, because all potential polymers implicated in the final cut (CHMP2A-CHMP3 or CHMP2A or CHMP2B) can form dome-like end-caps *in vitro* or *in vivo* [46,55,58••] (Figure 2). The model predicts that CHMP4 polymers, which may induce a first narrowing of the neck, recruit CHMP2A-CHMP3 or CHMP2A or CHMP2B to build-up a dome-like polymer. The successive narrowing of the helical filament and its affinity for membrane will 'mold' the membrane [63] and induce neck constriction. This could theoretically constrict the neck up to a diameter of 6 nm, which would be energetically favorable for spontaneous fast fission [64•] (Figure 3). It is yet unclear how such ESCRT-III assemblies may influence lipid redistribution and fission. Even though ESCRT-III-catalyzed membrane fission *in vitro* was observed without VPS4 ATPase activity [33•,34••], it is likely that VPS4 plays an active role beyond recycling ESCRT-III [35,45••,65–67].

A modification of the dome-model proposes the symmetric ring-like arrangement of ESCRT-I-ESCRT-II complexes in the bud neck of intraluminal endosomal vesicles that provide the platform for ESCRT-III filaments projecting into the pore in a whorl-like arrangement that themselves serve as docking site for a dome-like polymer or evolve into a dome-like polymer that executes fission [68,69].

ESCRT-III-induced lipid phase separation may play an additional role [70] thus contributing to constrictive line tension at the edge of membrane domains and permitting spontaneous fission [21]. It is thus possible that the ESCRT-III-dome may function as a scaffold that induces local lipid redistribution and phase separation. As a final touch VPS4 ATP hydrolysis may couple mechanical stress imposed on the membrane with physical properties of

Figure 3



Model for ESCRT-driven membrane fission. In case of HIV-1 CHMP4B (or CHMP4A, to a lesser extend CHMP4C) can be recruited to the budding site *via* a classical ESCRT-I-ESCRT-II-CHMP6 sequence or directly *via* Alix or *via* a yet unknown process. Membrane recruitment will induce CHMP4B filament formation that assembles inside the bud neck; this may induce a first constriction of the neck **(a)**. This first constriction may set the stage for CHMP3-CHMP2A or CHMP2B recruitment, which assemble upon interaction with CHMP4B **(b)**. CHMP2A-CHMP3 or CHMP2B polymers could grow into dome-like structures that attract the neck membrane and constrict it. Upon completion of assembly, VPS4 may start to disassemble the ESCRT-III coat, which may further destabilize the membrane and thus catalyze fission concomitantly with disassembly.

lipid bilayers. Thus the main principles of ESCRT-III-driven membrane fission may be similar to dynamin-catalyzed fission.

Viruses budding with ESCRTs

Hallmarks of ESCRT-dependency of enveloped virus budding are the presence of functional late domains in structural proteins as well as the inhibition of virion release by either dominant negative VPS4, ESCRT-III or Alix [16,17,71,72]. Accordingly, a large number of enveloped RNA viruses employ ESCRTs for their release including all retroviruses [17,36,73,74]. Furthermore most members of the negative strand non-segmented ssRNA viruses recruit ESCRTs for budding from the plasma membrane. This includes Rhabdoviruses [75], Filoviruses [76–78] and most Paramyxoviruses, although some (Nipah, Measles and HRSV) may bud in an ESCRT-independent way [79]. However, no evidence for ESCRTs has yet been reported for Bornaviridae budding. In addition, Arenaviruses, negative strand segmented ssRNA viruses bud ESCRT-dependent [80,81] (Table 1).

Viruses budding without ESCRTs

Orthomyxoviruses (influenza virus), negative-sense segmented ssRNA viruses, bud independently of the ESCRT machinery from the plasma membrane (Table 1) [82]. Influenza virus budding is especially interesting because fission is induced by the viral transmembrane protein M2, which functions otherwise as an ion channel. M2 localizes to the neck of the bud and contains a highly conserved amphipathic helix within its cytoplasmic tail that affects membrane curvature in a cholesterol-dependent way. In fact, the short amphipathic helix sufficed to induce budding from GUVs [83••]. The M2 peptide may destabilize the membrane thereby providing the force for changes in membrane curvature [84]. Thus the function of M2 resembles aspects of fission reactions catalyzed by hydrophobic insertion [13••], although M2-driven fission is controlled by the local cholesterol concentration and no protein scaffolds seem to be required.

Although Coronaviridae, positive-strand ssRNA viruses also bud independent of ESCRTs (Table 1), no definite viral or cellular fission factor has yet been described. Coronaviruses obtain their viral envelope containing the glycoprotein S, the major membrane glycoprotein M and a minor membrane protein E by budding through membranes of the ER-Golgi intermediate compartment (ERGIC) [85]. The E protein contains one or two transmembrane regions and was suggested to play a role in membrane bending during assembly and fission [86,87]. However, E may not be essential for MHV (mouse hepatitis virus) and SARS-CoV virion production [88,89]. On the other hand, E-depleted TGEV (transmissible gastroenteritis coronavirus) produces non-infectious immature virions that are stuck in the secretory pathway [90], indicating that the final mechanism still needs to be determined.

Furthermore a number of ssRNA viruses, togaviridae, bunyaviridae and flaviviridae distinguish themselves from other ssRNA viruses by having their glycoproteins organized into protein coats that cover the viral membrane [91,92]. Such coats may drive budding and may contribute to the final membrane fission process by aiding bud neck constriction. Budding may follow physically based budding principles described above, which stipulate that membrane destabilization leading to scission

Table 1

ESCRT-dependent and independent budding of ssRNA enveloped viruses

	Species	Late domain	Virus protein	Cell protein	dn ESCRT
(+) **ESCRT**[a]					
Rhabdoviruses	VSV[b]	PPPY (PSAP)	Matrix M	Nedd4	VPS4
	Rabies virus	PPEY	Matrix M	Nedd4	?
Filoviruses	Ebola virus	PTAP	Matrix VP40	Tsg101	VPS4
		PPEY	Matrix VP40	Nedd4	
	Marburg virus	PPEY	Matrix VP40	Nedd4	VPS4
		PSAP	NP	Tsg101	
Paramyxoviruses	PIV-5[c]	FPIV	Matrix M	n.d.	VPS4
	Mumps virus	FPIV	Matrix M	n.d.	VPS4, CHMP4B
	Nipah virus	YMYL	Matrix M	n.d.	?, VPS4 independent
	Sendai virus	YLDL	Matrix M	Alix	VPS4?
	Measles virus	?	?	?	VPS4 independent
	HRSV[d]	?	?	?	VPS4 independent
Bornaviruses	Borna virus	?	?	?	?
Arenaviruses	LCMV[e]	PTAP	Matrix Z	Tsg101	?
	Lassa virus	PTAP	Matrix Z	Tsg101	VPS4
	LCMV	PPPY	Matrix Z		?
		PPPY	Matrix Z		
	Mopeia virus	?	Matrix Z (NP?)	Alix	?
(–) **ESCRT**					
Orthomyxoviruses	Influenza virus				
Coronaviruses	MHV[f]				
	SARS-CoV				
Budding with a protein coat					
Flaviviruses	YFV[g]		NS3	Alix	?
	JEV[h]		NS3	Tsg101	?
	Dengue virus				?
Hepaciviruses	Hepatitis C			HRS, CHMP4B	VPS4, ESCRT-III
	Hepatitis G				
Pestviruses	Swine fever virus			?	?
Bunyaviruses[i]	Hanta virus				
Alphaviruses	SFV[j]			?	?
Rubivirus	Rubella virus			?	?

(+) ESCRT-dependent; (–) ESCRT-independent binding; dn, dominant negative effect of ESCRT expression.

[a] The description of late domains and ESCRT dependency of budding has been reviewed previously [75,76,78,79,81,114].

[b] Vesicular Stomatitis virus.

[c] Parainfluenza virus.

[d] Human Respiratory Syncytium virus.

[e] Lymphocytic Choriomeningitis virus.

[f] Mouse hepatitis virus.

[g] Yellow fever virus.

[h] Japanese encephalitis virus.

[i] Bunyaviruses are split into five genera, Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus, Tospovirus.

[j] Semliki Forest virus.

may occur when the whole viral particle is wrapped by proteins.

Togaviridae, genus alphaviruses (Semliki Forest virus (SFV), Chikungunya, Ross River, and Venezuelan Encephalitis virus) and rubivirus (Rubella virus) are plus sense ssRNA viruses. Rubella virus and alphaviruses anchor their RNA synthesis in membranes of a cell organelle known as the cytopathic vacuole (CPV) that derives from modified endosomes and lysosomes [93,94]. Budding is then organized from the plasma membrane and driven by nucleocapsid-E2 glycoprotein interactions [95], which together with E1 forms a protein shell covering the viral membrane [96,97]. Notably, SFV budding is not affected by dominant negative VPS4 indicating its independence of the ESCRT machinery [98] (Table 1).

Bunyaviridae (5 genera Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus, Tospovirus) have segmented, negative-sense ssRNA genomes. The virions bud into the lumen of the Golgi and are released *via* vesicular transport from the plasma membrane [99] (Table 1). The large cytoplasmic domain of the Gn glycoprotein may act as a surrogate matrix protein of hantaviruses [100,101], while the extracellular glycoproteins assemble into coats composed of tetrameric patches (Hanta virus) [102] or icosahedral lattices (Phlebovirus) [103–105]. Although little is known about virion release, the formation of

the glycoprotein coat may drive budding and release [102].

Flaviviridae, with its genera flavivirus (Dengue virus, yellow fever, west Nile virus, Japanese encephalitis virus), Hepacivirus (Hepatitis C virus (HCV), Hepatitis G virus) and pestivirus (swine fever) contain a positive sense ssRNA genome. HCV buds form endosomal membranes and despite the fact that most non-structural proteins of Flaviviridae have been implicated in virus morphogenesis none of them has an assigned role in membrane fission [106,107]. Immature virions are transported in vesicles from the ER to the Golgi where furin cleavage of prM induces the formation of mature virions, which are released from the plasma membrane *via* vesicular transport [107]. This latter process depends on ESCRTs [108,109] and dominant-negative VPS4 or ESCRT-III components as well as siRNA knockdowns (Tsg101, Alix, CHMP4B, VPS4) inhibited HCV production (Table 1) [110,111] albeit without affecting the accumulation of intracellular infectious particles [112]. Thus ESCRT-dependency may be required for HCV release *via* the exosomal secretion pathway [113] restricting the role of ESCRTs to post-budding transport and release steps rather than membrane fission at ER membranes.

Conclusions

Much progress has been made over the last decade to understand how enveloped viruses pinch off from their host cells. In fact much insight into ESCRT function has come from studying enveloped virus budding. Today we know some of the principles that govern ESCRT-III function, but a detailed picture of how it catalyzes fission in conjunction with the ATPase VPS4 is still lacking. The effect of ESCRT-III polymers on lipids and the generation or maintenance of membrane domains is yet unexplored. It is tempting to speculate that some aspects of ESCRT-III-driven fission will resemble those of dynamin-catalyzed fission; these include the role of the helical protein scaffold, although one is assembled on the outside of a membrane neck (dynamin) and the other one on the inside of a membrane neck (ESCRT-III), thus requiring completely different modes of inducing neck narrowing. However, the final constriction may be achieved by using mechanical forces generated either by GTPase (dynamin) or ATPase (VPS4) activities. Furthermore membrane tension and rigidity, which affect the kinetics of dynamin-catalyzed fission, may also play a role during ESCRT-driven fission reactions.

It is yet unclear why some of the enveloped viruses bud independently of ESCRTs. Obviously their genomes would be flexible enough to include short late domain sequences to recruit ESCRTs. In the absence of ESCRTs, one or several factors, viral or yet unknown cellular factors, may set the stage for membrane fission. In case of influenza virus, the mechanism of hydrophobic insertion by parts of M2 plays a crucial role to deform the membrane neck leading to fission. In a potential third class of viruses such as for example flaviviridae, the formation of a glycoprotein coat may be an important contributor to fission. The glycoprotein network could generate bending forces on the membrane that may lead to bud neck narrowing and eventually fission, which, however, might involve additional cellular or viral factors.

Acknowledgements

Work in the laboratory of the authors is supported by the ANR (Agence Nationale de la Recherche) (W.W.), the FINOVI Foundation (W.W.), the LabEx GRAL (W.W.) and the Institut Curie (P.B.). G.E. is supported by a post doctoral fellowship from the ANRS (*Agence Nationale de Recherche sur le Sida* et les hépatites virales).

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