Get on the exosome bus with ALIX

James H. Hurley and Greg Odorizzi

Exosomes have a growing inventory of functions, but the mechanism of protein sorting into exosomes has been unclear. Now, a signal sequence first described in viral budding provides just such a cargo sorting mechanism, revealing closer-than-expected parallelism between exosome biogenesis and the ESCRT-dependent endolysosomal pathway.

The cells of multicellular organisms interact continuously with one another to respond and adapt to their surroundings. Secreted neurotransmitters and growth factors are the classical intercellular messengers. Cells also secrete signalling molecules embedded within vesicles that influence differentiation, immunity, coagulation and metastasis[1]. Shedding microvesicles are released from cells by budding directly from the plasma membrane. Exosomes are another class of extracellular vesicles that are derived from the endomembrane system[1]. They are created when the limiting membrane of endosomes buds into the lumen, resulting in structures known as multivesicular bodies (MVBs). When MVBs fuse with the plasma membrane (Fig. 1), exosome release ensues. Alternatively, MVBs can fuse with lysosomes to deliver their contents for degradation. The most ancient pathway of MVB biogenesis is orchestrated by ESCRT (endosomal sorting complex required for transport)[2,3]. On page 677 of this issue, Baietti *et al.*[9] reveal a role for ESCRT complexes in exosome biogenesis, and provide insight into the process of exosome cargo sorting.

The ESCRT system is the best-understood pathway for MVB biogenesis, but alternative pathways also exist. The ESCRT-driven MVB pathway is triggered when membrane proteins are ubiquitinated and internalized in early endosomes. The ESCRT complexes then carry out ubiquitinated cargo selection and membrane budding. ESCRT-0, -I and -II all directly bind to ubiquitin; thus, cargo proteins must be ubiquitinated to be recognized by the ESCRTs and enter MVBs. Evidence for this comes from the disappearance of intralumenal vesicles (ILVs) in yeast cells engineered to block the ubiquitination of endosomal membrane proteins[4]. In the few examples in which cargo enters ESCRT-dependent MVBs without ubiquitination[5], sorting is thought to be a passenger effect.

Until recently, ESCRTs were not thought to have a role in the formation of exosomes. One influential model for exosome biogenesis invoked a lipid-driven mechanism, in which neutral sphingomyelinase liberates ceramide[6]. Ceramide has a small headgroup that favours its presence in the inner leaflet of highly curved vesicles. The ceramide headgroup forms extended hydrogen bond networks, which cluster ceramide into microdomains that favour budding. This model is attractive in terms of the biophysics of membrane budding, but it does not explain how cargo is selectively loaded into the vesicles. Evidence for ESCRT function in the biogenesis of extracellular signalling vesicles began with two recent reports[7,8] demonstrating that ESCRTs are involved in the shedding of microvesicles directly from the plasma membrane. One group showed that an arrestin-related protein, ARRDC1, bound directly to ESCRT-I and directed microvesicle release from human cells[7]. Another found that ESCRTs are required for microvesicle release from the *Caenorhabditis elegans* plasma membrane[8].

Now, Baietti *et al.*[9] show how a cargo molecule, syndecan, gets sorted into exosomes, and reveal an unexpected role for the ESCRTs in exosome biogenesis. Syntenin is a soluble protein recruited to the plasma membrane by binding to the cytosolic domain of syndecans, which are cell-surface transmembrane co-receptors for adhesion molecules and growth factors. Baietti *et al.* show that syntenin interacts with ALIX, an ESCRT-III-binding protein[10] that is also a member of the exosome proteome. Syntenin has three $LYPX_nL$ motifs in its unstructured N-terminus that bind ALIX. The lack of ordered structure in this region makes the motifs accessible for interaction with the ALIX V-domain. The $LYPX_nL$ motif was discovered as a late domain in HIV-1 (human immunodeficiency virus) and EIAV (equine infectious anaemia virus)[11]; LYPXL and other late-domain motifs are also found in unstructured regions in viral Gag proteins. Late domains are short peptide sequences required for the efficient release of nascent virions and come in three varieties: P(S/T)AP, PPXY and $LYPX_nL$, where n = 1 or 3. The viral P(S/T)AP motif seems to have been captured from the ESCRT-0 subunit Hrs, and the PPXY motifs from the substrates and adaptors of Nedd4 family ubiquitin ligases. The $LYPX_nL$ motif was an outlier, in that no known human proteins had a functional interaction with ALIX through this motif. Now, not one, but two uses for this motif have been revealed in human physiology. Not only are $LYPX_nL$ motifs involved in syntenin targeting into exosomes, but another new report shows that the G-protein-coupled receptor Par1 is downregulated through the recognition of an $LYPX_nL$ motif in its second intracellular loop by ALIX (ref. 12). The syntenin LYPXL-containing sequence has a dissociation constant K_d = 3 μM for ALIX, similarly to the highest affinity interactions observed with viral sequences[13,14].

Regardless of whether ESCRT-I, Nedd4 family proteins or ALIX is recruited first, all three of the above-mentioned pathways converge when the key ESCRT-III subunit CHMP4 is recruited[2,3]. CHMP4, working with downstream ESCRT-III subunits and the AAA^+ ATPase VPS4, is required for the scission of the narrow membrane neck that connects the virion and the plasma membrane. The ALIX pathway is the simplest and most direct route to CHMP4 recruitment, as the Bro1 domain of ALIX itself binds directly to a C-terminal helix of CHMP4. In a pivotal test, knockdowns of the CHMP4 isoforms A, B and C, and VPS4 isoforms A and B, decreased exosome release. Expression of an ATPase-crippled VPS4 allele has been used as a dominant negative in many studies of ESCRT function. Indeed, previous experiments showing that VPS4-dominant-negative (VPS4-dn) expression did not reduce exosome release in the Oli-neu cell line[6] were

James H. Hurley is at the Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA . Greg Odorizzi is at the Department of Molecular, Cell and Developmental Biology, University of Colorado, Boulder, Colorado 80309, USA.
e-mail: James.hurley@nih.gov; Odorizzi@colorado.edu

part of the basis for concluding that exosome biogenesis was ESCRT-independent. Baietti *et al.*[9] also find no effect of VPS4-dn expression, in contrast to the knockdown phenotype. The different phenotypes observed on expression of VPS4-dn and VPS4 knockdown might be consistent with a non-catalytic role for VPS4 in this pathway. In a 'one-shot' ESCRT-mediated budding event not involving recycling of the ESCRT-III machinery, there might be no need for VPS4 catalytic activity.

Rab5-positive early endosomes are the main gateway into the MVB pathway. In their GTP-bound state, the Rab family of small GTPases controls trafficking in the endocytic and secretory pathways by recruiting specific effector proteins onto membrane surfaces to drive vesicle docking and fusion at target membranes. Baietti *et al.* found that overexpression of a constitutively active, GTP-locked mutant form of Rab5 ($Rab5^{Q79L}$) blocked the release of syndecan-containing exosomes[9]. However, Rab5 regulates early endosome biogenesis, not fusion at the plasma membrane. Instead, Rab27A is thought to regulate plasma membrane fusion with exosome-laden MVBs. Furthermore, knockdown of Rab7 inhibited the release of syndecan-containing exosomes. A direct role for Rab7 in exosome release seems perplexing, as it regulates the fusion activity of late endosomes and MVBs, and it functions downstream of Rab5 to regulate endosomal maturation. Through a process termed Rab conversion, Rab7 replaces Rab5 at endosomal membranes, thereby 'switching off' fusion with incoming transport vesicles and switching on fusion with lysosomes. Thus, one explanation for the results seen by Baietti *et al.*[9] is that exosome release is inhibited somehow through chronic activation of Rab5, and that Rab7 normally provides feedback inhibition of Rab5.

A key finding in this study is that ALIX not only packages cargo to enter vesicles, but also triggers vesicle formation[9]. In the absence of cargo, total exosome production declines. In other words, the exosome bus doesn't leave the depot without its passengers. This places the syntenin LYPXL motif in the company of ubiquitin, which, until now, had a unique place as the only cargo signal known to activate ILV formation. How deeply involved is ALIX in ILV formation itself? ALIX binds directly to CHMP4, the central player in the scission of the ILV from the limiting membrane. Sensitivity to CHMP4 depletion is, so far, a universal hallmark of all ESCRT-dependent events. The direct interaction of ALIX with CHMP4 positions ALIX close to the heart of the ESCRT membrane remodelling reaction. ALIX functions as a dimer, the size and structural organization of which has many features in common with the ESCRT-II complex. ESCRT-I and -II have been directly visualized at ILV bud necks *in vitro*, and the structure of an ESCRT-I–II collar at the bud neck has been modelled[15]. It would not be surprising if ALIX had a direct bud-forming activity similarly to that of ESCRT-II, although this would need further investigation. These budding events are thought to work in concert with lipid reorganization, unifying the lipid- and ESCRT-driven models for budding.

Inherent sequence motifs, such as $LYPX_nL$, differ fundamentally from the removable ubiquitin signal. For instance, ubiquitin is covalently attached following receptor activation and can be removed to allow cargo to escape from the pathway. These levels of regulation are not available for sequence signals that are hard-wired into proteins. For cargo destined for constitutive exosome loading following biosynthesis, this lack of regulatory flexibility is not a problem. A more interesting puzzle is posed by the case of Par1 (ref. 12), where premature downregulation needs to be avoided. These points and others will be interesting to follow up as we explore the new world of ubiquitin-independent cargo-loading into MVBs.

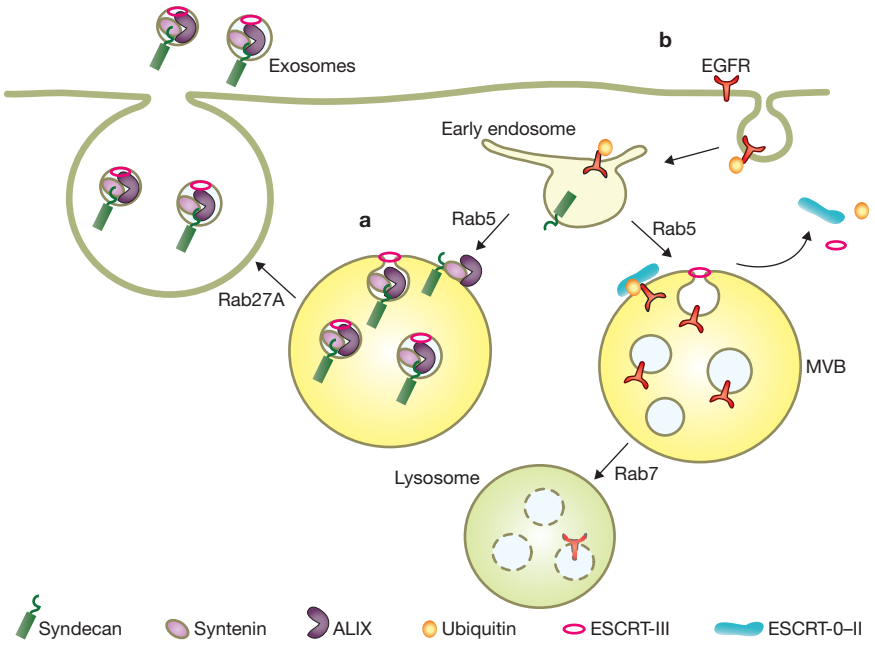

Figure 1 Roles of multivesicular bodies in exosome biogenesis and lysosomal protein sorting. (**a**) Syntenin (lilac) binds to ALIX (purple) directly through its LYPXL motif. ALIX recruits ESCRT-III complex proteins (pink), leading to intralumenal vesicle (ILV) formation. Multivesicular bodies (MVB, yellow) fuse with the plasma membrane and exosome release is mediated by a Rab27A-dependent pathway. (**b**) Cell surface receptors (such as the EGF receptor, EGFR, red) can be ubiquitinated and internalized through the endosome pathway. ESCRT complexes recognise and bind to the ubiquitin molecule (orange) on the receptor, and mediate formation of ILVs containing the ubiquitinated cargo. MVBs containing these ILVs then fuse with lysosomes (green) to mediate cargo degradation.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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