The reverse logic of multivesicular endosomes

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The EMBO Conference on Endocytic Machineries in Control of Cell Signalling and Tissue Morphogenesis held last October highlighted advances in our understanding of endocytic trafficking. A centrepiece was the remarkable plasticity and sorting options of the maturing late endosome compartment.

Fifty years after the first description of curious “multivesicular bodies” in micrographs of rat oocytes (Sotelo & Porter, 1959), it might seem odd that the precise mechanistic basis for the formation of these ubiquitous intracellular organelles remains as perplexing as their morphology. The biogenesis of multivesicular bodies (MVBs)—also known as late multivesicular endosomes—represents a subset of the remarkable endosomal transformations that occur in eukaryotic cells. Situated between early peripheral rab5 GTPase-positive endosomes and the terminal lysosome compartment, MVBs are characterized by the presence of intraluminal vesicles (ILVs) and sometimes also onion-like whorls of internal membrane (Fig 1). The internal packaging of the limiting membrane to generate ILVs is widely accepted to partition certain transmembrane molecules that were present originally at the cell surface into the MVB lumen. The spatial remodelling isolates receptors from the surrounding cytosol and can terminate signal propagation as a prelude to preparing receptors for destruction by the lysosomal compartment. In fact, stimulation by epidermal growth factor (EGF) increases the number and size of MVBs (White *et al*, 2006), whereas incapacitating the core MVB biogenesis machinery leads to excessive and prolonged signal

propagation (Vaccari & Bilder, 2005). However, before becoming fully committed to ILV production, nascent MVBs sort transmembrane proteins and lipids within emanating tubules to other intracellular destinations, such as the *trans*-Golgi network, recycling endosomes, or back to the plasma membrane (Fig 1).

At the recent EMBO Conference on Endocytic Machineries in Control of Cell Signalling and Tissue Morphogenesis, held in Chania, Crete and organized by Harald

Stenmark and Gillian Griffiths, MVB biology was a conceptual thread linking much of the lively discussion. Perhaps the most remarkable aspect of MVB function is the necessarily coupled inward deformation of the encapsulating endosomal membrane and the packaging of designated cargo into these dimpling territories to produce ILVs. It is intriguing because the process is topologically distinct from most intracellular membrane budding events, in which forming spherical or tubular carriers are oriented toward the surrounding cytosol, not away from it. The molecular identity of the principal machinery that coordinates cargo concentration is known: a cascade of factors termed endosomal sorting complexes required for transport (ESCRTs) dock and assemble on the endosome membrane to produce ILVs. Considerable structural and biochemical information is available and the initial constituents ESCRT-0, ESCRT-I and ESCRT-II are known to be involved in corraling cargo and recognizing ubiquitin, which is a switch for sorting most—but not all—transmembrane proteins into ILVs. The chief task of the ESCRT-III machinery is to extrude the sorted cargo into the endosome lumen, which is almost certainly linked to the capacity of ESCRT-III proteins to form long, membrane-attached, polymeric ‘ring-like’ filaments (Hanson *et al*, 2008).

Fig 1 | Endocytic sorting structures and pathways. Schematic of the main types of sorting endosome and the *trans*-Golgi network. The arrows indicate the temporal relationship of the different sorting trajectories. ESCRTs, endosomal complexes required for transport; ILV, intraluminal vesicle; LBPA, lysobisphosphatidic acid; MVB, multivesicular body.

But how does ESCRT-III really work? Why are there more than 10 related, but different, gene products in mammals? What is the function of the striking involution spirals? The physical problem to deal with is the convergence of the rim of inwardly bulging membrane, to allow a membrane scission event that will liberate a free ILV into the MVB interior. Scott Emr (Cornell U.), who studies ESCRTs in *Saccharomyces cerevisiae*, argued that there is a defined, interlinked three-stage assembly process of circular ESCRT-III filaments. He proposed a 'purse-string' model for ESCRT-III filament constriction through the processive removal of a membrane-tethered edge monomer by the AAA-type ATPase Vps4. How the ends of the spirals remain adjacent during the removal of the ESCRT-III subunits is unknown, but a more pressing question is how the model can deal with the recent finding that ESCRT-III can produce ILVs *in vitro* in the absence of Vps4 (Wollert *et al*, 2009). An alternative view is that successive smaller concentric rings or spirals of ESCRT-III polymer are laid down against a larger outer template (Im *et al*, 2009).

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In reality, MVB biogenesis involves two coupled but separate processes: directed involution of surface membrane away from the cytosol and membrane scission. The first involves cargo sorting and membrane remodelling, but the second entails overcoming biophysical energy barriers to drive bilayer fusion. It is becoming clear that regulated, localized structural imperfections within the bilayer lead to initial hemifusion—which is important to prevent the leakage of luminal contents. A mechanistic clue to the dichotomy between cargo selection and inward budding and membrane scission came from Howard Reizman's (U. Geneva) analysis of sphingolipid requirements for MVB formation in yeast. Using a recombinant strain, with an ectopic ceramide synthase that produces 24-carbon-chain sphingolipids instead of the normal C26, leads to a strong arrest in ILV formation and vacuole trafficking. Electron micrographs of this strain show flattened

MVBs with multiple internal structures that have possibly failed to pinch off and create free ILVs. These data indicate that it is possible to uncouple the linked invagination and cargo selection steps from final ILV fission, and that the latter requires an appropriate membrane lipid environment. A possible role for the large number of ESCRT-III subunits is to have different, temporally ordered functions; some could operate late as fusion scaffolds or accelerators by producing appropriate fusion-favouring stresses in areas containing the necessary lipids.

Not all internal vesicles seem destined for destruction and some transmembrane proteins actually appear to move out of ILVs. Jean Gruenberg (U. Geneva) talked about the remarkably different vesiculation and fusion reactions that seem possible within the MBV compartment. While ESCRTs drive inward vesiculation and lysosomal targeting, depending on an acidified MVB lumen, the ESCRT-I accessory protein Alix does not affect transport to lysosomes but to other cellular destinations. One possibility is that if ILVs can be reinserted into the perimeter membrane in a 'back fusion' process, repeated rounds of ILV scission and fusion can fine-tune sorting—and signalling—in the maturing endosome over time.

There are still no intraluminal protein components known to regulate fusion, and currently the only possible regulator that is positioned within ILVs—but also in the MVB-limiting membrane—is the rare lipid lysobisphosphatidic acid (LBPA), which binds to Alix. LBPA alone can promote the formation of ILVs; could these structures then have different protein and lipid compositions—and, ultimately, sorting fates—than ESCRT-dependent ILVs? It turns out that LBPA is mostly restricted to late rab7-positive mature MVBs, whereas ESCRTs are also present on incipient MVBs, which actively sort macromolecules to other intracellular destinations. This seems intuitively correct, because back fusion should logically follow ILV production. Coupling between the temporally resolved maturing endosomes might occur through the secretory carrier membrane protein SCAMP3—which is a multiply ubiquitinated tetraspanin protein that binds physically to ESCRT-0 and ESCRT-I factors, but only affects EGF receptor sorting later, in the mature MVB (Aoh *et al*, 2009). Because SCAMP3 engages ESCRT-I and is present on maturing endosomes, it could explain the dependence of back fusion on both ESCRT-I and Alix.

Some pathogenic viral genomes and toxins seem to exit endosomes through the retrofusion of ILVs with the outer MVB membrane, which could also be the route followed by translation-silencing siRNA duplexes—which enter the cell endocytically—to access the cytosol. Surprisingly, it has recently been described that endogenous miRNAs are found in so-called GW bodies, which are closely associated with MVBs (Gibbings *et al*, 2009). MVBs are linked functionally to RISC-miRNA complex assembly and activity, indicating that these endosomes could turn out to be a central locus of RNAi regulation.

MVBs in signalling and development

Marcos González-Gaitán (U. Geneva) described that *Drosophila* MVBs can guide daughter cell fate after a single round of asymmetric cell division. The sensory bristles covering the surface of the adult fly cuticle are composed of four morphologically different cell types that arise in the pupa from two rounds of asymmetric cell division of a parental sensory organ precursor (SOP). Notch signalling distinguishes the two daughter progeny, termed pIIa and pIIb cells, which will ultimately adopt dramatically distinct cellular properties. The mother cell expresses both the receptor Notch and its transmembrane ligand Delta. Prior to SOP cell division, internalized Notch and Delta are found in Sara and early rab5-positive endosomes that, upon division, are asymmetrically delivered to the pIIa cell, which responds to Notch activation; that is, Sara endosomes come from the SOP cell and are carried into the pIIa to begin signalling shortly after cytokinesis. Experimental manipulations that cause the Sara-positive endosomes to partition incorrectly into pIIb result in an inappropriate execution of a pIIa fate program.

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Most intriguingly, Notch signalling correlates with the maturation of Sara endosomes into MVBs, wherein the extracellular domain of Notch (NECD) is positioned at the limiting membrane. However, the transcriptionally active intracellular domain (NICD) is not apparent on these organelles.

Notch cleavage depends on the Delta ligand, which is also present in Sara endosomes, leading to a beguiling model for intraluminal, Delta-dependent cleavage of Notch in MVB to ensure the early establishment of the correct pIIa fate. The topological reversal of orientation upon receptor incorporation into ILVs would allow ILV-presented Delta to *trans*-stimulate Notch in the limiting membrane. Yet, the trafficking of both Notch and Delta is regulated by ubiquitination and both are directed to MVBs, so whether—and the mechanism by which—only one is sorted into ILVs remains unknown. Notch activation is more commonly thought to occur transcellularly; the physical force of endocytic uptake of the NECD bound to Delta on the plasma membrane of the signal-sending cell drives the cleavage and release of the NICD. How a similar mechanically dependent event can occur in the context of ILVs that contain Delta and engage Notch remains to be established, but it is reassuring that Notch signalling is modulated by the ESCRT machinery (Vaccari & Bilder, 2005). During development, MVBs might thus operate as specialized signalling platforms that bias cell fate determination.

The gathering of cargo into ESCRT-containing regions is typically governed by ubiquitin, which reflects the early role of ESCRT-0 and ESCRT-I in sequestering ubiquitin-modified cargo away from the recycling tubules that radiate from maturing early endosomes. Willem Stoorvogel (Utrecht U.) confirmed that in immature dendritic cells—which do not present antigen-loaded MHC class II molecules to T cells, but rather degrade the complex—ubiquitin regulates the selective entry of MHC class II into MVBs that contain the antigenic peptide loading cofactor DM. However, in mature dendritic cells, which are dedicated to MHC class II presentation, T-cell contact triggers the cessation of MHC class II ubiquitination and leads to the generation of a morphologically and

compositionally distinct, tetraspanin CD9-positive MVB population, which is expelled to produce extracellular exosomes for the delivery of membrane-embedded proteins to T cells. These two populations differ in that only the former delivers contents to lysosomes. The different MVB types seem to coexist, but the relative abundance of each changes as a function of dendritic cell maturation. As MHC class II packaging into ILV is elevated in T-cell-triggered dendritic cells, despite a decrease in ubiquitination, a fundamentally different mode of cargo sequestration and packaging into ILV probably occurs.

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Transient fusion of mature MVBs with lysosomes normally allows ILV digestion through the formation of a short-lived, hybrid degradative organelle. However, Mark McNiven (Mayo Clinic, Rochester, USA) suggested that tubulovesicular transport elements actively deliver receptors from late rab7-positive endosomes to degradative lysosomes. Dynamin 2, which usually promotes clathrin-coated vesicle scission from the cell surface during initial receptor uptake, has an unexpected late role in the degradation of activated EGF receptors. EGF induces the recruitment of dynamin 2 to endosomes, which occurs from 30–60 min through a novel direct interaction with CIN85, an SH3 domain adaptor. The disruption of this interaction leads to an accumulation of EGF and receptor in the lumen of late rab7-positive endosomes. In addition, this endosomal compartment morphs from spherical vesicles to exceptionally long receptor-enriched tubules, a restructuring that is accompanied by a marked delay in receptor degradation and consequent

persistent downstream signalling. Whether dynamin 2 severs vesicles that are rich in receptor for their delivery to the lysosome, and whether Alix and back fusion are involved in this process, remains to be established. Stubby tubules project from EGF-receptor-containing MVBs 30–60 min after stimulation, but the receptor is not enriched in these structures (Aoh *et al*, 2009). Thus, an equally interesting possibility is that by releasing tubules and vesicles, endosomal dynamin 2–CIN85 oversees the redistribution of other MVB surface constituents that must obligatorily egress before the endosome can traverse a checkpoint that allows MVB–lysosome fusion.

Owing to the swift pace and sophistication of current research, it might be taken for granted that a comprehensive mechanistic and functional description of MVB dynamics exists—it does not. What is obvious is that the MVB is a morphologically and compositionally malleable, operationally versatile compartment that defies a simple stereotyped classification. In addition to arguably representing the biggest mechanistic puzzle in contemporary trafficking research, the rich data presented in Chania were a clarion articulation that the MVB compartment undoubtedly has several more fascinating secrets tucked away inside.

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