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# Zooming into plant ubiquitin-mediated endocytosis

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Endocytosis in plants plays an essential role, not only for basic cellular functions but also for growth, development, and environmental responses. Over the past few years, ubiquitin emerged as a major signal triggering the removal of plasma membrane proteins from the cell surface and promoting their vacuolar targeting. Detailed genetic, biochemical and imaging studies have provided initial insights into the precise mechanisms and roles of ubiquitin-mediated endocytosis in plants. Here, we summarize the present state of knowledge about the machinery involved in plant ubiquitin-mediated endocytosis and how this is coordinated in time and space to control the internalization and the endosomal sorting of endocytosed proteins.

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## Introduction

Eukaryotic cells are continuously sensing external and internal stimuli in order to survive. As such, the ability to dynamically reorganize the composition of the plasma membrane (PM) is critical for the proper development and survival of a cell. Endocytosis is the process of internalizing patches of PM as a vesicle that fuses with early endosomes (EE), and of that targets endocytosed material towards the lysosome/vacuole via late endosomes (LE). Endocytic trafficking plays an essential role in the control of the abundance of receptors, transporters and other PM proteins [1]. The endocytic system and the mechanisms driving the regulation of PM protein internalization and sorting have been extensively characterized over the past decades. Several internal signals or post-translational modifications have also been shown to contribute to the endocytic trafficking of membrane proteins in non-plant models [2].

Ubiquitination is mediated by the consecutive action of an ubiquitin (Ub)-activating (E1), an Ub-conjugating (E2), and an Ub ligase (E3) enzymes, and results in the covalent attachment of Ub to a target lysine [3]. Ub, in the form a polyUb chain, is a well-recognized signal leading to the destabilization of proteins via proteasome-mediated degradation [3]. Ubiquitination of proteins can however trigger a wide variety of proteasome-independent cellular functions, using different linkages between Ub moieties within a chain [4]. Notably, monoUb and chains involving lysine (K)-63 from Ub (hereafter called K63 polyUb chains) are signals governing the internalization and the intracellular sorting of integral PM proteins, in a process called Ub-mediated endocytosis (UbME) [5]. The study of endocytosis and Ub-mediated trafficking in plants is more recent and has mostly been driven by analogies with what has been described in other organisms [6], although several plant-specific features arose during evolution. In the present review, we will discuss the recent advances made on the cellular aspects of plant UbME.

## The first clues about ubiquitin-mediated endocytosis in plants

A few plant PM proteins, such as the PIN2 auxin efflux carrier and the FLS2 flagellin receptor, were early shown to be ubiquitinated [7,8]. Treatment with the proteasome inhibitor MG132 enhanced the total levels of both PIN2 and FLS2, as well as the PIN2 ubiquitinated pool, and was originally interpreted as an evidence for proteasome-mediated degradation. The actual demonstration for the role of Ub in plant PM protein endocytosis and vacuolar degradation resulted from parallel work on the IRT1 root iron transporter, the BOR1 boron transporter and PIN2 [9–11]. All three proteins were shown to be post-translationally modified with Ub moieties, and putative ubiquitinated lysine residues identified in cytosolic loops. The substitution of target lysines to non-ubiquitinatable arginine residues in IRT1, BOR1, and PIN2 shed light on the functional importance of UbME. Expression of ubiquitination-defective forms of these proteins triggered their accumulation at the PM, and failure to be degraded in the vacuole in response to environmental cues such as gravistimulation or boron excess [9–11]. These observations pointing to a role of Ub in endocytosis were backed up by alternative approaches using artificially ubiquitinated cargos (carrying in-frame fusions with Ub). The sole presence of Ub on a cargo indeed appears as a sufficient signal for PM protein sorting to the vacuolar lumen of plant cells [12–14].

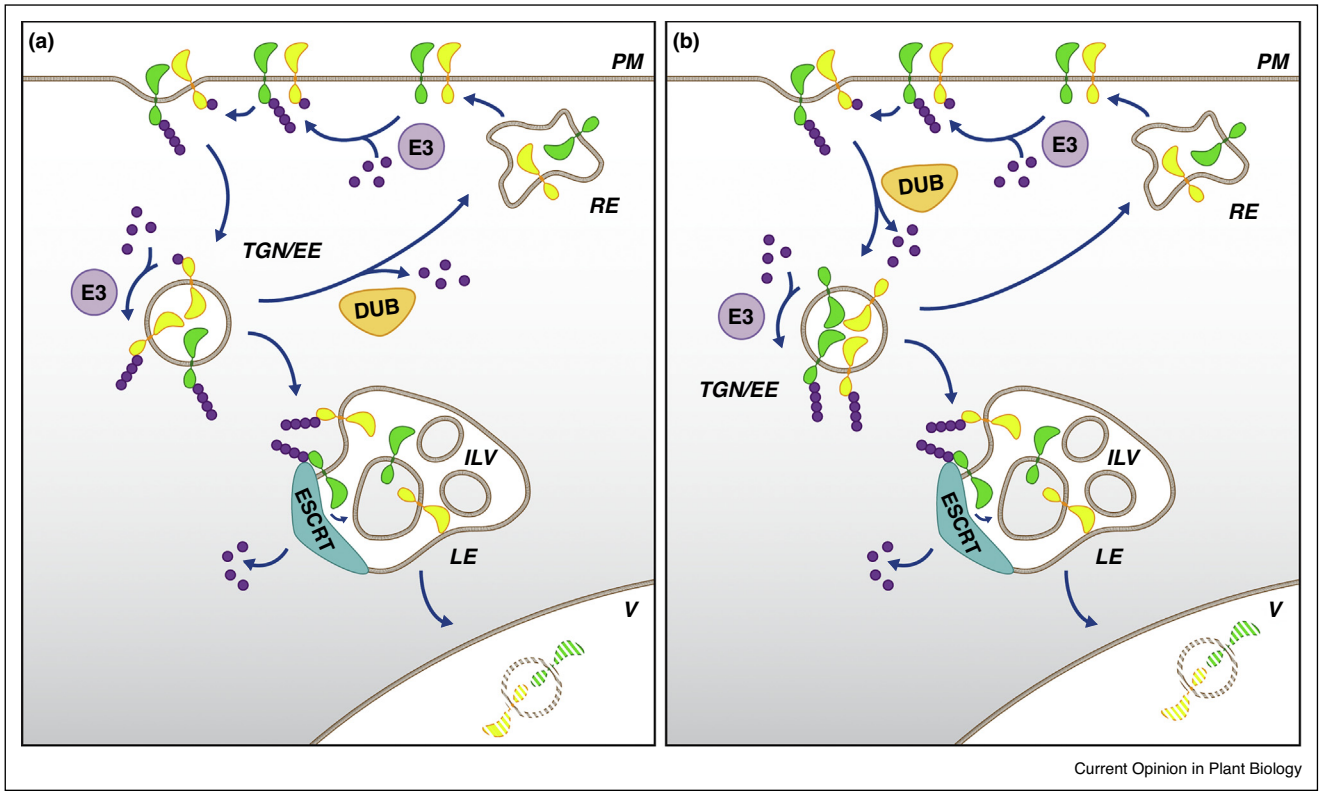
Since then, other PM proteins were demonstrated to undergo UbME. These include the BRI1 brassinosteroid

receptor [13••], the PHT1;1 phosphate transporter [15••,16], and the membrane-associated PYL4/PYR1 ABA receptors [17••,18,19•]. In addition, a growing number of plant PM proteins were shown to be either ubiquitinated [20•], or to interact with E3 Ub ligase enzymes [21–27], but the final proof that these proteins are controlled by UbME is often missing. Although very likely endocytosed in an Ub-dependent fashion, one should keep in mind that Ub triggers a wide variety of additional cellular functions targeting PM proteins, including ER-associated degradation of misfolded proteins, autophagy, regulation of kinase activity, etc. The unambiguous demonstration of UbME relies on direct experimental evidence showing that a cargo is not properly endocytosed when ubiquitination targets are substituted to arginines or in a mutant defective for its cognate E3 Ub ligase.

## Ubiquitination types and endocytic routes

Among the many possible forms of Ub modifications found in eukaryotes, a single Ub moiety or a K63-linked chain are sufficient to promote internalization using clathrin-dependent routes and/or endosomal sorting of a plant cargo (Figure 1) [12–14]. Consistently, plant proteins shown to undergo UbME are decorated with either monoUb or K63 polyUb chains. Using a combination of anti-Ub antibodies recognizing all types of Ub modifications, or polyUb chains only, IRT1 was indeed shown to be multimonoubiquitinated on several lysine residues [9]. An ubiquitination-defective IRT1 variant shows increased cell surface localization and is unable to reach the *trans*-Golgi network (TGN), which also serves as EE in plants [28], and where wild-type IRT1 is found. MultimonoUb-dependent internalization from the cell surface also controls the PM pool of the Phot1 blue light photoreceptor under low blue light, but does not control its degradation [26]. In contrast, BOR1 is linked to di-Ub modifications in response to boron that promote its endosomal sorting and vacuolar degradation [10]. PIN2 and BRI1 are decorated with K63-linked Ub chains and both require K63 polyUb for their proper endosomal

**Figure 1**




Dual role of Ub in internalization and endosomal sorting of plasma membrane proteins. **(a)** MonoUb and K63-linked Ub chains drive the internalization of PM proteins. Both Ub forms drive clathrin-dependent endocytosis but may also contribute to clathrin-independent pathways. Deubiquitination by DUB enzymes allows the recycling of endocytosed cargo proteins. Cargos carrying K63-linked chains at the TGN/EE are recognized by the ESCRT complex, sorted into ILVs, and routed towards the vacuole for degradation. A fraction of monoubiquitinated cargos likely undergoes K63 polyubiquitination by a TGN/EE-localized E3 Ub ligase to promote vacuolar targeting. **(b)** This model depicts the deubiquitination of internalized ubiquitinated cargos before reaching the TGN/EE. A TGN/EE-localized E3 is required to reubiquitinate with K63-linked Ub chains the pool of PM protein destined for degradation in the vacuole. PM, plasma membrane; TGN/EE, *trans*-Golgi network/early endosome; RE, recycling endosome; LE, late endosome; ILV, intraluminal vesicle; V, vacuole; E3, E3 Ub ligase; DUB, deubiquitinase; ESCRT, endosomal sorting complex required for transport. Purple dots represent ubiquitin moieties.

sorting [11,13••]. Inability to be modified by K63-linked chains prevents the targeting of PIN2 and BRI1 to the vacuole. For BRI1 however, ubiquitination also contributes in part to its internalization from the cell surface, as visualized by the increased cell surface residence of ubiquitination-defective BRI1 by TIRF imaging [13••], and is negatively regulated by the ELT1 protein in rice [29].

Overall, plant cargo proteins differently use ubiquitination to drive their intracellular dynamics. The type of Ub (i.e. monoUb, multimonoUb or K63 polyUb) likely dictates the pace of Ub-dependent internalization and sorting, through the differential avidity for Ub-binding proteins routing them into or along the endocytic pathway. In addition, the contribution of UbME to the internalization of a cargo is highly variable. Ubiquitination is crucial to internalize IRT1, only partly contributes to BRI1 removal from the cell surface, and appears dispensable for the early steps of PIN2 and BOR1 endocytosis [9–11,13••]. This highlights the existence of alternative Ub-independent internalization routes for some cargos such as BRI1 that, altogether, likely contribute to the robust and integrated control of endocytosis and fine adjustment of cell surface protein abundance.

## The plant ubiquitin-dependent internalization machinery

The RSP5 HECT E3 ligase from *Saccharomyces cerevisiae* is considered as responsible for the ubiquitination of most if not all internalized membrane proteins in yeast, with the help of arrestin adaptors [5]. Compared to higher eukaryotes, plant HECT E3s have been downsized with only seven found in the Arabidopsis genome (28 HECT E3s are encoded by the human genome), and arrestins are completely absent [30,31]. In plants, the specificity in cargo ubiquitination lies in the dramatic expansion of the non-HECT E3 repertoire with ~1500 present in Arabidopsis. A few E3s, mostly from the RING or U-box families, were demonstrated to interact with or to ubiquitinate plant PM proteins. These include the IDF1, NLA, RGLG1/2, and RSL1 RING E3s, which control ubiquitination of IRT1, PHT1;1, PIN2 and PYL4, respectively [11,16,18,32,33]. Many other examples exist in the literature [21–27], but in most cases the formal demonstration that such E3s impact on UbME is still awaited.

The recruitment of Ub cargos into endocytic structures (e.g. clathrin-coated pits) requires endocytic accessory adaptor proteins, the best characterized of which are Epsins [34]. These adaptors contain a Ub-interacting motif, and are able to associate with clathrin, AP2 adaptor complex and PI(4,5)$P_2$ via their epsin N-terminal homology (ENTH) or AP180-N-terminal homology (ANTH) domains [34]. In contrast to their yeast and mammalian counterparts, plant E/ANTH proteins do not contain conserved Ub-interacting motifs, which are required for the interaction with ubiquitinated proteins [35,36]. This likely suggests that ubiquitinated cargos are recognized at the PM by other plant-specific accessory adaptors yet to be characterized, presumably recruiting ubiquitinated proteins to AP-2 and/or TPLATE endocytic adaptor complexes [37–39]. While it is clear that UbME uses clathrin-mediated endocytosis [9,14,40], reports exist in mammals of Ub-dependent clathrin-independent internalization for the EGF receptor [41]. Considering the emerging importance of clathrin-independent processes in the endocytosis of plant cargos known to undergo UbME, such as BRI1 [42], future efforts will be needed to precisely match internalization mechanisms to internalization pathways.

Ubiquitination is a reversible post-translational modification, counterbalanced by deubiquitinase enzymes (DUBs), potentially allowing the recycling of internalized cargos [43]. This is exemplified by the TRKA nerve growth factor receptor that undergoes TRAF6-dependent K63 polyUbME and can be deubiquitinated by the K63 polyUb-specific CYLD DUB [44,45]. To date, no DUB has been associated with recycling-based deubiquitination of cargo in plants.

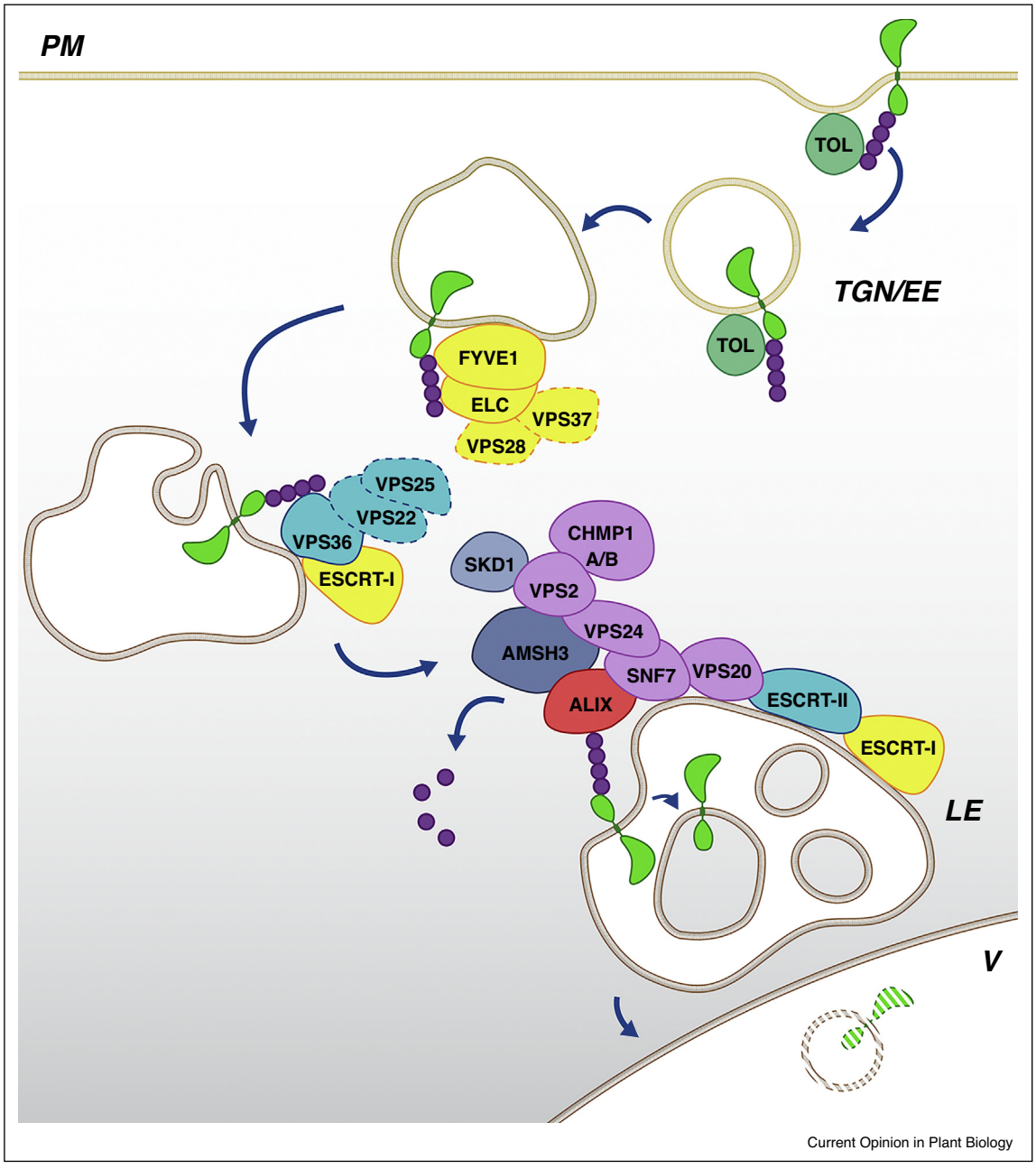
The sequence of events following Ub-triggered internalization is still very obscure. The accepted model is that a cargo reaching the TGN/EE and carrying Ub moieties will be recognized by the ESCRT complex and will be routed towards the vacuole, unless deubiquitinated (Figure 1, left panel). Such vacuolar targeting of Ub cargos, by default, has recently been challenged for the yeast JEN1 glucose transporter using advanced imaging. The RSP5 HECT-type E3 ligase K63-polyubiquitinates JEN1 at the cell surface, with the help of the ROD1 arrestin, to trigger glucose-induced internalization of JEN1 [46,47]. JEN1 is then rapidly deubiquitinated after endocytosis by an unknown DUB, before being re-decorated with K63-linked chains by TGN-localized RSP5/ROD1 to allow progression towards the vacuole [46]. This suggests that a second level of control for the degradation of PM proteins occurs in the TGN. Such scenario may also apply to plant PM proteins and suggests that first, deubiquitination involves poorly-specific DUB(s) recognizing Ub modifications rather than the cargo itself, consistent with the relatively low number of DUBs (~50) found in the Arabidopsis genome, and that second, TGN/EE-localized E3s can K63 polyubiquitinate cargos destined for degradation (Figure 1, right panel).

## Endosomal sorting and vacuolar targeting of plant ubiquitinated cargos

Ub-mediated endosomal sorting and vacuolar/lysosomal targeting involves the endosomal sorting complex required for transport (ESCRT) multi-subunit complex

in LE. In non-plant eukaryotes, four distinct ESCRT sub-complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) and various accessory components act in the recognition of ubiquitinated cargos using Ub-binding domains, and in their sorting into LE intraluminal vesicles (ILVs) [48]. The detailed composition and function of the plant ESCRT machinery is described in this issue by Isono and Kalinowska.

**Figure 2**




Sequential recognition of ubiquitinated cargos by the endosomal sorting machinery. Ubiquitinated cargos are likely recognized at the cell surface or early after internalization by the TOL proteins, which act as ESCRT-0 in plants. The case of a cargo carrying a K63-linked Ub chain and being targeted to the vacuole is represented. Other ESCRT sub-complexes are sequentially recruited along the endocytic pathway via protein–phospholipid or protein–protein interactions. The exact nature of endosomes where ESCRT are recruited is unclear considering the constant maturation of the endocytic system, contrasting with the static description provided by early and late endosomes. The maturation of endosomes along the endocytic pathway is represented by the modification of the shape and of the membrane color, underlying modifications in phospholipid and protein composition. The ESCRT-I complex and its subunits are shown in yellow, ESCRT-II in turquoise and core ESCRT-III subunits in pink. Purple dots represent ubiquitin moieties.

While most of the genes encoding ESCRT complex components exist in plants, the canonical VPS27 and HSE1 ESCRT-0 subunits are absent from the *Arabidopsis* genome and appear to be opisthokont-specific [6]. The TOL family of protein in plants however possesses all functionally relevant features for recognition and sorting of ubiquitinated cargos, including ability to bind Ub and clathrin, and localization to TGN/EE [49]. Consistently, TOLs were shown to recognize and sort PIN2 in endosomes towards the vacuole. TOL6 was also observed at or near the PM, indicating that the sorting of ubiquitinated cargos may be initiated very early after internalization (Figure 2). This is consistent with recent studies in *Caenorhabditis elegans* where ESCRT-0 directly associates with clathrin-coated pits via endocytic adaptors, although not influencing internalization kinetics [50].

Plants have only three of the canonical ESCRT-I subunits (VSP23, VPS28 and VPS37). The VPS23 plant homolog ELC directly binds Ub and is located in LE marker-positive endosomes, where it interacts with homologs of ESCRT-I in Arabidopsis [51]. Besides the canonical ESCRT-I machinery, plants also possess an atypical ESCRT-I member. The FYVE1 protein (also named FREE1) binds Ub, localizes to LE and is essential for the formation of ILVs [40,52]. FYVE1 is recruited to the ESCRT-I complex through its interaction with ELC, and both associate with PYL4 and IRT1 [17••,19•,40,52]. Consistently, genetic interference with *ELC* or *FYVE1* expression yields ubiquitinated protein accumulation in the membrane fraction, stabilization of ubiquitinated PYL4, and impaired trafficking of IRT1 and PIN2 [17••,19•,40,52,53].

Very little is known about plant ESCRT-II subunits. Only the Arabidopsis VPS36 protein has recently been demonstrated to bind Ub using a GLUE domain, and to be important for the proper trafficking of several PM proteins towards the vacuole [54].

The plant ESCRT-III complex is important for proper endosomal sorting of several cargos [55]. During the sorting process in ILVs, the cargo is deubiquitinated by ESCRT-III-associated DUB(s). In plants, the K63 and K48 polyUb-specific DUB AMSH3 is a good candidate for the deubiquitination step [56]. AMSH3 interacts with the VPS2.1 and VPS24.1 subunits from ESCRT-III, and is recruited to LE by the Arabidopsis ALIX protein that bridges the interaction between ESCRT-III, AMSH3, and presumably the K63 polyUb-conjugated cargo [15••,57••,58]. ALIX and AMSH3 proteins are not only important for vacuolar biogenesis, but also for proper sorting of PHT1;1, BRI1 and PIN2 [15••,56].

Altogether, the Ub-binding proteins from ESCRT complex not only allow to ferry ubiquitinated cargos along the endocytic pathway to reach the lytic vacuole, but also participate in the formation of ILVs thus having a profound role of trafficking in general.

## Conclusions and perspectives

With recent advances in our understanding of UbME in plants, it has become increasingly clear that plants harbor some unique features in the mechanisms and factors governing UbME. In addition, the static organization of ubiquitinated cargo sorting depicted in textbooks will certainly have to be revisited in the future. Endosomes are indeed constantly maturing along the endocytic pathway, far from being limited to the sole TGN/EE and LE. We currently have little resolution on endosomal intermediates and how the UbME machinery is dynamically recruited along the endocytic pathway. The precise spatial and temporal recruitment profiles of the UbME machinery to sites of internalization and during sorting using high resolution live imaging, combined to more systematic co-localization of endosomal proteins and implementation of correlative light-electron microscopy, will provide a better picture of the sequential events at stake.

## Authors contribution

G.D. and G.V. wrote the paper.

## Conflict of interest

The authors declare no conflict of interest.

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