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Vacuoles in mammals

A subcellular structure indispensable for early embryogenesis

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A vacuole is a membrane-bound subcellular structure involved in intracellular digestion. Instead of the large "vacuolar" organelles that are found in plants and fungi, animal cells possess lysosomes that are smaller in size and are enriched with hydrolytic enzymes similar to those found in the vacuoles. Large vacuolar structures are often observed in highly differentiated mammalian tissues such as embryonic visceral endoderm and absorbing epithelium. Vacuoles/lysosomes share a conserved mechanism of biogenesis, and they are at the terminal of the endocytic pathways, Recent genetic studies of the mammalian orthologs of *Vam*/*Vps* genes, which have essential functions for vacuole assembly, revealed that the dynamics of vacuoles/lysosomes are important for tissue differentiation and patterning through regulation of various molecular signaling events in mammals.

Introduction

Eukaryotic cells develop membrane-bound organelles that provide specialized environments for biochemical and biophysical processes essential for cellular functions. Vacuoles are one member of the organelles. The term "vacuole" originates from the transparent morphology of this organelle, implying that the structure is "empty," being devoid of the cytoplasmic materials. Light microscopy studies have revealed that a typical plant cell vacuole often occupies more than 90% of the cellular volume (**Fig. 1**). Vacuoles also prominently occur in fungal cells: they occupy approximately a quarter of the cell volume in *Saccharomyces cerevisiae*.[1] Filamentous fungi also possess well-developed vacuoles.[2] Fission yeasts such as *Schizosaccharomyces* exhibit smaller but numerous vacuoles within the cells.[3]

Animal vacuoles are commonly far less morphologically developed than those in plants and fungi. Animal cells possess hydrolytic enzyme enriched lysosomes, which are usually much smaller than plant and fungal vacuoles. In this regard, the vacuolar/lysosomal architecture in animal cells is similar to that in fission yeast. However, recent studies have revealed that some animal cells possess well-developed prominent vacuoles. In this article, I describe animal cells that develop "vacuoles" with morphological signatures and the role of these organelles in cell and tissue physiology.

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Membrane Flow Toward Vacuoles: A Conserved Mechanism in Different Species

Cells take up extracellular material by invagination of a small portion of the cell membrane, which then pinches off to form a vesicle that travels through the cytoplasm and interacts with a series of membrane compartments. This process is known as endocytosis (**Fig. 2**). The yeast vacuole is at the terminal of the endocytic pathways, where the endocytosed materials are accumulated.[4] In animal cells, the endocytic pathways are well characterized. Plant cells also exhibit endocytic activities and deliver the extracellular molecules to the vacuoles.[5]

The intracellular membrane compartments actively exchange their membranes and contents, yet keeping their identities. The basic logics for intracellular transport have been evolutionarily conserved in various species of fungi, plant, and the animal kingdom. The dynamic exchange processes among organelle membranes are tightly regulated by cellular machinery composed of small GTP-binding proteins like arf and rab proteins, v- and t-SNARE molecules, and tethering complexes.[6,7]

Yeast genetic studies have revealed that more than 50 genes, known as *VPS* (*vacuolar protein sorting*) genes, are involved in vacuolar protein transport and localization. Orthologs of *VPS* are found in plants and mammals. Thus, the basic mechanisms for vacuole- and lysosome assembly are similar in fungi and animals. In addition to *VPS*, many yeast genes, including *PEP* (*peptidase*) and *VAM* (*vacuolar morphology*), have been identified. The orthologs of *VPS*, *PEP*, and *VAM* genes are present in plants as well as animals and some of these genes can functionally substitute the endogenous yeast genes.[8-10] Mammalian *VPS* homologs are implicated in lysosome-related hereditary complications.[11]

Endocytic Pathway in Visceral Endoderm, an Embryonic Epithelium

The endocytic pathway is thought to downregulate various signal transduction pathways by compartmentalizing and degrading the signaling molecules. Although this view has been well established at the cellular level, the significance of vacuolar/lysosomal signal regulation is poorly understood at the level of

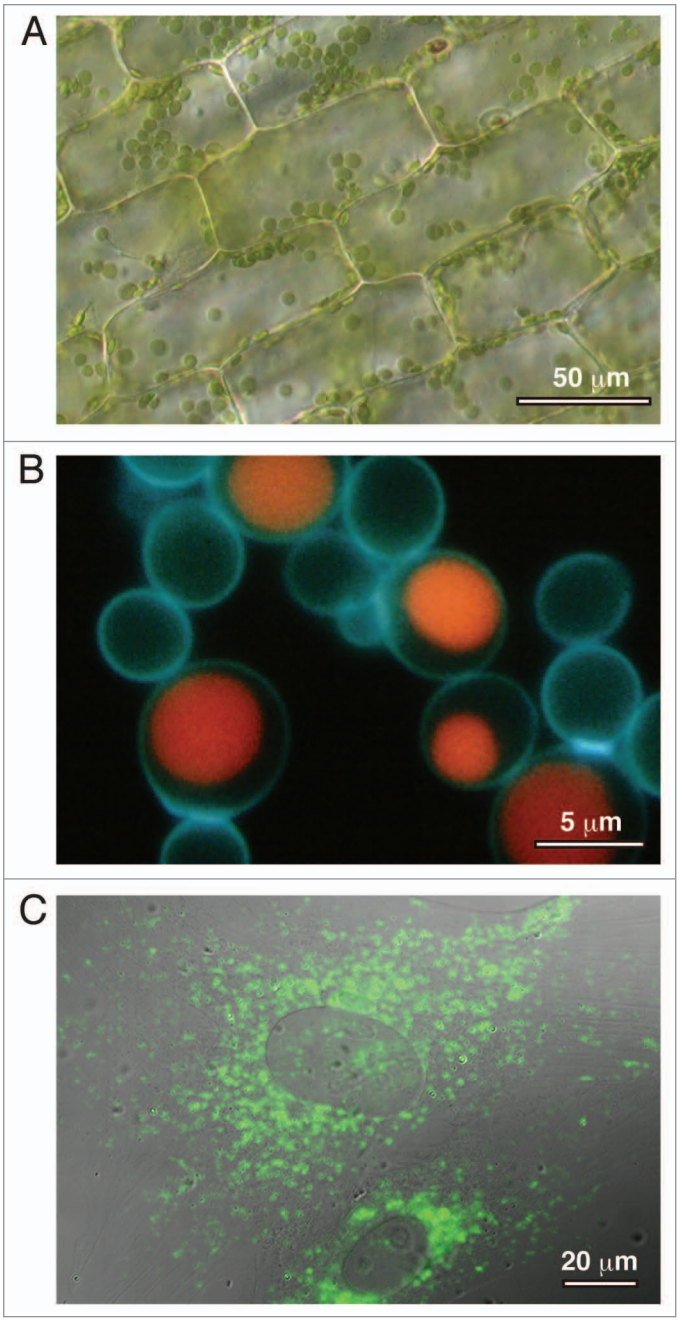

Figure 1. Architecture of lysosomes and vacuoles in plant, yeast, and animal cells. (**A**) Leaf of a flowering aqueous plant, *Egeria densa*. The vacuoles occupy approximately 90% of the cell volume and push the cytoplasmic green chloroplasts toward the cell wall. (**B**) Yeast cells in stationary phase show a single large vacuole accumulating red *ade* pigment (red). (**C**) Mouse embryonic fibroblasts show numerous small compartments with lysosome associated membrane protein 2 (lamp2; green).

tissues. This article reviews the physiological relevance of endocytosis in the mammalian system, especially in the context of cell differentiation and tissue organization that is directly regulated by both activation and silencing of various signal cascades.

Yeast Vam/Vps41 protein is a subunit of the HOPS (homotypic fusion and vacuole protein sorting) tethering complex involved in vacuolar assembly.[12-14] Along with Ypt7, a small GTP-binding protein, the HOPS tethering complex mediates specific membrane recognition between vacuole and both homotypic vacuole as well as endosome. Deletion of either the *VAM2* or *YPT7* genes in yeast results in fragmentation of large vacuoles and partially aberrant localization of vacuolar proteins,[12,15-18] indicating that the HOPS complex and its regulators are required for vacuolar assembly in yeast cells (**Fig. 3**).

The HOPS subunit orthologs and its regulator (Ypt7) are widely distributed in various organisms, including animals and plants.[19-21] The Vam2/Vps41 protein is implicated in the maintenance of nervous system integrity in nematodes, and in fruit fly eye pigmentation. A mutation in *rab7*, a gene encoding the ortholog for *YPT7*, was shown to be responsible for the pathogenesis of Charcot-Marie-Tooth disease type 2B (CMT2B): degeneration of peripheral neurons in humans.[22] These observations suggested that the HOPS proteins influence the physiology of multicellular organisms by controlling endosome/lysosome function. However, the relationship between the cell and tissue phenotypes remains to be established.

Our reverse-genetic studies showed that either *mVam2* or *rab7* functions are required for early embryogenesis in the mouse. The targeted deletion of either gene leads to early embryonic death at peri-gastrulation stages.[23,24] Notably, mutant cells actively proliferate with no obvious degeneration. However, at the systemic level, the embryo morphology is severely affected. In the rab7-deficient embryos, the embryonic mesoderm initially differentiates, but fails to migrate distally to form a primitive streak, a structure essential for establishing the three germ layers. In addition, the embryos lose the extraembryonic mesoderm components such as the allantois and amnion.[24] In contrast, *mVam2*-mutant embryos can organize the extraembryonic mesoderm structures in a normal fashion, but the mutant embryos are defective in differentiation/maintenance of the embryonic mesoderm and the neural ectoderm, showing a severe anterior-truncation phenotype.[23] Although mVam2- and rab7-mutants show the contrasting phenotypes, these studies showed that gastrulation, a key event of mammalian embryogenesis, requires the function of the organelle assembly factors.

Embryonic fibroblasts lacking either *mVam2* or *rab7* functions show severe defects in endocytic transport from early endosome to late endosome, yet internalization of cell surface and extracellular molecules remains largely unaffected. These cellular phenotypes correspond well to those of the yeast mutants (**Fig. 3**). In addition, the lysosome compartments of the mutant fibroblasts are smaller than those of wild-types. The reduced lysosome size is also observed in yeast with *VAM* deletion.[15] However, as described earlier, animal cells exhibit smaller lysosomal compartments; therefore, the morphological phenotype is not apparent in the fibroblasts.

"Vacuole" in Embryonic Tissue and its Function During Gastrulation

Large vacuolar structures in visceral endoderm (VE), an embryonic tissue of pregastrulae, have been described in previous

electron microscopic studies.[25,26] The large vacuoles (apical vacuoles) participate in the endocytic pathway as they are labeled by tracer molecules.[27,28] The apical vacuoles are the terminal organelles of the fluid-phase endocytosis, and accumulate lysosomal membrane proteins, including lysosomal associated membrane proteins (lamps), syntaxin-7, and lysosomal proteinases cathepsins. Thus, apical vacuoles and lysosomes have similar characteristics in animal cells.[23,24,29]

Both mVam2 and rab7 are required for the assembly of apical vacuoles. In the mutant embryos, the VE cells lack the apical vacuoles but accumulate numerous fragmented membrane compartments which are positive for endosomal markers. The mutant cells are capable of taking up cell-surface and extracellular materials and transporting them to the endocytic compartments positive for an early endosome marker sorting nexin 1 (SNX1). However, the mutant cells fail to deliver the engulfed material to lamp2-positive, late endosomal compartments. In addition, endosome-endosome fusion in the mutant cells is severely impaired. Thus, the materials endocytosed at different time points are well separated within the cytoplasm, indicating that the accumulated fragmented vesicles are derivatives of those endosomes.[23,24] These morphological phenotypes associated with the loss of mammalian *vam*$^{-/-}$ function is similar to that found in the yeast vacuolar assembly.

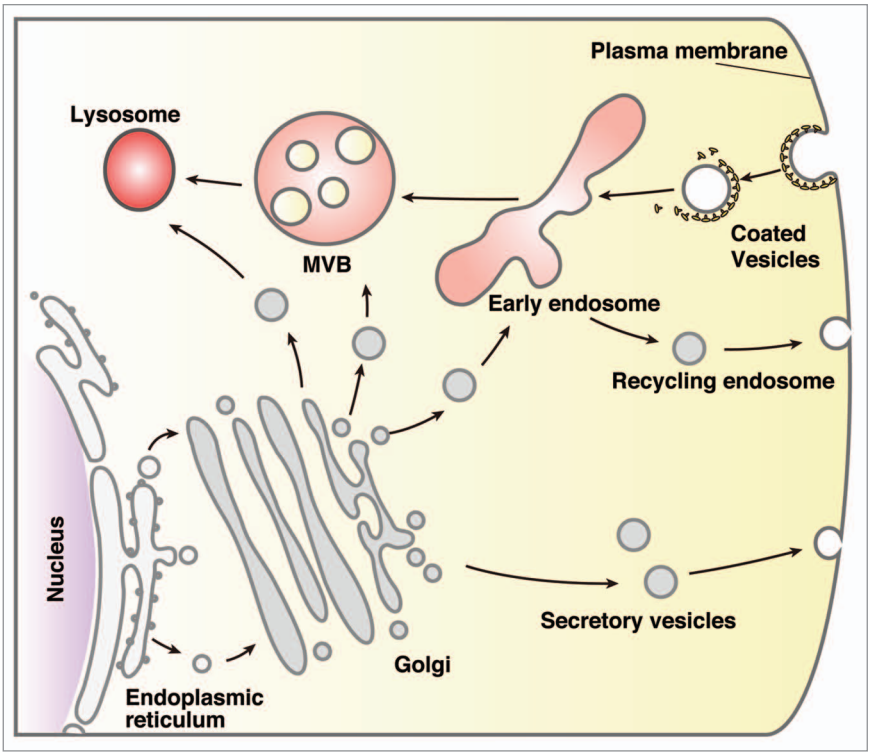

Figure 2. Endocytic and exocytic membrane dynamics composed of various membrane organelles. The early endosomes, which receive the internalized materials, gradually mature, removing some components to be recycled back to the cell surface/extracellular spaces, and become late endosomes. The late endosomes, then acquire a digestive nature including an acidic interior environment and lytic enzymes, and develop into fully matured lysosomes. The endocytic pathway is highly regulated and provides membrane as well as luminal contents to the lysosomes and vacuoles. The vacuolar proteins are synthesized on ribosomes on the endoplasmic reticulum (ER), translocated into the ER lumen, and transported to the Golgi apparatus. This intracellular trafficking route constitutes the early stages of exocytosis. In the Golgi apparatus, the proteins destined for endosomes and vacuoles are sorted out from those to be directed to the cell surface and/or to be secreted. Therefore, the early secretory and vacuolar pathways are the essential processes for vacuole assembly. In addition, the protein sorting in the Golgi apparatus is indispensable for establishing organelle identities and function.

Endocytosis Controls Molecular Signaling and Developmental Patterning

VE is an absorbing epithelium overlying the epiblast (embryo proper) and extraembryonic ectoderm. Rodent embryos obtain nutrition from uterus fluid and the maternal circulation that are separated from the embryo proper by the VE epithelial layer. Early embryogenesis is regulated by multiple cytokines provided from maternal tissues, and transcellular signaling occurs across the VE cells. Obviously, these functions are critically dependent upon endocytosis. Indeed, the VE actively endocytoses various materials from the maternal circulation, and develops large vacuoles between the apical plasma membrane and the nucleus.

The *mVam2*-mutant embryos show severe defects in tissue patterning at the peri-gastrulation stage, as well as defective subcellular morphogenesis. Various signaling cascades such as TGFβ, BMP, Wnt, and FGF signaling, control the spatial organization of embryos. In the *mVam2*-deficient embryos, the spatial and temporal patterns for TGFβ and Fgf activities remain unaffected; however, the BMP signaling is ectopically activated. Mouse embryos establish a specific repertoire of VE at the distal end of the egg cylinder (referred to as distal visceral endoderm; DVE) at embryonic day 5.2 (E5.2). In the subsequent developmental stages, the DVE moves toward the future anterior side to form the anterior visceral endoderm (AVE), which defines the anterior-posterior axis before gastrulation. This axial determination is one of the paramount events of mammalian patterning,[30] and it is regulated by a balance between BMP and TGFβ signaling activity.[31] The BMP signaling components (activated receptors and ligands) are endocytosed and delivered to the lysosomes and apical vacuoles, in fibroblasts and visceral endoderm, respectively, to terminate the signaling. However, in the absence of mVam2, the BMP signaling complex remains activated, leading to excessive BMP signaling, which ultimately results in defective embryo patterning.[23]

Assembly of the Apical Vacuoles: Microautophagy

Delivery of endocytosed materials to the large apical vacuoles involves quite unique membrane dynamics. In most cases, so far

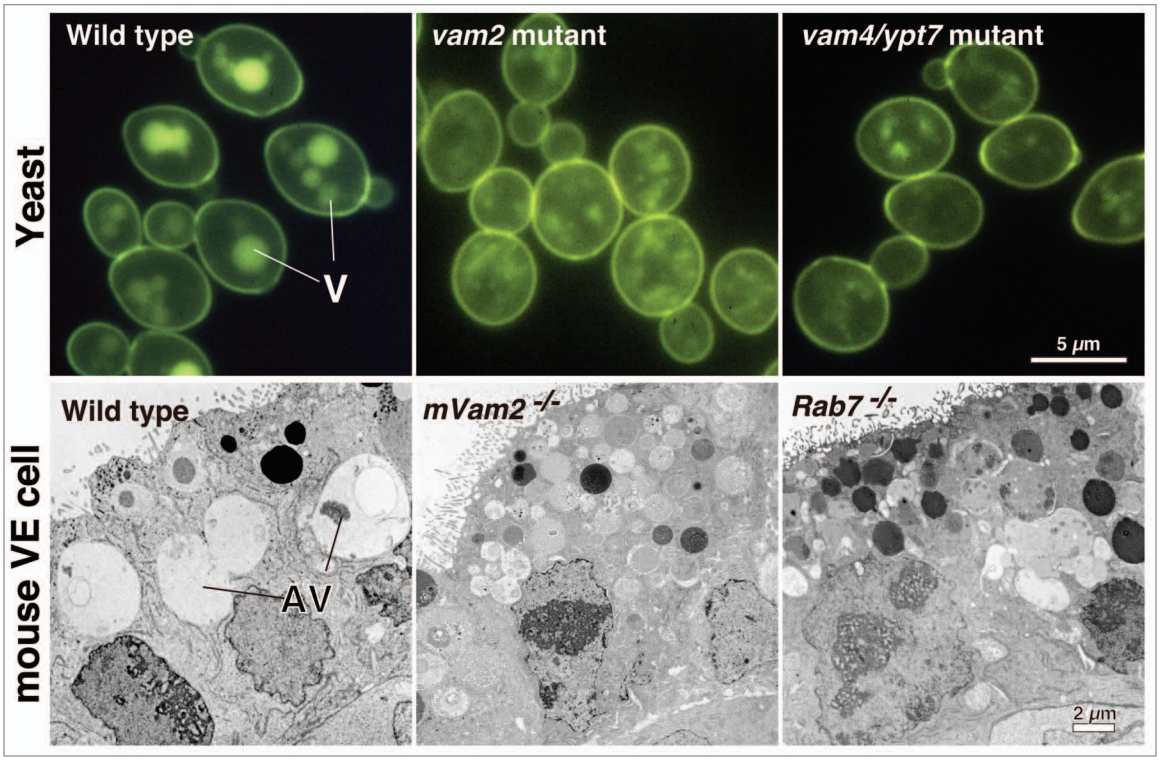

Figure 3. Vacuolar morphology in yeast and mouse visceral endoderm. (Upper panels) *Saccharomyces cerevisiae* harbouring the *vam* mutations were labeled with a fluid-phase endocytic marker, lucifer yellow CH (for vacuoles) and aniline blue WS (for cell wall) and viewed under a fluorescence microscope. The wild type yeast cells exhibit a few large vacuoles (V), however, loss of *VAM* genes causes fragmentation of the vacuoles, where the endocytic markers are accumulated. (Lower panels) Wild-type mouse visceral endoderm (VE) cells show large apical vacuoles (AV) at the apical side of the nucleus. Upon loss of *mVam2* or *rab7* gene function, the apical vacuoles show fragmented morphology.

studied, the mixing of contents of 2 distinct membrane compartments occurs via a fusion of the 2 distinct membranes to form a continuous membrane. However, the large apical vacuoles can be assembled by another scenario, wherein the large apical vacuoles swallow the smaller, pre-vacuolar endosomes entirely, without forming a continuous membrane, and then digest the endosomes within the vacuole.[24] This rather unique membrane process is known as microautophagy, by which peroxisomes and the nucleus are delivered to the vacuoles in yeast cells. In mammalian cells, microautophagy has been less frequently reported, and its relevance has not been elucidated. Rab7 and mVam2 are required for microautophagy in the VE cells, and the loss of either protein results in defective gastrulation. Therefore, the microautophagic delivery of endosomes is pertinent for early embryogenesis.[32]

Large vacuolar structures are often observed in highly differentiated mammalian tissues. The newborn rodent ileum, which is the absorbing epithelium facing the digestive tract, develops large compartments at the apical side of the cytoplasm.[33-35] The ileum of neonates is specialized to absorb milk nutrients, and it develops an intracellular compartment known as the supranuclear vacuole.[36] The supranuclear vacuoles possess several lysosomal proteins and digest the milk endocytosed from the lumen of the digestive tract. These features imply that large subcellular compartments are components of the endocytic pathway, and are most likely involved at the terminal of the pathway.[37,38]

Microautophagy in the ileum has not been well characterized. Because the ileal and visceral endoderm are the absorbing epithelia with high activity for endocytosis, they may share a similar mechanism for vacuolar assembly. Further studies on endocytic membrane dynamics in the ileal cells as well as other epithelium are required to identify the cellular mechanisms that sustain the nutritional and barrier functions of absorbing epithelial tissues. Avian hypoblast cells and germ wall cells often exhibit large vacuolar structures known as the yolk sphere, which contain materials of varying electron density.[39,40] However, membrane dynamics have not been well studied in these tissues. The hypoblast, the equivalent of rodent visceral endoderm in human and chick, plays important regulatory roles in early embryogenesis through active regulation of multiple signal transduction cascades and supplying nutrients.[41] Similar microautophagic membrane dynamics may occur in the hypoblasts for fulfilling the endocytic tasks.

Involvement of Early Endocytic Stages for Embryogenesis

In addition to the protein machinery, lipids also play a central role in determining the organelle identity. Phosphoinositides (PtdIns), enriched in the cytosolic leaflets of organelle membranes, show an organelle-specific distribution and provide the location cue. PtdIns are characterized on the basis of the

number and position of phosphate moieties in the inositol ring. Phosphorylation and de-phosphorylation of PtdIns are catalyzed by specific enzymes which reside in the distinct subcellular compartments, therefore, PtdIns function as specific markers for each subcellular compartment.[42]

Phosphatidyl inositol 3-phosphate [PtdIns(3)P] plays a role in the early stages of the endocytic pathway. PtdIns derived from the Golgi and plasma membrane reach the endosomes via the synthetic and endocytic pathways, respectively, and are modified by the class III PtdIns kinase, Vps34, resulting in the accumulation of PtdIns(3)P in the early endosome. PtdIns(3)P shows high affinity for a Zinc-finger motif known as a FYVE domain and recruits a set of proteins containing the FYVE motif, which include Fab1, YOTB, Vac1, and EEA1 ("FYVE" is an acronym for the names of these proteins). These FYVE containing proteins are indeed involved in the assembly and dynamics of endosomes through interacting with the endosomal membranes.

The function of Vps34 PtdIns 3-kinase is required for mouse development at pregastrulation,[43] implicating PtdIns-mediated membrane dynamics in an essential role in this critical developmental stage. In addition, the *Vps52* gene is required for embryonic growth and organization at the perigastrulation stage.[44] These findings suggest a regulatory link between cellular architecture and global embryonic patterning. In the later developmental stages, proper embryogenesis is dependent on the functions of multiple Vps-related proteins, including SNX13,[45] Hβ58/Vps26,[46,47] CHMP5/Vps60,[48] and Hgs,[49,50] further demonstrating that regulation of membrane trafficking is involved in tissue morphogenesis.

The PtdIns(3)P associated with the early endosomes is modified further by a PtdIns kinase, which adds another phosphate moiety at the 5-position of PtdIns(3)P. This enzymatic reaction leads to consumption of PtdIns(3)P on the endosomes, and accumulation of PtdIns(3,5)P2, which cause loss of EEA1 and rab5 proteins from the transient endosomes. Then by an undetermined mechanism, the late-endosomal rab7 is recruited to the nascent late endosomes. This endosome conversion is dependent on the switch of PtdIns(3)P to PtdIns(3,5)P2 and subsequent replacement of rab5 with rab7. It is an intriguing possibility that rab7 itself, or its binding partners, specifically recognize PtdIns(3,5)P2 on the membrane, although this mechanism has not been fully substantiated yet.

Conversion of PtsIns(3)P to PtdIns(3,5)P2 is mediated by PIPKIII and Fab1, in mammalian and yeast cells, respectively. Loss of this key enzyme results in severe defects in the endosome/vacuole function, including acidification, endocytic and biosynthetic trafficking. One of the most apparent phenotypes is that the lysosome/vacuole shows enlarged morphology. PtdIns(3,5)P2 is required for membrane budding, without which the vacuole/lysosome continue to enlarge in size due to an imbalance of inflow and outflow of the membranes. Alternatively, inward invagination of membranes, known as multivesicular body formation, requires the presence of PtdIns(3,5)P2. Indeed, proteins involved in the MVB formation contain the PtdIns(3,5)P2 recognition motif. In either situation, the production of PtdIns(3,5)P2 or consumption of PtdIns(3)P is essential for maintaining lysosomal/vacuolar integrity.

Again, the importance of PIPKIII and its orthologs is well conserved among the 3 kingdoms. Yeast *fab1* mutants exhibit giant vacuoles.[51] In *Arabidopsis*, 2 PIPKIII enzymes with a PtdIns(3)P recognition motif are encoded by 2 genes, and double mutants show accumulation of aberrantly huge vacuoles in pollen.[52] Alteration of PIPKIII function in somatic cells results in defective endocytosis and vacuolar acidification.[53] PIPKIII is required for the proper assembly of the apical vacuoles in the VE cells of the mouse embryo.[54] PIPKIII mutant embryos develop a gigantic vacuole in the visceral endoderm cells. The abnormally enlarged vacuoles carry lysosomal proteins, including lamp1, suggesting that the biosynthetic pathway from the Golgi apparatus proceeds normally. However, an endocytic tracer like FITC-dextran is not efficiently delivered from the extracellular medium to the abnormally large vacuoles. Importantly, the PIPKIII mutant embryos are defective in gastrulation: they are able to initiate mesoderm differentiation; however, they fail to extend the primitive streak and organize the extraembryonic mesoderm structures, thus the mutant embryos are defective in the progression of the subsequent developmental program.[54] An intestine-specific deletion of the PIKIII function in mouse results in malnutrition after birth and pathological appearance of an ileum that resembles the human Crohn's disease morphology. These findings suggest that the 2 distinct polarized absorptive epithelia, visceral endoderm and intestine, have similar molecular mechanisms for assembling endomembrane systems.[54]

Conclusion

Vacuoles are considered to be rather specific for plants and fungi, however, even animal cells often exhibit lysosomal compartments with a prominent appearance. The physiological and molecular roles of mammalian vacuoles are described in this article. There is increasing evidence that the significant vacuolar/lysosomal architecture is directly reflecting the importance of their function, especially in cell differentiation and tissue-modeling in the early stages of embryogenesis. Cell signaling regulates multiple critical events in all the developmental stages and organogenesis. In the adult animals, tissue regeneration and maintenance are regulated by proper doses of signaling and underlying controlling mechanisms may be involved in pathological complications such as carcinogenesis, immune function, and neural transmission. Future studies on vacuole function and endocytic compartment architecture in highly differentiated and specialized cells in mammals would offer additional insight.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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