The role of ESCRT proteins and phosphoinositides in MVE biogenesis, endocytic trafficking and disease

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"Human consciousness arose but a minute before midnight on the geological clock. Yet we mayflies try to bend an ancient world to our purposes, ignorant perhaps of the messages buried in its long history. Let us hope that we are still in the early morning of our April day."

- Stephen J. Gould (1941-2002)

This thesis is dedicated to my Grandpa, Cornelis 'Oop' Heiman (1918-1995)
- For love and pride, for creating our opportunities

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Abbreviations

In alphabetical order

Akt	-	Serine/threonine kinase (also known as protein kinase B)
ALS	-	Amyotrophic lateral sclerosis
AP complexes	-	Adaptor protein complexes
Atg	-	Autophagy related genes
CCV	-	Clathrin coated vesicle
CHMP	-	Charged multivesicular body protein / Chromotin modifying protein
CI-M6PR	-	Cation-independent mannose-6-phosphate receptor
CME	-	Clathrin mediated endocytosis
ECV	-	Endosomal carrier vesicle
EE	-	Early endosome
EEA1	-	Early endosomal antigen 1
EGF	-	Epidermal growth factor
EGFR	-	Epidermal growth factor receptor
EM	-	Electron microscopy
ER	-	Endoplasmic reticulum
ERK	-	Extracellular-signal-regulated kinase
ESCRT	-	Endosomal sorting complex required for transport
FTD	-	Frontolobular dementia
FTLD-U	-	Frontotemporal lobar degeneration with ubiquitin deposits
FYVE	-	Conserved in Fab1, YOTB, Vac1, EEA1
GFP	-	Green fluorescent protein
GLUE	-	GRAM-like ubiquitin-binding in Eap45
HCRP1	-	Hepatocellular carcinoma related protein 1
HD	-	Huntington's disease
HIV	-	Human immunodeficiency virus
HOPS	-	Homotypic fusion and vacuole protein sorting
Hrs	-	Hepatocyte growth factor regulated tyrosine kinase substrate
IF	-	Immunofluorescence
ILV	-	Intraluminal vesicle
Lamp1/2	-	Lysosomal associated protein 1 and 2
LBPA	-	Lyso-*bis*phosphatidic acid
LC3	-	Microtubule-associated protein 1 light chain 3
LE	-	Late endosome
MAPK	-	Mitogen-activated-protein kinase
MEK	-	Map-Erk Kinase
MHC	-	Major histocompatibility complex
MIM	-	MIT interacting motif
MIT	-	Microtubule interacting and transport
MTMR	-	Myotubularin-related proteins
MVE	-	Multivesicular endosome
NCE	-	Non-clathrin endocytosis
PI3K	-	Phosphatidylinositol 3-kinase
PKB	-	Protein kinase B

PLC-PKC	-	Phospholipase C- protein kinase C
PX	-	Phox homology
PtdIns(3)P	-	Phosphatidylinositol 3-phosphate
PtdIns(3,5)P$_2$	-	Phosphatidylinositol (3,5)-bisphosphate
PtdIns(4,5)P$_2$	-	Phosphatidylinositol (4,5)-bisphosphate
RILP	-	Rab7-interacting lysosomal protein
RTK	-	Receptor tyrosine kinase
siRNA	-	small interfering RNA
SNARE	-	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNX3	-	Sorting nexin 3
STAM	-	Signal-transducing adaptor molecule
TDP-43	-	TAR-DNA-binding protein 43
TEM	-	Transmission electron microscope
TGN	-	Trans Golgi network
Tsg101	-	Tumor susceptibility gene 101
Tnf	-	Transferrin
UBD	-	Ubiquitin binding domain
UEV	-	Ubiquitin E2 variant
UIM	-	Ubiquitin interacting motif
Vps	-	Vacuolar protein sorting

List of Publications

I   Bache K.G., **Stuffers S.**, Malerød L., Slagsvold T., Raiborg C., Lechardeur D., Wälchli S., Lukacs G., Brech A., Stenmark H. (2006)
The ESCRT-III subunit hVps24 is required for degradation but not silencing of the epidermal growth factor receptor. *Mol. Biol. Cell.* 2006 Jun; 17(6):2513-23.

II   Malerød L., **Stuffers S.**, Brech A., Stenmark H. (2007)
Vps22/EAP30 in ESCRT-II mediates endosomal sorting of growth factor and chemokine receptors destined for lysosomal degradation. *Traffic.* 2007 Nov;8(11):1617-29.

III   Filimonenko M., **Stuffers S.**, Raiborg C., Yamamoto A., Malerød L., Fisher E.M., Isaacs A., Brech A., Stenmark H., Simonsen A. (2007)
Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *J. Cell Biol.* 2007 Nov 5;179(3):485-500.

IV   Progida C., Malerød L., **Stuffers S.**, Brech A., Bucci C., Stenmark H. (2007)
RILP is required for the proper morphology and function of late endosomes. *J Cell Sci.* 2007 Nov 1;120(Pt 21):3729-37.

V   **Stuffers S.**, Sem-Jacobsen C., Stenmark H., Brech A. (2008)
Multivesicular endosome biogenesis in the absence of ESCRTs. *Submitted.*

VI   **Stuffers S.**, Stenmark H., Brech A. (2008)
Time-resolved ultrastructural detection of phosphatidylinositol 3-phosphate. *Manuscript.*

Introduction

In order to survive, eukaryotic cells must be in constant communication with the exterior environment. Not only is the cell dependent on the uptake of nutrients and the transmission of metabolic, neuronal and proliferative signals, it also has to protect itself and the organism from microbial invasion. As the vital barrier and the primary interface of the cell with its exterior environment, the limiting membrane is important for the detection of and accurate response to extracellular signals (e.g. from receptors, ions, nutrients and pathogens) and the entry of molecules into the cell, thereby contributing to long-term cell survival.

Endocytosis

Mammalian cells have developed a number of mechanisms to internalize small molecules, macromolecules and particles from the cell surface and target them to specific organelles within the cytoplasm. Collectively, these processes are referred to as "endocytosis", which comprises phagocytosis ("cell eating"), pinocytosis ("cell drinking"), clathrin-dependent receptor-mediated endocytosis and clathrin-independent endocytosis. Endocytosis is an important mechanism of the cell to control uptake of nutrients, regulation of cell-surface receptors, cholesterol homeostasis, antigen presentation, neurotransmission and the maintenance of cell polarity and migration, to name a few. Aberrations in endocytic processes have been associated with a large number of disease processes (Mukherjee et al., 1997). The specific and efficient sorting of membrane proteins and receptors is of great importance for proper performance and hence the long-term survival of the individual cell and the organism as a whole. For instance, nutrient receptors (e.g. transferrin (Tnf) receptor, certain vitamins) are generally recycled, which enables them to undergo several rounds of internalization thereby guaranteeing the cell nourishment. Growth factor receptors (e.g. epidermal growth factor receptors), on the other hand, are sorted in a well-controlled manner in order to regulate their signaling and are preferably degraded at a certain stage. Uncontrolled, extensive signaling from these receptors can lead to overgrowth and cell proliferation, which are hallmarks of cancer. Additionally, pathogens (e.g. viruses and symbiotic microorganisms) and toxins have the ability to exploit endocytic pathways to gain entry into the cell, reach their target and perform their virulent or toxin action.

The detailed understanding of the endocytic machinery and the sorting and trafficking of molecules and pathogens by, and their interaction with this pathway will be important for us to gain insight in the pathogenesis of diseases in which endocytosis is involved. Hopefully our insight will ultimately lead to more specific drug-targeting and treatment as well as improved and cost-efficient disease management.

Clathrin-dependent receptor-mediated endocytosis
Clathrin-dependent receptor-mediated endocytosis is the fastest, highest regulated and most well-characterized of all endocytic pathways. Facilitated by cargo-specific adaptor molecules (e.g. adaptor protein (AP) complexes) (Motley et al., 2003), a clathrin lattice assembles at the plasma membrane (Ahle and Ungewickell, 1989). Integral membrane proteins are concentrated into these specialized clathrin-coated regions, and ligand binding accelerates receptor internalization and triggers the membrane to bud inward and pinch off by the constricting action of dynamin forming clathrin-coated vesicles (CCVs) (Heuser and Kirschner, 1980). CCVs transport their cargo through the cytosol and, after removal of their clathrin-coats, fuse with each other and/or with early endosomes. The early endosome (EE) serves as the first sorting station in the endocytic pathway, segregates cargo destined for recycling to the cell surface and cargo designated for degradation via multivesicular endosomes (MVEs) and late endosomes (LE) to lysosomes. Endosomes are closely interrelated and highly dynamic structures. By recruiting distinctive sets of endocytic proteins to their membranes, endosomes 'mature' and acquire their characteristics of discrete compartments (Rink et al., 2005). They ultimately fuse with lysosomes thereby delivering their contents to the hydrolytic environment of these organelles for degradation (Futter et al., 1996). Even though boundaries between two distinguishable compartments in the endocytic pathway are generally blurred, both at the molecular and at the ultrastructural level, compartments are most commonly divided into early endosomes (EEs), multivesicular endosomes or endosomal carrier vesicles (MVEs/ECVs), late endosomes (LEs) and lysosomes, successively (Box1: Endocytic compartments in mammalian cells). Communication between endocytic organelles requires actin- and microtubule-based motility and it has long been proposed that endosomes influence their own motility via direct interaction with motor-proteins (Matteoni and Kreis, 1987; Nielsen et al., 1999).

BOX 1 Endocytic compartments in mammalian cells

Early endosomes (EEs)

Morphology

Compartment of highly complex and pleiomorphic organisation; cisternal, tubulo-vesicular; diameter of the irregular shaped vacuole lies between 200-500 nm; vacuole is electron lucent with only a few ILVs; electron-dense coats sometimes visible.

Functional description

Highly dynamic structures with high homotypic fusion capability; first sorting station to which endocytosed molecules are delivered; rapid sorting of cargo for recycling or degradation.

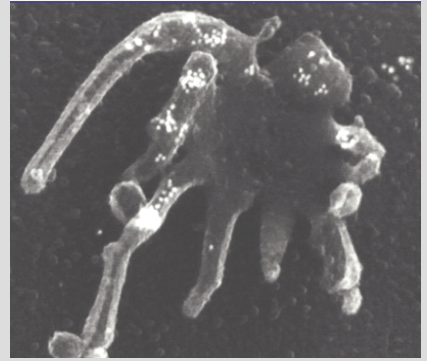## Position in the cell

Mostly in the cell periphery

Abundant proteins and lipids

High: Rab5, PtdIns(3)P, EEA1, Rab4
Low: ESCRT-0, Clathrin
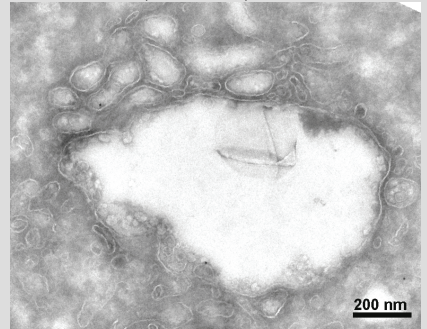Other: ARF6, cellubrevin, Rab4, COP-I, recycling receptors

Entry of EGF-receptor / cargo

2-5 minutes (uptake at 37°C, depending on cargo and cell type)

pH 6.0-6.8

EE, J. Heuser, Nat. Cell Biol. 2001

200 nm

Enlarged, but typical EE (cryo-immuno-EM)
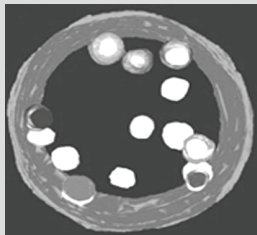
J.L. Murk et al.,PNAS 2003, EM tomography
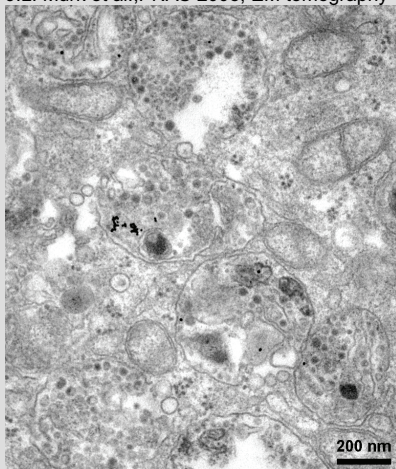
Multivesicular endosomes (MVEs) / Endosomal carrier vesicles (ECVs)

Morphology

Regularly shaped and spherical; containing large amounts of densely packed ILVs; MVE diameter 300-400 nm, diameter of ILVs 50-80 nm

Functional description

Intermediate sorting station for cargo on the way to degradation; in certain cell types, cell specific functions, e.g. antigen presentation

Position in the cell

More towards lysosomes (perinuclear), throughout the cytoplasm

Abundant proteins and lipids

High: ESCRT-0, clathrin, PtdIns(3)P (on ILVs), CD63, cholesterol
Low: Rab5, Rab7, ESCRT-I, LBPA (on ILVs)

Entry of EGF-receptor / cargo

10-12 minutes (uptake at 37°C depending on cargo and cell type)

pH < 6.0

200 nm

MVEs in different stages of maturation (EM)

Late endosomes by conventional EM; LEs can contain both ILVs and onion-shaped membranes. (By conventional EM)

Late endosomes (LEs)

Morphology

Pre-lysosomal compartments; more pleiomorphic in shape than MVEs, average size 200-500 nm; can contain both multivesicular regions and onion-like internal membranes

Functional description

Sorting stations,e.g. for mannose-6-phosphate receptor to cycle back to TGN after delivering lysosomal enzymes, and for MHC-II molecules

Position in the cell

Perinuclear, concentrated near the microtubule organizing center

Abundant proteins and lipids

High: Rab7, ESCRTs, CD63, LBPA, LAMP1, LAMP2, Rab9
Low: PtdIns(3)P, cholesterol
Other: CI-MPR (NB: is also found in TGN)

Entry of EGF-receptor / cargo

15-30 minutes (uptake at 37°C, depending on cargo and cell type)

pH: around 5.5

Lysosomes

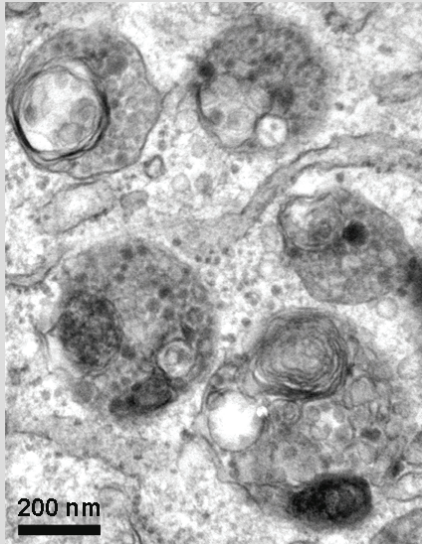## Morphology

Globular shape; lumen is electron-dense, onion-like internal membranes; can contain amorphous material and to some extent ILVs in different stages of degradation

Functional description

Lysosomes carry hydrolases that degade nucleotides, proteins, lipids, phospholipids, and remove carbohydrate, sulfate, or phosphate groups from molecules; can undergo homotypic fusion and heterotypic fusion with LEs; functional overlap with LEs

Position in the cell

Mostly perinuclear

Abundant proteins and lipids

High: CD63, LBPA, LAMP1, LAMP2
Other: Hydrolytic enzymes

Entry of EGF-receptor / cargo

30 minutes and longer (uptake at 37°C)
pH: 4.5-5
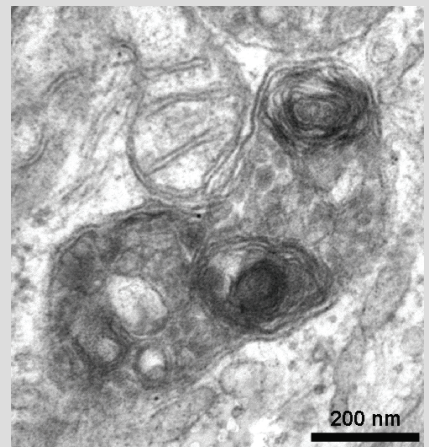
Lysosome, containing onion-shaped internal membranes; in this image fusing with another compartment .(By conventional EM)

NOTE The endocytic pathway is a system of great plasticity and dynamics. It is impossible to recognize the structures solely on the basis of morphology or position in the cytoplasm. Endosomes are closely interrelated, so even when specific markers are used it can be difficult to distinguish between compartments. It is worth noting that MVEs and LEs are sometimes referred to as the same structures in scientific publications. Not mentioned here are the **recycling endosomes** (positive for Rab 11 and Rab8, Tnf receptor containing) which provide an indirect and slower recycling route from the EE to the plasma membrane. These data were collected from a number of leading articles in the field. (References: (Futter et al., 1996; Griffiths et al., 1989; Gruenberg and Maxfield, 1995; Gruenberg, 2001; Gruenberg and Stenmark, 2004; Kornfeld and Mellman, 1989; Mellman, 1996; Sachse et al., 2002a; Tjelle et al., 1996)

As they mature, endosomal compartments become more acidic, with EEs having a pH of 6-6.8, late endosomes 5-6, and lysosomes 4.5-5 (Mellman, 1996). This pH-dependent gradient enables the cellular machinery to manage cargo-specificity, as it allows receptor-ligand complexes, with their individual molecular profiles, to dissociate at different stages of the endocytic pathway (Skarpen et al., 1998).

Receptor signaling and sorting

Receptor tyrosine kinases (RTKs) and their ligands (e.g. growth factors) play essential roles in a wide variety of cellular processes. Ligand-binding to RTKs initiates conformational alterations that trigger the dimerization of receptors and sequentially enables transmission of signals across the plasma membrane in order to activate the receptors' intrinsic tyrosine kinase activity. Several tyrosine residues in the cytoplasmic tail of the receptor are phosphorylated, thereby creating binding sites for adaptor protein (AP) complexes and additional regulatory proteins. Furthermore, alterations at the receptor-site initiate a large number of concurrent biochemical cascades that transmit extracellular signals through the cytosol to target organelles. The three best characterized signaling pathways that are induced by activation of RTKs are i) the Ras-mitogen-activated-protein kinase (Ras-MAPK) pathway, which translocates signals to the nucleus for the phosphorylation of specific transcription factors leading to changes in gene expression and protein activity, ii) the phosphatidylinositol 3' kinase-protein kinase B (PI3K-PKB/Akt) pathway, which mediates proliferation and cell survival, and iii) the phospholipase C-protein kinase C (PLC-PKC) pathway, which regulates the release of intracellular Ca^{2+} from the endoplasmic reticulum (ER) and the activation of protein kinase C (PKC) . Additionally, signaling cascades can be activated after the internalization of receptor-ligand complexes into endosomal compartments (Vieira et al., 1996), which allows more precise temporal and spatial signal regulation and the specific targeting of signaling complexes to their site of action (Miaczynska et al., 2004b).

Ligand-induced receptor endocytosis and subsequent down-regulation is an important mechanism for preserving the fragile balance between the 'positive' signals that the cell benefits from and the 'excessive' or 'negative' signals that can potentially harm the cell and result in disease. Concomitant with receptor activation, ligand binding initiates a multi-step process that ultimately leads to receptor degradation and signal attenuation. Numerous

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features are required for the receptor to be trafficked correctly and mammalian cells have developed a vast amount of elaborate mechanisms for this purpose. Molecular tags are required for accurate targeting, as well as adaptor proteins that can recognize both the cargo-tag and the target. Several structurally distinct tags (signals) have been discovered that initiate rapid internalization and target cargo for clathrin-dependent endocytosis and degradation in lysosomes (Bonifacino and Traub, 2003). An example of an RTK that is targeted for lysosomal degradation in order to regulate its signaling processes is the epidermal growth factor receptor (EGFR). Endocytosis of the EGFR is the most frequently used model for studying mechanisms and kinetics of the endocytic pathway, its morphology and its signaling processes (Carpenter and Cohen, 1976).

The precise mechanism that regulates the internalization of EGFR remains to be elucidated, but the C-terminus of the EGFR contains a number of motifs that are capable of interaction with the clathrin AP-2 complex which provides a link to clathrin coated pits (Sorkin et al., 1995). In addition, one of the major interacting proteins of the adaptor Grb2, c-Cbl, has been associated with the regulation of EGFR internalization and degradation (Levkowitz et al., 1998). c-Cbl is an E3 ubiquitin ligase and interaction with phosphorylated tyrosine residues of the cytoplasmic tail of EGFR leads to its activation and mediates ubiquitination of the receptor by recruitment of E2 ubiquitin conjugated enzymes (Levkowitz et al., 1999). Even though ubiquitination of EGFR has been shown to be non-essential for its internalization into the cell (Huang et al., 2006), the attachment of mono-ubiquitin to one or several lysine residues (multi-ubiquitination) in the cytoplasmic tail of the receptor is known to serve as an important intracellular sorting signal for the degradative pathway (Barriere et al., 2006; Haglund et al., 2003; Huang et al., 2006; Huang et al., 2007; Levkowitz et al., 1999; Umebayashi et al., 2008).

CCVs containing EGF-receptor complexes un-coat and rapidly fuse with EEs. Due to the mildly acidic pH in EEs, EGF-receptor complexes do not dissociate substantially (Sorkin et al., 1988). Whereas a fraction of the complexes recycle from the EE back to the cell surface, mono-ubiquitinated EGF-receptor complexes are internalized from the limiting membrane of the endosome and accumulate in the intraluminal membranes of MVEs, known as intra-lumenal vesicles (ILVs). Once incorporated into ILVs the EGF-receptor complexes are most likely prohibited from recycling and are destined for rapid proteolysis by way of fusion of the MVE with primary lysosomal vesicles (Futter et al., 1996) (Figure 1).
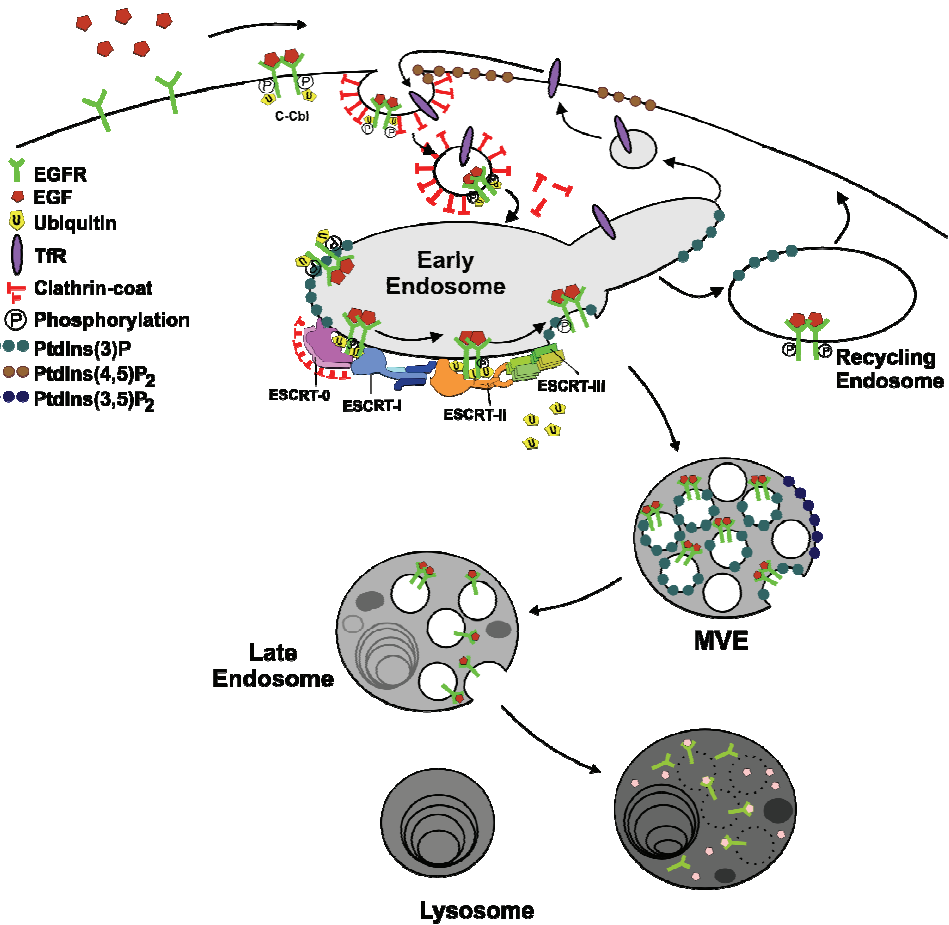
Figure 1   EGFR trafficking
Ligand binding to monomeric EGFR leads to dimerization and autophosphorylation. Subsequently, c-Cbl is phosphorylated and recruited to the receptor where it induces monoubiquitination. Ubiquitinated EGFRs accumulate in clathrin coated vesicle (CCV) and are internalized. Before fusing with the early endosome (EE), the vesicle loses its clathrin coat. In the EE receptors are seggregated: whereas unubiquinated receptors recycle to the plasma membrane, ubiquitinated receptors trigger an endosomal sorting cascade consisting of ESCRT-0, -I, -II and –III, which ultimately triggers multivesicular endosome (MVE) formation and the sorting of receptor-complexes into intraluminal vesicles (ILVs). Before the ILVs form, ESCRT complexes are recycled to the cytoplasmic pool. MVEs mature into late endosomes (LEs), fuse with lysosomes and deliver the EGFR-containing ILVs to the proteolytic interior of the lysosome where they are degraded. (Adapted from **PAPER III**)

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More recent studies have shed light on the mechanisms of sorting of ubiquitinated membrane proteins for lysosomal degradation. A key role in this process is allocated to a multimeric protein complex that contains several ubiquitin-binding domains (UBDs) and will be discussed in the following section.

The ESCRT machinery

Lysosomal targeting of ubiquitinated endocytic cargo is partly mediated by the endosomal sorting complex required for transport (ESCRT) machinery, which is well-conserved across the eukaryotic lineage (Leung et al., 2008). Initially, a subset of at least 13 class E vacuolar protein sorting (*vps*) proteins required for proper endosomal function and MVE biogenesis were discovered in yeast (Odorizzi et al., 1998) (Table 1). Depletion of either one of these proteins in yeast leads to the formation of class E compartments, multicisternal prevacuolar compartments (analogous to LEs in mammalian cells) (Raymond et al., 1992). The class E *vps* proteins were found to assemble and form three protein complexes, named ESCRT-I (Katzmann et al., 2001), ESCRT-II (Babst et al., 2002b) and ESCRT-III (Babst et al., 2002a), which interact with each other and associate transiently with the endosomal membrane to carry out their function. Subsequent studies in mammalian cells and yeast have led to the identification of a fourth complex that functions upstream of ESCRT-I, and is termed ESCRT-0 (Bache et al., 2003b; Bache et al., 2003a; Bilodeau et al., 2003; Katzmann et al., 2003; Williams and Urbe, 2007). In addition, a number of ESCRT-associated proteins were discovered that mostly associate with ESCRT-III and play a role late in the machinery (Williams and Urbe, 2007).

The ESCRT machinery is recruited to endosomal membranes in an ordered manner. In the presence of mono-ubiquitinated EGFR the activity of the early endosomal protein Rab5 is modulated (Barbieri et al., 2004) which leads to the formation of a micro-environment enriched in active Rab5 and recruited Rab5 effectors (Zerial and McBride, 2001). One of the effectors clustering in these Rab5 domains is the Class III PI(3)K catalytic subunit hVps34, which phosphorylates the lipid phosphatidylinositol in the 3' position and synthesizes phosphatidylinositol 3-phosphate [PtdIns(3)P] on the early endosomal membrane (Christoforidis et al., 1999). Recruitment of ESCRT-0 to the endosomal membrane is initiated by PtdIns(3)P via an interaction with the FYVE-motif of the ESCRT-

0 subunit Hrs (Raiborg et al., 2001). The current model, also referred to as the 'conveyer belt model' (Figure 2), proposes ESCRT-0 to recruit ESCRT-I to the membrane through interactions between the P(S/T)AP motif of Hrs and the UEV (ubiquitin E2 variant) domain of the ESCRT-I subunit Tsg101 (Bache et al., 2003b; Katzmann et al., 2003; Lu et al., 2003). ESCRT-I, in turn, recruits ESCRT-II to the machinery, presumably via the interaction of Vps28/ESCRT-I with Vps36/ESCRT-II. Additionally, the Vps36 subunit of ESCRT-II has the intrinsic capacity to bind to PtdIns(3)P through its GLUE domain (Slagsvold et al., 2005). Ultimately, the Vps25 subunits of ESCRT-II facilitate recruitment of the ESCRT-III components to the membrane via interaction with Vps20/ESCRT-III. The ESCRT-III subunits have the ability to cycle on and off membranes forming polymeric filaments on the surface of endosomes (Babst et al., 2002a). Dissociation of the ESCRTs from the membrane is facilitated by the AAA+ ATPase Vps4 and allows the recycling of ESCRT proteins (Scheuring et al., 2001).

Complex	Human Homologue	Yeast Homologue	Domain/Motif	Function in endocytic traffic
ESCRT-0	HRS STAM1, STAM2	Vps27 Hse1	UIM, FYVE, PSAP, VHS UIM, VHS, SH3	PtdIns3P, Tsg101, cargo interaction Interaction with Hua1 and Rsp5
ESCRT-I	TSG101 VPS28 VPS37A, B, C, D MVB12A, B	Vps23 Vps28 Vps37 Mvb12	UEV, CC, Steady-box CC	Cargo and Hrs interaction Interaction with Vps36 (ESCRT-II)
ESCRT-II	EAP30; SNF8 EAP20 EAP45	Vps22 Vps25 Vps36	CC, WH PPXY, WH GLUE / NZF, WH	Interaction with CHMP6 (ESCRT-III) Cargo, PtdIns3P and ESCRT-I interaction
ESCRT-III	CHMP6 CHMP4A, B, C CHMP2A, B CHMP3	Vps20 Snf7; Vps32 Vps2, Did4 Vps24	Charged, CC, MIM Charged, CC, MIM Charged, CC, MIM Charged, CC, MIM	Interaction with Vps25 (ESCRT-II) Membrane deformation, inward vesiculation
Associated	Vps4A, B; SKD1 ALIX/AIP1 CHMP5 CHMP1A, B LIP5 Nedd4 UBPY AMSH ? ?	Vps4 Bro1; Vps31 Vps60 Did2; Vps46 Vta1 Rsp5 Doa4 Ubp7 Hua1 Rup1	AAA ATPase, MIT Bro1 Charged, CC Charged, CC 2xMIT C2, WW, HECT UBP, Rhod MIT, JAMM	ESCRT disassembly and recycling UBPY recruitment, ESCRT-III interaction ESCRT-III like protein ESCRT-III like protein Positive regulator of Vps4 Cargo ubiquitination (ubiquitin ligase) Cargo deubiquitination (enzyme) Cargo deubiquitination (enzyme) Links Rsp5 to Hse1 Complex with Rsp5 and Ubp2

Table 1   Class E Vps proteins and complexes

Ubiquitinated cargo is initially selected at the endosomal membrane by Hrs and STAM1/2 of ESCRT-0, which both contain ubiquitin interacting motifs (UIMs) (Raiborg et al., 2002; Urbe et al., 2003). A recently discovered splice variant of Eps15, Eps15b, also contains a UIM and is associated with Hrs, possibly providing additional binding sites for ubiquitinated cargo (Roxrud et al., 2008). In addition, Hrs recruits clathrin which forms

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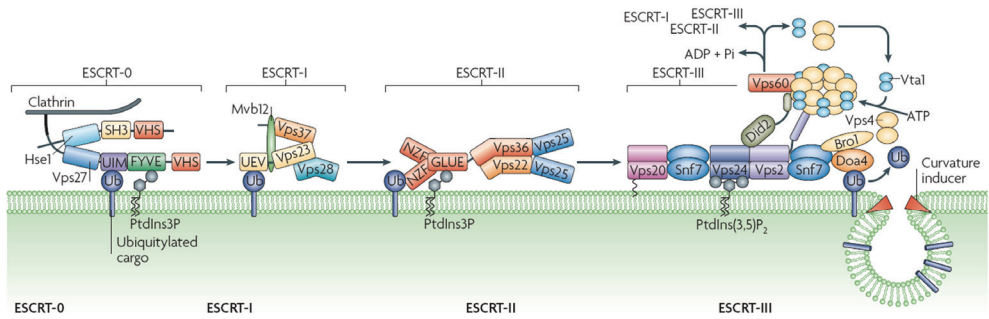
Figure 2   A schematic outline of the conveyer belt model
(Adapted from Williams R.L. and Urbé S., Nat Rev Mol Cell Biol. 2007 May; 8(5):355-68). The FYVE domain of Hrs/Vps27 binds PtdIns(3)P on the endosomal membrane and leads to the recruitment of the other components of ESCRT-0. The UIM domains of Hrs/Vps27 and STAM/Hse1 recognize and bind ubiquitinated cargo for sorting into ILVs of MVEs. ESCRT-0 recruits ESCRT-I to the membrane by interactions with the UEV domain of Tsg101/Vps23. Membrane-bound ESCRT-I recruits ESCRT-II via interactions between Vps28 and Vps36. In mammalian cells the GLUE domain of Vps36 binds to endosomal PtdIns(3)P and to ubiquitinated cargo. Membrane-bound ESCRT-II recruits the downstream ESCRT-III complex via interactions between Vps25 and Vps20. Additionally, Vps24 is capable of binding to endosomal PtdIns(3,5)P$_2$.ESCRT-III forms a polymeric lattice on the membrane and recruits the de-ubiquitinating protein UBPY/Doa which removes ubiquitin-moieties from the cargo. The ESCRT-III lattice is disassembled by the ATPase Vps4 in complex with Vta1 before sorting of cargo into the ILV.

bilayered coats on endosomes which leads to the formation of Hrs micro-domains (Sachse et al., 2002b). These clathrin coats are different to the ones at the plasma membrane and have a crucial function in the clustering of Hrs on the endosomal membrane and hence the specialized recognition of ubiquitinated membrane proteins and their efficient sorting (Raiborg et al., 2006). Also ESCRT-I and -II bind ubiquitinated cargo: through the ubiquitin E2 variant (UEV) domain in the Tsg101 subunit of ESCRT-I and the GLUE domain of Vps36 in ESCRT-II (Slagsvold et al., 2005). How exactly the ubiquitinated cargo that is trapped in Hrs micro-domains ends up in ILVs of MVEs remains unclear. It has been proposed that ubiquitinated cargo is "handed off" from ESCRT-0 sequentially to ESCRT-I, -II, and –III complexes (Hurley and Emr, 2006). Despite the slight variety of UBDs, all motifs recognize the same hydrophobic patch on the ubiquitin surface implying that one ubiquitin moiety can interact with only one ESCRT at a time. Consequently, it seems more likely that cargoes cluster on the endosomal membrane, recruiting multiple ESCRTs and concentrating the machinery in micro-domains (Hurley and Emr, 2006). Whereas ESCRT-0, ESCRT-I and ESCRT-II have the capability to interact with ubiquitinated proteins, ESCRT-III recruits the de-ubiquitinating enzymes UBPY/Doa4 and AMSH that remove ubiquitin

moieties from the cargo before sorting into ILVs of MVEs (Agromayor and Martin-Serrano, 2006; Row et al., 2007; Zamborlini et al., 2006). Besides a role in the sorting of membrane proteins, the ESCRT machinery appears to be essential for the inward vesiculation and thus the biogenesis of MVEs in yeast. In higher eukaryotes, however, it is not clear whether these processes are exclusively dependent on an interaction with the ESCRT machinery and how sequential recruitment results in inward vesiculation.

The conveyer belt model gives a coherent summation of the available data (Figure 2). However, a number of aspects of the organization of cargo recognition and MVE biogenesis cannot yet be explained, and therefore alternative models have recently been proposed (Nickerson et al., 2007).

Phosphoinositides

The lipid composition of a typical cell membrane is much more complex than originally thought. Membranes are composed of a perplexing variety of 500-1000 different lipid species which can assemble into dynamic domains of distinctive composition. By recruiting and concentrating accurate protein congregations, these dynamic domains enable the lipid bilayer to regulate a large number of cellular processes, such as intracellular transport and signaling events (Kobayashi et al., 1998a).

Phosphoinositides are derivatives of the lipid phosphatidylinositol that can undergo rapid cycles of phosphorylation and dephosphorylation at the 3', 4' and 5' positions of their inositol sugar head group. This modification process is dynamically regulated by lipid kinases and phosphatases, respectively, and mediates highly localized changes in the levels of phosphoinositides. In this way the temporal and spatial regulation of effector proteins is carefully controled. Phosphoinositides are present in small quantities but have crucial regulatory functions in guiding membrane trafficking and in cell signaling as regulators of nuclear functions, cytoskeletal dynamics, and signal transduction (Di Paolo and De Camilli, 2006; Roth, 2004). The seven different phosphoinositides are located in distinct membrane domains, where they associate with specific trafficking and signaling events (LeRoy and Wrana, 2005) (Figure 1). The phosphoinositide PtdIns(4,5)P_2, for instance, facilitates the

recruitment of adaptor proteins that initiate the formation of clathrin-coated pits at the plasma membrane, whereas PtdIns(3)P plays an important role in endocytic membrane trafficking and autophagy (Rusten and Stenmark, 2006). The different effector proteins of phosphoinositides are recruited to phosphoinositides via lipid binding domains that specifically recognize the head group of the phosphoinositide and are responsible for a wide variety of regulatory functions to be carried out.

PtdIns(3)P is restricted to the endocytic pathway and is most abundant on EEs and on the internal membranes of MVEs (Gillooly et al., 2000). Studies in yeast have shown that PtdIns(3)P is transported to the vacuole (the yeast equivalent of the lysosome) for turnover (Wurmser and Emr, 1998). The effector proteins of PtdIns(3)P contain either a FYVE (for: conserved in Fab1, YOTB, Vac1 and EEA1) (Gaullier et al., 1998; Patki et al., 1998) or a Phox homology (PX) domain (Cheever et al., 2001; Ellson et al., 2002; Simonsen and Stenmark, 2001; Song et al., 2001; Xu et al., 2001). Binding of these domains to PtdIns(3)P is very specific and even though the exact function of each of the 38 predicted FYVE-domain-containing proteins remains to be elucidated, many of these proteins seem to play a role in membrane trafficking, regulation of cytoskeletal function and signal transduction (Hayakawa et al., 2007). PX domain-containing proteins, of which more than 100 different proteins are predicted to exist in the human genome, have a wide variety of functions and include t-SNAREs for tethering, mammalian sorting nexins involved in membrane trafficking events, kinases implicated in cell survival, and proteins that play a critical role in the assembly of the neutrophil oxidase complex.

Regulation of the levels of phosphoinositides by phosphatases and kinases is evidently important for endocytic traffic and proper cell functioning. Mutations in myotubularin-related proteins (MTMRs), a family of proteins recently shown to specifically dephosphorylate PtdIns(3)P (Blondeau et al., 2000; Taylor et al., 2000) lead to severe disorders such as myotubular myopathy and demyelinating neuropathy.

Aims of the study

The main objective in this thesis was to gain insight in how ESCRT proteins, both individually and in complex, and the phosphoinositide PtdIns(3)P, are involved in MVE biogenesis, the endocytic trafficking of cell surface receptors, and in cell processes related to pathogenesis.

The more specific aims of the individual papers were as follows:

Paper I: **To elucidate the role of Vps24 (ESCRT-III) in the trafficking and signaling of EGFRs.**
The role of Vps24/ESCRT-III in EGFR trafficking and silencing and MVE biogenesis had not yet been investigated in mammalian cells. Therefore we were interested to analyze the functions of Vps24/ESCRT-III in endosomal sorting and receptor signaling in mammalian cells and to compare these to the important role of Tsg101/ESCRT-I in these processes.

Paper II: **To elucidate the role of Vps22/ESCRT-II in the signaling and degradative sorting of ubiquitinated receptors.**
In yeast ESCRT-II mutants, translocation of cargo from the limiting membrane into ILVs is found to be inhibited. As mammalian ESCRT-II was originally characterized as a complex modifying RNA polymerase-II (a non-endosomal function), we were interested to study whether the function of ESCRT-II in membrane traffic is conserved from yeast to mammalian cells and whether it plays a role in receptor signalling and membrane dynamics.

Paper III: **To analyze the possible role of ESCRTs in the autophagic clearance of protein aggregates.**
ESCRTs are known to play an important role in the endocytosis of ubiquitinated membrane proteins and the proper formation of MVEs. Yet, little is known about the correlation of functional MVEs and autophagic degradation, and the possible role of ESCRTs therein. With mutations of an

ESCRT-III subunit (CHMP2B) recently associated with two neurodegenerative phenotypes, and autophagy shown to be an important pathogenic mechanism in neurodegenerative diseases, we were interested to study the possible link between ESCRTs and autophagy and to relate this to neuronal pathogenesis.

Paper IV: **To establish whether RILP is required for endocytic trafficking of receptors and endosome formation.**

RILP interacts with two ESCRT-II subunits as well as with the dynein-dynactin motor complex, possibly providing a mechanistic link between endosome motility and MVE biogenesis. We were interested to study the roles of endogenous RILP in the endocytic sorting of receptors and the formation of MVEs.

Paper V: **To elucidate the involvement of the ESCRT machinery in MVE biogenesis.**

ESCRT proteins appear to be essential for the biogenesis of MVEs in yeast. However, it is not clear whether ESCRT-independent pathways of MVE biogenesis exist in higher eukaryotes. Therefore we were interested to study how multiple depletions of key subunits of all ESCRTs influences MVE formation.

Paper VI: **To analyze the spatial and temporal localization of PtdIns(3)P in the endocytic and autophagic pathway by immunofluorescence and electron microscopy.**

PtdIns(3)P plays an important role in the recruitment of various effector proteins in the endocytic pathway. We were interested to functionally track PtdIns(3)P, in combination with an endocytic cargo and a subset of endosomal markers.

Summary of the included papers

An outline of the included publications is presented in the following sections. The full articles are printed at the end of this thesis.

Paper I: The ESCRT-III subunit hVps24 is required for degradation but not silencing of the epidermal growth factor receptor

Since it had previously been demonstrated that the ESCRT machinery is greatly conserved from yeast to man (Williams and Urbe, 2007) an important and logical follow-up was to elucidate and map the functions of the ESCRTs and their individual components in mammalian cells. Expression of an N-terminal fragment of mouse Vps24 (a component of ESCRT-III) in human cells was shown to inhibit ligand-induced EGFR degradation (Yan et al., 2005), the same effect observed when ESCRT-I subunit Tsg101 (Bache et al., 2003a; Bishop et al., 2002) was depleted. However, the effects of Vps24/ESCRT-III depletion on EGFR degradation, signaling and the relationship between the two had yet to be clarified. In this study we wanted to compare the roles of the 'upstream/early' ESCRT-I protein Tsg101 and the 'downstream/late' ESCRT-III protein Vps24 in these processes. Additionally, since ESCRTs are thought to be coupled mechanistically to the process of membrane invagination (Bache et al., 2003a; Gruenberg and Stenmark, 2004; Lloyd et al., 2002), we were interested to study the consequence of Vps24/ESCRT-III depletion on MVE morphology. We found that Vps24 is essential for ligand-induced degradative EGFR trafficking, as EGFR is retained in EEs in the absence of Vps24 and down regulation is delayed. Even though activation of the MAP kinase pathway downstream of activated EGFR was sustained when Tsg101 was depleted, the depletion of Vps24 had no such effect. This demonstrates that while both have a similar effect on EGFR degradation, the roles of ESCRT-I/Tsg101 and Vps24/ESCRT-III are distinct, with Vps24 not being required for silencing of EGFR signaling. We wanted to address two potential mechanisms that contribute to the regulation of receptor signalling, i.e. i) the dissociation of the ligand-receptor complex by a more acidic environment (low pH) (Skarpen et al., 1998) and ii) the internalization of EGFR into ILVs of MVEs which disrupts signaling into the intracellular space and targets the ligand-receptor complex for degradation by fusion with lysosomal hydrolases. Indeed, by using fluorescence ratio imaging we found that depletion of Tsg101 and Vps24 differentially

affects the pH of EGF-containing endosomes. Whereas similar to the control cells, FITC-EGF localized mostly to compartments with a pH of 4.5 in Vps24 depleted cells, FITC-EGF was more often localized to compartments with a pH of 6.4 in cells depleted of Tsg101. This suggests that Tsg101 depletion causes a delay in endocytic trafficking which hinders the normal accumulation of EGF in acidic endosomes, possibly leading to sustained activation of MEK1/2 and ERK1/2. Next, we studied the morphology of endosomal compartments in cells depleted for Vps24 by electron microscopy and observed an accumulation of significantly smaller MVEs which did contain ILVs, but were fewer and less homogenous in shape. EGFR (labeled by immuno-gold) mostly localized to intraluminal membranes even when Vps24 was depleted, suggesting that sorting of EGFR does not require Vps24. In conclusion, we found that two ESCRT-subunits, which are both required for degradative trafficking, play differential roles in the silencing of EGF-activated MAP-kinase activity. In addition to their linear trafficking association, these proteins may have more extensive cellular functions which are organized in a less linear fashion.

Paper II: Vps22/EAP30 in ESCRT-II mediates endosomal sorting of growth factor and chemokine receptors destined for lysosomal degradation

Even though yeast ESCRT-II mutants are known to inhibit the sorting of cargo from the limiting membrane into ILVs (Babst et al., 2002b), ESCRT-II appeared to be dispensable for degradation of ubiquitinated major histocompatibility complex (MHC)-I molecules in mammalian cell (Bowers et al., 2005). Also, in contrast to other ESCRTs which are known to play a role in HIV budding, HIV-1 appears to utilize a pathway in which ESCRT-II is not involved (Langelier et al., 2006). Hence, we were interested to determine the role of ESCRT-II in EGFR signaling and trafficking by depleting cells of the ESCRT-II subunit Vps22/EAP30 using siRNA. We found that the levels of the two additional ESCRT-II subunits, Vps25 and Vps36, were strongly reduced in cells depleted of Vps22, indicating that Vps22 is crucial for the integrity and stability of the ESCRT-II complex. Vps22, like other ESCRTs proteins, localized to EEs and MVEs that are involved in the trafficking of internalized EGFR. In addition, we found that ESCRT-II was required for the efficient degradation of EGF and EGFR in lysosomes. As a possible explanation for this, we observed that in cells depleted of Vps22/ESCRT-II the sorting of ubiquitinated cargo into the ILVs of MVEs was significantly inhibited, a result that we verified both by quantitative immunoelectron microscopy and by utilizing a newly developed biochemical assay for ILV

sorting. When we studied the morphology of Vps22-depleted cells by electron microscopy, we observed, besides a redistribution of EGFR to the limiting membrane of MVEs, a more general alteration of endosome morphology. Two subtypes of EGFR-containing endosomes could be distinguished: one of which consisted of compartments resembling EEs, whereas the second subtype consisted of smaller structures with a multivesicular appearance. Since the requirement of ESCRT-II for cargo degradation may be restricted to RTKs, we studied the degradative sorting of another ubiquitinated receptor, the G-protein coupled chemokine receptor CXCR4, which was shown to require both Hrs and Vps4 for efficient down-regulation (Marchese et al., 2003). We showed that the degradation of CXCR4 was strongly inhibited in cells depleted of ESCRT-II, indicating that the ESCRT-II complex is involved in degrading a wider range of ubiquitinated receptor families. Since we had previously found a distinct role of ESCRTs in regulating EGFR signaling (**PAPER I**) we wanted to determine the role of Vps22/ESCRT-II in this process. Interestingly, we found that, similar to Vps24 (ESCRT-III) and in contrast to Hrs (ESCRT-0) and ESCRT-I, Vps22/ESCRT-II was not involved in silencing of EGFR signaling. We concluded that ESCRT-II plays an important role in the degradative sorting of at least two important receptor families and that Vps22-depletion leads to the delayed transport of EGFR along the endocytic pathway. Even though each ESCRT is required for the efficient trafficking of EGFRs, only ESCRT-0 and -I are needed for silencing of EGF signaling from endosomes.

Paper III: Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease

ESCRTs are known to play an important role in the endocytosis of ubiquitinated membrane proteins and, at least in part, in the biogenesis of MVEs. Mutations of the ESCRT-III subunit CHMP2B was recently associated with two neurodegenerative phenotypes, frontotemporal dementia (FTD) (Skibinski et al., 2005) and amyotrophic lateral sclerosis (ALS) (Parkinson et al. 2006). In addition, autophagy has been shown to play an important role in the pathogenic mechanisms in neurodegenerative diseases. We were interested to investigate a possible link between these factors. We found a dramatic accumulation of non-endosomal ubiquitin-aggregates in cells depleted of Tsg101/ESCRT-I and Vps24/ESCRT-III, which labeled positive for p62 and Alfy, two proteins associated with autophagic degradation. We hypothesized three likely mechanisms to cause formation of these aggregates, i.e. increased protein synthesis, decreased proteasome activity and decreased

autophagic degradation, and investigated these possibilities. We found that, whereas protein synthesis and proteasome activity remained unchanged, autophagic degradation was strongly inhibited in cells depleted for Tsg101 and Vps24. To further study this we used a double-tagged mCherry-GFP-LC3 construct (Pankiv et al., 2007) which allowed us to confirm that transport of the autophagy-marker LC3 to acidic lysosomes was inhibited in ESCRT-depleted cells. Since this is most likely caused by an inhibition in the formation of autolysosomes, our data indicated that functional MVEs were required in autophagy. We were interested to study i) the precise stage of the pathway in which the impediment occurred and ii) further characterize the ubiquitin-positive, autophagic aggregates. We utilized electron microscopy analysis and found that clusters of autophagosomes formed and that GFP-LC3- and LBPA-positive amphisomes were more numerous in cells depleted of Tsg101 and Vps24. This suggests an impediment at a later stage in the autophagic pathway, most likely an inhibition of the fusion between amphisomes and lysosomes. The ubiquitin-, p62-positive aggregates found in ESCRT-depleted cells were either membrane-free or contained clusters of small vesicular-tubular elements and larger structures of typical endosomal morphology. Previously, Holm et al. described the presence of ubiquitin- and p62-positive cytoplasmic inclusions in the hippocampus and neocortex of FTD patients who had a mutation in the ESCRT-III CHMP2B gene (Holm et al., 2007). We overexpressed the CHMP2B[intron5] mutant construct in cells and investigated whether these inclusions were comparable to the aggregates we found in our ESCRT-depleted cells. Indeed, we found similar p62- positive aggregates in cells expressing the CHMP2B[intron5] mutant. In addition, overexpression of the CHMP2B[intron5] mutant in HeLa-GFP-LC3 cells led to increased levels of GFP-LC3, indicating that autophagic degradation is inhibited by the expression of CHMP2B mutants. To further characterize the possible link between ESCRT-proteins, autophagy and neurodegenerative disease we studied i) TDP-43, the major ubiquitinated protein associated with FTD and ALS, and ii) expanded Huntingtin (Htt) polyQ inclusions associated with Huntington's disease. We found i) TDP-43 to accumulate in the ubiquitin- and p62-positive aggregates which were formed in ESCRT-depleted cells and ii) a strong reduction in the clearance of Htt polyQ aggregates Vps24-depleted cells, indicating that ESCRT-III is required in this process. Our data showed, for the first time, an important link between TDP-43-positive inclusions, Htt-polyQ aggregate clearance and therefore neurodegenerative disease, and proteins involved in MVE formation and autophagy.

Paper IV: RILP is required for the proper morphology and function of late endosomes

During their maturation into MVEs and simultaneously LEs, endocytic compartments are trafficked along microtubules from the periphery of the cell towards the perinuclear region. However, the possible link between the maturation process and the motility of these compartments has not yet been elucidated. Recently, a potential candidate arose, when the Rab7-interacting lysosomal protein (RILP) was shown to interact both with the ESCRT-II subunits Vps22 and Vps36 and with the dynein-dynactin motor complex (Progida et al., 2006; Wang and Hong, 2006). We wanted to investigate whether endogenous RILP is indeed involved in endocytic trafficking of receptors by studying the effects of siRNA-mediated depletion of RILP on several facets of cellular functions. We found that RILP and Vps22 are most likely to exist in a complex *in vivo*, since depletion of RILP caused co-depletion of Vps22 and vice versa. In addition, depletion of RILP caused the degradation of EGFR to be delayed, and EGFR to accumulate in EEA1-positive early endosomes. This indicated that, like Vps22, RILP is required for trafficking of EGFR from EEs to LEs. Shuttling of the cation-independent mannose-6-phosphate receptor (CI-M6PR) between the trans-Golgi network (TGN) and endosomes was not affected by depletion of RILP, although lysosomal degradation of CI-M6PR was markedly inhibited. Endocytosis and recycling of the nutrient receptor transferrin (Tnf), on the other hand, was not affected by depletion of either RILP or Vps22, indicating that the blockage of transport out of EEs was not of a general nature. The data obtained so far, suggested that RILP was mostly involved in the degradative sorting of receptors, most likely by regulating the degradative function of LEs, perhaps by mediating the fusion of LEs with lysosomes. RILP-depleted cells showed a strong increase in labelling to the late endosomal markers LBPA and Lamp1, suggesting that RILP was involved in the degradation of late-endosomal proteins and lipids, possibly by controlling the maturation- and/or fusion-process of LEs. Interestingly, when comparing RILP-depleted and control cells at a higher resolution (by electron microscopy), we found a striking difference in the morphology of LEs. Not only were endosomes clearly clustered, they were nearly devoid of intraluminal membranes. We concluded that RILP is required for i) trafficking of EGFR, and most likely other (ubiquitinated) receptors, for lysosomal degradation and ii) the biogenesis of MVEs, perhaps by coordinating this process with dynein-mediated endosome motility.

Paper V: Multivesicular endosome biogenesis in the absence of ESCRTs

Previous studies have shown that the depletion of single components of the individual ESCRTs leads to defective degradative trafficking of ubiquitinated cargo and alterations in endosome/MVE morphology. We wanted to elucidate the function of the ESCRT machinery in MVE biogenesis and EGFR sorting by multiply-depleting cells for key subunits of all ESCRTs simultaneously thereby supposedly leaving the cells devoid of their ESCRT machinery. We found that quadruple depletion (4xKD) of Hrs/ESCRT-0, Tsg101/ESCRT-I, Vps22/ESCRT-II and Vps24/ESCRT-III caused a dramatic alteration in the morphology of the endocytic pathway, which we defined to 3 main types of distinct structures: i) strongly enlarged MVE-like structures, either containing few ILVs or resembling 'fusion products' of several MVEs, ii) large areas of densely packed membrane folds and sheets and iii) autophagosomes and amphisomes. The morphology was most similar to the morphology found in cells single depleted of Tsg101/ESCRT-I, but was much more dramatically altered and featured the enlarged 'collection' MVEs which were only observed in 4xKD cells. Additionally, ILVs in enlarged and normal-sized MVEs showed an altered morphology, with a larger diameter and less homogenous shape. Even if ILVs were still formed in 4xKD cells, EGFR was not sorted into these ILVs and remained predominantly on the limiting membrane of endocytic structures. Observing such dramatic structural rearrangements, we questioned whether endogenous endocytic proteins were properly distributed in the endocytic compartments. We investigated this both by IF and EM using a range of endocytic and associated markers, and found that even though early and late endocytic markers were strikingly redistributed, components of the endocytic pathway remained clearly differentiated. Next, we utilized EM quantitative analysis to investigate the extent to which normal endocytic compartments could still be formed after quadruple-depleting cells of the ESCRT machinery. We found that the formation of EGF-induced endocytic structures was inhibited, as we had previously observed in singly depleted cells, but that EGF-independent MVEs were still formed, even in the absence of the ESCRT machinery. Our data supported the idea of the existence of distinct subpopulations of MVEs and we were interested to find hallmarks of these diverse subgroups. We investigated two candidates for the regulation of MVE biogenesis, the late-endosomal lipid LBPA (Matsuo et al., 2004) and the sphingolipid ceramide (Trajkovic et al., 2008), in combination with our quadruple depletion. However, results only led to the speculation that these two candidates most likely play an important role in MVE biogenesis, but that besides these, additional mechanisms for the formation of ILVs do exist. In conclusion, we found that even though the morphology of

endocytic structures is dramatically altered in cells depleted of all ESCRTs, components of the endocytic pathway stay clearly differentiated and MVEs can still be formed. The formation of ILVs still occurs, indicating that ESCRT-independent pathways for ILV formation exist. Our data support a model in which ESCRT proteins, besides their function in the sorting of membrane proteins, play an important role in the regulation of membrane dynamics. Importantly, our data also support the hypothesis that distinct subgroups of MVEs exist.

Paper VI: Time-resolved ultrastructural detection of phosphatidylinositol 3-phosphate

PtdIns(3)P plays an important role in the recruitment of various effector proteins in the endocytic pathway. FYVE domains bind PtdIns(3)P efficiently *in vitro* but isolated FYVE domains often fail to localize to endosomes when expressed in cells (Hayakawa et al., 2004). The ultrastructural localization of phosphoinositides is difficult to study. However, it is of interest to clarify the functional localization of PtdIns(3)P in the endocytic pathway in the context of distinctive mechanisms for ILV formation and subpopulations of MVEs, as it has previously been reported that PtdIns(3)P and LBPA localize to distinct endosomal compartments (Gillooly et al., 2000). Therefore we attempted to functionally track the distribution of PtdIns(3)P at the ultrastructural level during a defined time window using a monomeric dimerizable FYVE probe (GFP-2xFv-FYVE(Hrs)), which binds with high affinity to PtdIns(3)P after induced dimerization. We found that the probe localized to early and late endocytic compartments according to the time period of dimerization, which indicated that PtdIns(3)P is turned over via the endocytic machinery. Dimerization of the probe in combination with EGF stimulation led to clustering of mainly early endocytic compartments, and left most of the probe localized to the limiting membrane of these endosomes. We observed that even though there was little colocalization between the probe and LBPA in the clusters, it appeared that in some more matured profiles GFP-2xFv-FYVE(Hrs) did colocalize with LBPA. Importantly, this suggested that PtdIns(3)P and LBPA positive ILVs can reside within the same types of MVEs. The clustered endosomes did contain coats positive for the PtdIns(3)P-binding protein Hrs, indicating that recruitment of Hrs to the endosomal membrane is not impeded by the binding of GFP-2xFv-FYVE(Hrs). In addition, we investigated the localization of PtdIns(3)P to the autophagic pathway and found very little colocalization with the autophagic marker p62. We concluded that the dimeriser-inducible probe could potentially be useful for the time-resolved detection

of PtdIns(3)P at the ultrastructural level, but that its effect on endosome morphology after EGF stimulation need to be taken into account.

Discussion

In the articles presented in this thesis we have studied the roles of ESCRT proteins and PtdIns(3)P in the biogenesis of MVEs, endocytic traffic and in cell processes associated with disease. During the past decade, remarkable progress has been made towards the elucidation of mechanisms by which the ESCRT machinery operates. The many components of the ESCRT machinery are largely identified and structural studies have provided insight in how the complexes are assembled. Even though a number of significant questions regarding detail of these mechanisms remain unanswered, the role of ESCRTs in the lysosomal targeting of ubiquitinated receptors is relatively well-established. The functional role of ESCRTs in the biogenesis of MVEs, however, is rather obscure to date. Owing to increased focus on the topic and innovations in methods and adjoining fields, recent progress has been made in understanding some facets of inward vesiculation and MVE biogenesis, and we now seem to be on the verge of a major breakthrough. Nevertheless, joint efforts in further research will be needed to elucidate the mechanisms of this intriguing process. Exciting as well is the more recently established link between the molecular mechanisms of ESCRTs and pathogenesis. Its association with diseases highlights the importance of basic medical research and founds one of the major motivations to studying the ESCRT machinery.

MVE biogenesis

In yeast, depletion of any of the proteins of the ESCRT machinery results in the formation of typical class-E compartments (Odorizzi et al., 1998) which has led to the hypothesis that the ESCRT machinery plays a crucial role in the inward budding and accordingly the biogenesis of MVEs. Although the hypothesis has been carried through into higher eukaryotes, no direct evidence has been found so far and our understanding of the mechanisms driving inward vesiculation and MVE biogenesis remains limited. Yet, it is easy to imagine that with the higher complexity of the organism, additional mechanisms may have evolved.

In mammalian cell cultures, single depletion of either one of the ESCRTs causes a marked and specific alteration in endosome morphology. Depletion of Hrs/ESCRT-0 results in the formation of enlarged MVEs which contain few ILVs (Bache et al., 2003a; Razi and Futter, 2006), while depletion of Tsg101 leads to a profound rearrangement of the early endosome, in which enlarged vacuoles either seem to fold into multicisternal structures or tubulate (Doyotte et al., 2005). Moreover, even though MVE biogenesis still occurs, Tsg101 depletion leads to a strong reduction of the MVEs that were formed in response to stimulation with EGF (Razi and Futter, 2006). In **PAPER I** and **II** we show that depletion of the ESCRT-III subunit Vps24 or the ESCRT-II subunit Vps22, respectively, causes a less dramatic but significant change in endosome morphology. Vps22/ESCRT-II-depleted cells contain an increased number of early endosomal structures in addition to the clusters of small MVEs that are comparable to the smaller and clustered MVEs found in Vps24-depleted cells. Interestingly, even though depletion of ESCRT subunits leads to clear morphological changes, MVE biogenesis still occurs and the ultimate requirement of ESCRTs in this process remains unclear. In **PAPER V** we therefore aimed to optimize conditions for efficient removal of the ESCRT machinery by depleting cells of key subunits of all four ESCRTs simultaneously, using siRNA oligonucleotides against Hrs, Tsg101, Vps22 and Vps24. It has been established that ESCRTs lose their integrity and that the expression levels of the other subunits are decreased when either of these key subunits are depleted (**PAPER II** and (Babst et al., 2002a; Bache et al., 2003b; Bache et al., 2004)). We therefore assumed that by depleting its key subunits we abolished the function of the entire ESCRT machinery. The morphology of endocytic structures is dramatically altered in ESCRT-depleted cells and, interestingly, depletion of the ESCRT machinery inhibits the formation of EGF-induced endocytic structures, but still allows the biogenesis of EGF-independent MVEs. Our data support a model in which ESCRTs proteins, besides their role in membrane protein sorting, play an important role in the regulation of membrane dynamics. Importantly, our results indicate that distinct subpopulations of MVEs exist and that mainly EGF-induced MVE biogenesis depends on the ESCRT machinery. ILV formation, albeit not abolished, appears to be altered in ESCRT depleted cells illustrated by the observation of fewer and less densely packed ILVs that are not as homogenous in shape and size (**PAPER V**). Of significant relevance to alternative mechanisms for MVE biogenesis, an alternative pathway for MVE sorting was found for the melanosomal protein Pmel17, which is sorted into ILVs by a mechanism that is independent of ubiquitination and

insensitive to functional inhibition of ESCRT-0 and -I (Theos et al., 2006). Moreover, in a recent study an alternative mechanism for the formation of exosomes, the equivalents of ILVs in "secretory" MVEs, was described, which operates independently of the ESCRT machinery but requires the sphingolipid ceramide (Trajkovic et al., 2008).

Mechanisms of inward vesiculation

Data found to date are in favor of the hypothesis that ILVs are formed through distinctive mechanisms, some of which are ESCRT-dependent whereas others are ESCRT-independent (Falguieres et al., 2008; Gillooly et al., 2001; Kobayashi et al., 1998b; Mobius et al., 2003; Pons et al., 2008; Trajkovic et al., 2008) and **PAPER V**). The main candidates that are currently proposed to play a role in the inward vesiculation of endosomes are ESCRTs, PtdIns(3)P, sorting nexin 3 (SNX3), lyso-*bis*phosphatidic acid (LBPA), ceramide and cholesterol, and will be discussed in this section.

ESCRTs Primarily components of ESCRT-III are thought to play a role in ILV formation. In humans, the ESCRT-III family consists of 11 proteins that are recruited from the cytosol to the endosomal membrane where they assemble into large detergent insoluble polymers (Babst et al., 2002a; Williams and Urbe, 2007). ESCRT-III associates with the endosomal membrane through at least one or a combination of the following interactions: i) the Vps20/CHMP6 subunit of ESCRT-III binds to Vps25 of ESCRT-II (Yorikawa et al., 2005), ii) the ESCRT-associated protein AIP1/Alix can interact with Snf7/CHMP4 of ESCRT-III and Tsg101 of ESCRT-I possibly bridging the two proteins under certain conditions (Katoh et al., 2003; von Schwedler et al., 2003), and iii) its intrinsic ability to bind to membranes, regulated for instance by the autoinhibitory sequences located near the C termini (Muziol et al., 2006; Shim et al., 2007; Zamborlini et al., 2006) and/or the interaction of Vps24/CHMP3 subunit with PtdIns(3,5)P_2 (Whitley et al., 2003). A recent study demonstrates crucial roles for Tsg101 and AIP1/Alix as positive and negative regulators, respectively, of ILV formation *in vitro* (Falguieres et al., 2008) which suggests the importance of these two proteins in recruitment and potentially removal of ESCRT-III. Ultimately, the AAA+ ATPase Vps4 associates with the polymeric lattice through interaction of its N-terminal microtubule interacting and transport (MIT) domain with conserved sequence motifs, MIT interacting motifs (MIMs), located at the C-termini of CHMP1-3 of ESCRT-III, to disassemble the complex (Scott et al., 2005; Stuchell-Brereton

et al., 2007). The appropriate combination of Vps4 with its associated protein Vta1 and ESCRT-III subunits strongly initiates Vps4 ATPase activity, which is required for disassembly of the coat in which cargo is clustered and is thought to be the main thermodynamic driving force for the formation of ILVs (Azmi et al., 2008; Hurley and Yang, 2008; Sachse et al., 2004). However, the factors that cause membrane deformation and ultimately fission of the vesicle from the limiting membrane are still missing from the current model. A number of studies have recently shed light on the mechanisms by which ESCRT proteins potentially drive inward budding and hence, at least in part, are responsible for MVE formation (Falguieres et al., 2008; Ghazi-Tabatabai et al., 2008; Hanson et al., 2008; Lata et al., 2008; Teis et al., 2008). The ESCRT-III proteins hSnf7-1/CHMP4A and hSnf7-2/CHMP4B are reported to assemble into circular membrane-associated polymers that can drive deformation of the membrane to which they are attached (Hanson et al., 2008). Likewise, *in vitro* studies demonstrate Snf7 and Vps24 to spontaneously form ordered polymers which are disassembled by Vps4 (Ghazi-Tabatabai et al., 2008; Lata et al., 2008). The hSnf7/CHMP4 polymers are proposed to control the distribution of proteins in the membrane and thereby contributing to define the content and generate the ILV (Hanson et al., 2008). One problem in the search for the right candidate(s) for inward vesiculation is that all proteins known to induce membrane deformation act in the opposite direction, towards the cytoplasm, driving "positive" membrane curvature (McMahon and Gallop, 2005). However, the circular shape of the ESCRT-III polymers could potentially promote "negative" membrane curvature instead and consequently drive inward vesiculation. ESCRT-III contains two functionally distinct subcomplexes: the Vps20/CHMP6-Snf7/CHMP4 subcomplex is known to bind to the membrane, whereas the Vps2/CHMP2-Vps24/CHMP3 subcomplex binds to Vps20/CHMP6-Snf7/CHMP4 subcomplex and serves to recruit additional components and facilitate protein sorting (Babst et al., 2002a). The Snf7/CHMP4 is the major ESCRT-III subunit in the complex. It is reportedly seven times more abundant than Vps20/CHMP6 and at least twice more abundant than Vps24/CHMP3 and Vps2/CHMP2, which exist in a 1:1 stochiometry (Teis et al., 2008). In a recent model the Vps25 subunit of ESCRT-II is proposed to induce the conformational changes at the N-terminal of Vps20/CHMP6 that are required in order for Vps20/CHMP6 to nucleate the formation of Snf7/CHMP4 polymers on the membrane. Snf7/CHMP4-subunits continue to polymerize until a yet unidentified signal of sufficiency is provided which initiates the recruitment of Vps24/CHMP3, and possibly Vps2/CHMP2 concurrently, to the circular membrane-associated polymer in order to 'cap' and terminate the reaction (Figure 3).

Membrane deformation could be initiated either by the polymer itself or by the ubiquitinated cargo that is concentrated within the circular array. No significant amounts of ESCRT-III components are found on the intraluminal membranes of MVEs, which suggests the polymeric ring to form at the 'neck' of the imminent vesicle, and to disassemble before the vesicle pinches off. This newly proposed working model provides a nice framework, yet, further research is required to bridge the gaps and elucidate the functional role of ESCRTs in ILV formation and the mechanistic link between MVE biogenesis and sorting and signalling events.
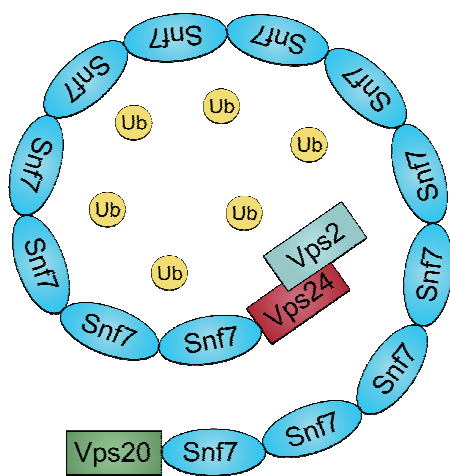
Figure 3. **Model for membrane deformation by circular arrays of ESCRT-III protein filaments.** Conformational changes at the N-terminal of Vps20/CHMP6 nucleate the formation of Snf7/CHMP4 polymers on the membrane. Polymerization of Snf7/CHMP6 continues until a signal of sufficiency is provided which initiates the recruitment of Vps24/CHMP3, possibly in complex with Vps2/CHMP2, to the array to 'cap' and terminate the reaction. Membrane deformation could be caused by the circular array or by the ubiquitinated cargo that is concentrated within the circular array. The exact mechanisms for promoting membrane deformation and inward vesiculation and how these are functionally coupled to sorting and signalling events are not yet known.

PtdIns(3)P / SNX3   PtdIns(3)P localizes to early endosomal membranes and is known to play a role in endocytic membrane traffic and the sorting of signaling receptors (Gillooly et al., 2000; Petiot et al., 2003). In addition, PtdIns(3)P is thought to control the formation of ILVs, at least in part, by recruiting FYVE- and PX domain containing proteins to the membrane of endosomes (Bright et al., 2001; Fernandez-Borja et al., 1999; Fili et al., 2006; Futter et al., 2001). The exact mechanisms behind this are not yet fully understood, but inhibition of PtdIns3-kinase with wortmannin, and consequently inhibition of PtdIns(3)P synthesis, causes a reduction in the formation of ILVs that seem unable to pinch off from the limiting membrane of MVEs (Fernandez-Borja et al., 1999; Futter et al., 2001). Moreover, the PX domain-containing protein SNX3 was recently found to be required for the formation of ILVs (Pons et al., 2008). The molecular mechanism by which SNX3 regulates membrane invaginations remains to be elucidated but is most likely to be indirect,

by the recruitment of yet additional proteins. Interestingly, SNX3 appears not to be involved in the degradative sorting of EGF receptor. Hrs, on the other hand, is essential for lysosomal targeting but is demonstrated to be dispensable for MVE biogenesis and PtdIns(3)P appears to control the complementary functions of Hrs and SNX3 in sorting and MVE biogenesis (Pons et al., 2008). How PtdIns(3)P decides which of the effector proteins to recruit at what time is not yet understood. The concentration of the phosphoinositide on the membrane along with the particular binding affinity of the effector protein or the availability and/or concentration of additional proteins for recruitment could be factors involved in this selection process. Nevertheless, these data highlight the crucial regulatory functions and high flexibility of phosphoinositides.

LBPA   The concept of ILVs of distinctive origins is strongly supported by data from previous studies demonstrating that at least two populations of internal membranes with different lipid composition can be separated from each other (Kobayashi et al., 2002). One of these fractions primarily contains 2-2'-LBPA, a phospholipid that was later shown to drive the pH-dependent, spontaneous formation of multivesicular liposomes (Matsuo et al., 2004). Its inverted cone-shaped structure and seemingly intrinsic capacity to stimulate membrane invagination make 2-2'-LBPA an ideal candidate to regulate internal membrane biogenesis. The invagination process is likely to be regulated by proteins *in vivo* and to date the main candidate seems to be the ESCRT-associated protein AIP1/Alix, which was reported to regulate the organization of LBPA-containing endosomes (Matsuo et al., 2004) and control ILV formation by acting as a negative regulator (Falguieres et al., 2008). Whether AIP1/Alix plays an exclusive role in combination with LBPA or also interacts with other ESCRT proteins in the formation of ILVs is not known. Interestingly, however, contrary to AIP1/Alix Tsg101 is demonstrated to be a positive regulator of the inward vesiculation and the two proteins appear to jointly coordinate the process of vesicle formation in a controled fashion (Falguieres et al., 2008). LBPA localization is restricted to the intraluminal membranes of MVEs and does not colocalize with PtdIns(3)P or cholesterol but appears to reside in a distinctive pool of ILVs ((Gillooly et al., 2000; Matsuo et al., 2004; Mobius et al., 2003) and **PAPER VI**). LBPA is poorly degradable which has led to the proposal of a functional mechanism in which LBPA-containing ILVs transport engulfed cargo back to the limiting membrane of the MVE, instead of towards the lysosome, in a process called "back-fusion" (LeBlanc, I et al., 2005). PtdIns(3)P-containing ILVs, on the other hand, would deliver their cargo to the lysosome for degradation. This model seems to

be an interesting alternative, however, with the current approaches it has not yet been possible to characterize the biochemical and biophysical properties of distinctive intraluminal membranes. It is worth noting that to date no LBPA has been detected in yeast, which indicates that higher organisms have evolved more complex membrane systems for higher efficacy and more specificity.

Ceramide   A recent study by Trajkovic et al. describes a mechanism for the formation of ILVs which requires the sphingolipid ceramide and is independent of the ESCRT machinery (Trajkovic et al., 2008). Ceramide is synthesized by the sphingomyelinase-dependent hydrolytic removal of the phosphocholine moiety of sphingomyelin. Cargo is demonstrated to segregate into distinct microdomains on the endosomal membrane, which are proposed to contain high concentrations of shingolipids from which ceramides are synthesized. Owing to its inverted cone-shaped structure, ceramide may, similar to LBPA, induce spontaneous negative curvature (Goni et al., 2005). Exosomes, the equivalents of ILVs in "secretory" MVEs (Fevrier and Raposo, 2004), are enriched in ceramide and cells depleted of neutral sphingomyelinase 2 (nSMase2) show reduced exocytosis of exosomes (Trajkovic et al., 2008).

Cholesterol   Cholesterol is one of three major components of lipid bilayers. It plays a crucial role in membrane organization and dynamics and is known to modulate the function of various membrane proteins either by specific modular interaction or by altering the membranes physical properties (Ikonen, 2008). Besides its requirement for the formation of clathrin-coated pits (Rodal et al., 1999), membrane cholesterol is necessary for the structure and function of caveolae and microdomains or rafts, which function as sorting platforms in endocytosis (Ikonen and Parton, 2000; Mukherjee and Maxfield, 2004). Mammalian cells attain the cholesterol required for the membrane synthesis through receptor-mediated endocytosis of cholesteryl esters-containing low-density lipoproteins (LDL). In lysosomes the cholesteryl esters are hydrolyzed to free cholesterol, which subsequently is used for membrane synthesis. Cholesterol levels are tightly regulated by means of feedback mechanisms and the endocytic pathway plays an important role in cellular cholesterol homeostasis. Membrane cholesterol is unevenly distributed amongst the diverse organelles of the cell and is especially abundant in the plasma membrane (Liscum and Munn, 1999). In compartments of the endocytic pathway cholesterol is demonstrated to be most abundant in the membranes of ILVs (Mobius et al., 2003). There it appears, like PtdIns(3)P, to localize

differently in MVEs than LBPA, again indicating that endosomes along the degradative pathway may contain at least two types of intraluminal membranes with different lipid compositions (Mobius et al., 2003). Whether cholesterol colocalizes with any of the other aforementioned candidates for ILV formation is not known and would be of interest to investigate. However, owing to its cone-shaped structure, cholesterol prefers positive curvature (Huttner and Zimmerberg, 2001) and is unlikely to initiate inward vesiculation by itself. Alternatively, rather than being the driving force of ILV formation, cholesterol more probably is a "passive" yet essential membrane component of the ILVs. Two different types of MVEs have been described, of which one appears to be cholesterol rich whereas the other contains LBPA (Mobius et al., 2003). These typical MVEs could either be representing MVEs at slightly different stages of maturation, or, more likely, characterize distinctive subpopulations of MVEs. Interestingly, LBPA is known to play a role in the transport of free cholesterol (Kobayashi et al., 1999) and was recently reported to control the cholesterol storage capacity of endosomes (Chevallier et al., 2008). The accumulation of cholesterol in late endocytic compartments, characteristic of the cholesterol storage disorder Niemann Pick disease, interferes with endosomal membrane dynamics and reduces bilayer fluidity (Kobayashi et al., 1999; Maxfield and Tabas, 2005; Sobo et al., 2007).

Furthermore, the phospholipid-binding protein annexin-1 is shown to be required for EGF-stimulated ILV formation, but not for inward vesiculation in unstimulated cells (White et al., 2005). Annexin-1 can mediate vesicle aggregation *in vitro* and so a possible role is proposed for annexin-1 to bring opposing membranes of the forming intraluminal vesicle together to promote scission (Futter and White, 2007).

Whether ILVs of distinctive origin can localize to the same MVE or rather localize to their specific MVE-subtype remains unclear and would be of great interest to clarify. Our data in **PAPER VI** suggest that LBPA positive ILVs and PtdIns(3)P positive ILVs can localize to the to the same MVEs. And in **PAPER V** we demonstrate that, unlike previously reported (White et al., 2005), LBPA positive ILVs are confined to the same MVE as EGFR positive ILVs that are most likely formed by mechanisms involving PtdIns(3)P (Pons et al., 2008). However, we could also identify a small quantity of MVEs that contained either LBPA-positive ILVs or EGFR-positive ILVs alone or ILVs of unidentified origin. On one hand, considering the specific cell-type associated functions of exosomes (van Niel et al., 2006), it seems clever for "homogenous" MVE subtypes to exist, whereas on the other hand

"heterogenous" MVEs would be more cost-efficient for the cell. As our data suggest some cells may adopt the golden mean and combine the two alternatives while, for instance, cell types with specific functions may lean towards more explicit MVE subtypes (Figure 4).
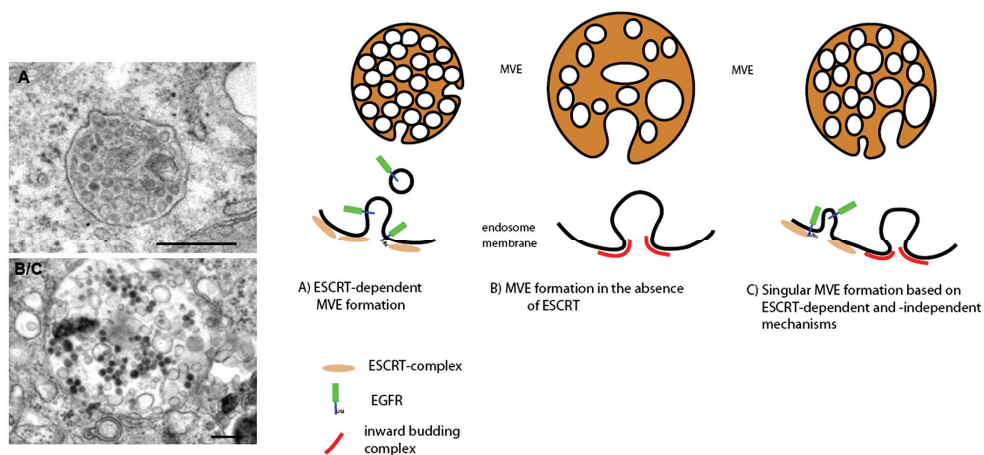
Figure 4   Models for MVE biogenesis
(**A**) In the presence of ESCRTs, formation of MVEs that contain homogenous ILVs occurs. The sorting of receptors, for instance EGFR, into these ILVs is facilitated by the ESCRT machinery. Presumably, the ESCRT-III complex is disassembled before inward budding and does not follow into the newly formed vesicle. (**B**) In the absence of ESCRT function, biogenesis of MVEs does still occur by (an) alternative mechanism(s), which results in the formation of a less homogenous ILV population. (**C**) It is not excluded that the proposed ESCRT-independent mechanism could function in parallel with the ESCRT-dependent process. Micrographs are depicting an MVE in HEp-2 control cells (**A**) and an enlarged MVE in cells depleted of all ESCRTs (**B/C**) and are corresponding to the model shown to the right. Scale bars, 200 nm.

Subpopulations of MVEs

An alternative or complementary possibility to MVE subpopulations is the existence of independent and parallel routes from the early endosome of which at least one is ESCRT-independent. By freeze-etch electron microscopy it is demonstrated that early endosomes (EEs) are complex structures of great pleomorphism, and consist of a central cisternal region from which heterogeneous tubules originate (Gruenberg, 2001). On the molecular level, EEs are known to be highly dynamic compartments that are composed of a mosaic of functional and structural lipid-protein domains (Gruenberg, 2001). For instance, the small GTPase Rab5 interacts with a multiplicity of effectors and is thought to build defined

effector-platforms on the EE-membrane which in turn can conduct their specific roles in membrane dynamic processes (Zerial and McBride, 2001). It is imaginable how distinctive lipid-protein domains execute their specific functions and form the cornerstone for parallel, distinctive subpopulations of MVEs and possibly LEs. For instance, ESCRT proteins are found to localize to confined endosomal microdomains which also contain ubiquitinated cargo and clathrin, whereas EEA1 and Tnf are found on distinct microdomains in Rab5Q79L mutant cells (Raiborg et al., 2002). Evidently, the localization to these particular microdomains could merely reflect the segregation of cargo for lysosomal targeting or recycling, but could alternatively denote the prospect of the formation of distinctive subpopulations of MVEs. Moreover, it has been demonstrated that a subgroup of EEs exists that is EEA1-negative and instead operates via APPL1 and APPL2, two Rab5 effectors (Miaczynska et al., 2004a). APPL-positive endosomes, like EEA1-positive endosomes, contain Rab5 and are accessible to both EGF and Tnf, but function with different kinetics and lower efficiency than the EEA1-containing compartments. There appears to be a certain degree of overlap between these distinctive compartments though and whether these "subpopulations" coalesce during maturation is not known. They appear to have different objectives however; whereas EEA1-containing EEs traffic cargo along the canonical endocytic route, the APPL-positive compartments function as intermediates in signaling between the plasma membrane and the nucleus (Miaczynska et al., 2004a). One more study reports the coexistence of two distinct populations of EEs of which one is dynamic, highly mobile and rapidly maturing whereas the other is static and matures more slowly (Lakadamyali et al., 2006). Both EEA1 and Rab5 are used as early endosomal markers and show approximately 80% colocalization. However, whether the Rab5-positive, EEA1-negative compartments contain APPL is not investigated in this study and it could, hence, refer to comparable subpopulation as proposed by Miaczynska et al. (Miaczynska et al., 2004a). Furthermore, EGFR and LBPA were previously described to localize to distinct subpopulations of MVEs (White et al., 2005). However, as previously mentioned, in **PAPER V** we used quantitative immuno-EM methods to demonstrate that EGFR and LBPA colocalize in more than 70% of the MVEs.

In conclusion, it seems most likely for ESCRT-dependent and -independent mechanisms of ILV formation, and possibly pathways, to function in parallel. ILVs of distinctive origin presumably coexist in MVEs and provide an alternative mechanism when the preferred mechanisms fail. In this respect it is worth noting that strongly enlarged MVEs form in cells

quadruple-depleted of all ESCRTs (**PAPER V**). In these cells sorting through the ESCRT machinery may be completely abolished and additional mechanisms for receptor sorting may be upregulated in order to rescue the integrity of the cell. The enlarged MVEs may thus reflect the upregulation of additional, ESCRT-independent machineries or pathways. Even though not much solid data on the topic has been found to date, it seems very plausible that cells provide back-up mechanisms in the pathways that are as important to their viability and integrity as the endocytic pathway.

Endosome maturation

Acquisition of endosome maturity depends both on the accurate spatial and temporal recruitment of proteins and lipids to the endocytic pathway and their removal, and appears to be a highly regulated process in which many factors are involved. For instance, a recent study reports *in vitro* vesicle budding into the endosome lumen to be dependent on membrane components and cytosolic factors, as well as on time, temperature, pH and energy (Falguieres et al., 2008). Additionally, movement of the endosomal compartment from the cell periphery to the perinuclear area appears to be coordinated with its maturation process and, ultimately, its fusion with the lysosome (Gruenberg and Stenmark, 2004). This minus-end-directed transport along microtubules is mediated by the motor protein dynein (Aniento et al., 1994). In **PAPER IV** we show Rab7-interacting lysosomal protein, RILP, to be the mechanistic link between endosome motility and MVE biogenesis. RILP is an effector of the small GTPase Rab7 and controls trafficking of late endosomes through its interaction with the dynein-dynactin motor complex (Cantalupo et al., 2001; Jordens et al., 2001). Additionally, RILP was found to interact with the ESCRT-II subunits Vps22 and Vps36 (Progida et al., 2006; Wang and Hong, 2006) and appears, like ESCRT proteins, to be crucial for degradative functions of LEs (**PAPER IV** and (Wang and Hong, 2006)). Importantly, RILP depletion leads to the clustering of endosomal structures that are virtually devoid of intraluminal membranes and to elevated levels of resident late endosomal lipids and proteins. This indicates that RILP is required for both the normal distribution of LEs and lysosomes and for the formation of ILVs (**PAPER IV**). The exact functional mechanism of RILP and ESCRT-II in MVE biogenesis and microtubule-dependent endosome motility are yet to be established. Depletion of RILP leads to co-depletion of ESCRT-II and vice versa and, hence, individual contribution of these factors to degradative sorting and MVE biogenesis could be discussed. However, the morphological differences

between endosomes in RILP-depleted cells (**PAPER IV**) and endosomes in Vps22-depleted cells (**PAPER II**) strongly indicate separate functional roles for these proteins. How exactly dynein-dynactin-motility and distinctive localization play a role in endosome maturation is not known, and is presumably a tactic to bring together MVEs and lysosomes and, consequently, to promote fusion. However, maturing MVEs can move to the perinuclear region before Rab5-Rab7 conversion (Driskell et al., 2007; Rink et al., 2005) and dynein may not be the only motor regulating lysosome motility (Loubery et al., 2008). This indicates the participation of yet additional mechanisms in endosome maturation and motility.

Receptor trafficking and signaling

Ligand-induced activation of the epidermal growth factor receptor (EGFR) leads to initiation of several intracellular signaling cascades. For instance, the Ras-MAPK pathway is activated in response to EGF stimulation, which results in the phosphorylation of MEK1/2 and consequently the activation and phosphorylation of ERK1/2 which then translocates into the nucleus and regulates the activity of several transcription factors (Murphy and Blenis, 2006). In **PAPER I** and **II** we demonstrate that depletion of ESCRT-II (Vps22) and ESCRT-III (Vps24), like depletion of ESCRT-0 (Hrs) or ESCRT-I (Tsg101) (Raiborg et al., 2008), results in retarded downregulation of EGFR. Interestingly, however, whereas depletion of ESCRT-0 or ESCRT-I leads to prolonged downstream signaling of EGFR, depletion of ESCRT-II (Vps22) or ESCRT-III (Vps24) does not appear to interfere with signal attenuation (**PAPER I** and **II**). In theory, receptor silencing will most likely be attained by one or a combination of the following mechanisms: i) dissociation of the receptor-ligand complex at low pH, ii) sequestration of the receptor-ligand complex into ILVs of MVEs or, iii) preventing access of signaling mediators to the cytosolic tail of the receptor. Firstly, by using fluorescence ratio imaging we demonstrate that EGF accumulates in endosomes with a close to neutral pH in Tsg101- depleted cells and in endosomes with a low pH in Vps24-depleted cells (**PAPER I**). Tsg101 depletion appears to cause a delay in endocytic trafficking and to hinder the normal accumulation of EGF in acidic endosomes thereby possibly impeding the dissociation of the receptor-ligand complex, consequently, resulting in prolonged signaling. Secondly, endosome morphology is more dramatically

altered after depletion of Tsg101, than after depletion of Vps22 or Vps24 (Doyotte et al., 2005; Razi and Futter, 2006) and **PAPER I** and **II**). In particular, Tsg101 is required for ILV formation (Falguieres et al., 2008; Razi and Futter, 2006), whereas Vps24 alone does not seem to be critical in this mechanism possibly due to functional support of the Vps20-Snf7-subcomplex of ESCRT-III. This raises the possibility that EGFR can signal from the multicisternal endosomes in Tsg101-depleted cells, but not from ILVs in Vps24-depleted cells. Contradictory, however, whereas EGFR is mostly localized to intraluminal membranes in Vps24-depleted cells, the sorting of ubiquitinated cargo into the ILVs of MVEs is significantly inhibited in cells depleted of Vps22/ESCRT-II and EGFR redistributes from the intraluminal membranes to the limiting membrane of MVEs (**PAPER I** and **II**). Even though this discrepancy raises doubt regarding the sequestration of the receptor-ligand complex into ILVs of MVEs as a possible mechanism of signal attenuation, one plausible explanation could be the differences in experimental set-up used in these studies. Instead of the approach of pre-embedded labeling with EGFR-gold that we used in the Vps24 study (**PAPER I**), we chose for an immunocytochemistry EM approach in the Vps22 study, labeling cryo-sections to EGFR and immuno-gold (**PAPER II**). Antibody binding most likely competes with ligand binding to the receptor and almost certainly alters the fate and kinetics of internalized EGFR. Therefore the internalization of directly labeled EGF would probably have been a more appropriate approach, even if the affinity of labeled EGF may be altered as well. A possible explanation for the attenuation of signaling even when the receptor is not sorted into ILVs, as was demonstrated in Vps22-depleted cells, could be that the access of signaling mediators to the cytosolic tail of the receptor is prevented, possibly by ESCRT-I subunits.

In the context of EGF signaling, it is worth noting that at low doses of EGF (1.5 ng/ml), EGFR is predominantly internalized through clathrin-mediated endocytosis (CME), whereas at higher doses ($\geq$ 20ng/ml) the receptor is internalized both through CME and non-clathrin endocytosis (NCE) (Sigismund et al., 2005). Interestingly, a recent study reports that EGFRs internalized through CME are not targeted for degradation, but instead are recycled to the plasma membrane, thereby determining the duration of signaling. NCE-internalized EGFRs, on the other hand, are trafficked through the canonical endosomal pathway which ultimately results in the degradation of receptors in lysosomes (Sigismund et al., 2008). However, when (part of) the degradative pathway falls short recycling of endocytosed EGFRs is enhanced (Sigismund et al., 2008) as was demonstrated in cells depleted of

Hrs/ESCRT-0 Tsg101/ESCRT-I (Babst et al., 2000; Doyotte et al., 2005; Razi and Futter, 2006). Interestingly, however, neither Vps22/ESCRT-II-depletion nor Vps24-depletion appears to have an effect on EGFR recycling (Raiborg et al., 2008). These data show good correlation between the effects of ESCRT depletion on EGFR recycling and prolonged signaling and support a model in which ESCRT-0 and ESCRT-I function relatively early in the endocytic pathway, at a stage where receptors recycling still is an option, whereas ESCRT-II and –III function at a later stage where the receptors' fate can no longer be altered.

Interestingly, inactivation of the Vps25 subunit of ESCRT-II is demonstrated to enhance activation of two other signaling pathways, Notch and Dpp, in *Drosophila* (Thompson et al., 2005). Whether these differential data merely account for the distinction of signaling pathways between organisms or perhaps reflect the existence of endosome subpopulations that express specific subsets of proteins important for regulating signaling and sorting of distinct receptors, is unknown and would be of interest to investigate.

ESCRTs and disease

With the improvement of methods for large genome-wide screenings and medical diagnostics, an increasing number of genes associated with human diseases is identified. Interestingly, these include genes encoding components of the ESCRT machinery and, so far, ESCRT subunits have been associated with three main categories of pathogeneses, i.e. neurodegeneration, cancer and infection (Table 2), which will be discussed in the following sections. Additionally, missense mutations in the ESCRT-III subunit CHMP4B have recently been found in families with autosomal dominant cataracts, in which it appears to contribute to lens transparency (Shiels et al., 2007). These data emphasize that ESCRTs are crucial players of a wide variety of physiological cell processes and that their aberration potentially results in diseases of distinctive origin. In addition to a role of the ESCRT machinery through the assurance of accurate and efficient endocytic traffic and signaling and MVE formation, ESCRT- and ESCRT- associated proteins may play more "individual", machinery-unrelated, roles in pathogenesis through their interaction with disease-related proteins or pathogens.

Complex	Subunit	Dysfunction/ Disease	Pathogenesis	References
Cancer				
ESCRT-0	Hrs (Vps27)	tumourigenesis and metastatic potential	Hrs depletion associates with upregulation of E-cadherin and reduced β-catenin signaling	(Toyoshima et al., 2007)
ESCRT-I	Vps37A (HCRP1)	Hepatocellular ca. (HCC) and metastasis	Growth inhibitory protein, suppressing proliferation, transformation and invasion; strongly reduced levels in HCC	(Xu et al., 2003)
	Tsg101 (Vps23)	Ovarian ca. (prognostic)	Upregulation of Tsg101: suppression of p21 expression and posttranslational regulation through MAPK signaling	(Young et al., 2007a; Young et al., 2007b)
	Tsg101 (Vps23)	Mammary ca.	Overexpression of Tsg101: increased signalling through MAPK	(Oh et al., 2007)
	Tsg101 (Vps23)	Papillary thyroid ca., gastrointestinal stromal tumors	Overexpression of Tsg101 (consequences unknown)	(Koon et al., 2004; Liu et al., 2002)
ESCRT-I/II	Erupted (Tsg101) / Vps25	Neoplastic transformation (ovary and imaginal discs), over-proliferation of adjacent WT cells	Enhanced Notch and growth factor signalling in mutant cells	(Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005)
ESCRT-III	CHMP3 (Vps24)	Prostate ca.	CHMP3 induces neuroendocrine cell differentiation	(Wilson et al., 2001)
	CHMP3 (Vps24)	Non-small cell lung ca.	CHMP3 has a functional role in neuroendocrine cell differentiation	(Walker et al., 2006)
ESCRT-0 associated	Hrs (Vps27)	Benign brain tumors (e.g. Schwannomas, meningiomas, ependymomas)	Interaction with neurofibromatosis 2 tumor suppressor protein schwannomin/merlin, regulating STAT signalling	(Gutmann et al., 2001; Scoles et al., 2000; Scoles et al., 2002)
ESCRT-III associated	CHMP1A	Ductal pancreatic ca.	Tumor suppressor, regulating tumor growth potentially through p53 signaling pathway	(Li et al., 2008)
Neurodegenerative disease				
ESCRT-III	CHMP2B (Vps2)	Frontotemporal Dementia (FTLD-U) and ALS	Disruption of endosomal trafficking, protein accumulation	(Parkinson et al., 2006; Skibinski et al., 2005)
ESCRT-I/III	Tsg101 (Vps23) / CHMP3 (Vps24) / CHMP2B	Neurodegeneration (FTLD-U, ALS, Huntington's disease (HD))	Reduced autophagic degradation, accumulation of Ub-protein aggregates containing TDP-43; reduced clearance of Huntingtin-positive inclusions	PAPER III
ESCRT-I-I-III	Vps28, Vps25, Vps32 (Snf-7), Vps4	Neurodegeneration (HD)	Reduced ESCRT functions aggravates polyglutamine-induced neurotoxicity in HD model; all ESCRTs required for autophagy	(Rusten et al., 2007)
ESCRT-III	CHMP4B (Snf7-2) / CHMP2B	Neurodegeneration (FTLD-U, ALS)	Accumulation of autophagosomes; failure of mutant CHMP2B to dissociate properly leading to dysfunctional ESCRT-III on late endosomes	(Lee et al., 2007)
ESCRT-I associated	Tsg101 (Vps23)	Spongiform neurodegeneration (hallmark of prion disease)	E3 ubiquitin-protein ligase Mahogunin ubiquitinates Tsg101; depletion of Mahogunin disrupts endosomal trafficking	(Kim et al., 2007)
ESCRT-I associated	Tsg101 (Vps23)	Charcot-Marie-Tooth disorder (CMT1C)	Interaction with SIMPLE; SIMPLE plays a role in the lysosomal sorting of plasma membrane proteins	(Shirk et al., 2005)
ESCRT-III associated	CHMP1B	Hereditary spastic paraplegia	Interaction with spastin; spastin mutation results in disruption of endosomal trafficking	(Reid et al., 2005)
Infections				
ESCRT-0	Hrs (Vps27)	*Mycobacterium marinum*	Inability to recruit Hrs contributes to the arrest of phagosomal maturation induced by pathogenic mycobacteria	(Vieira et al., 2004)
ESCRT-I-III and associated	Tsg101, Vps25/Vps36, Vps24/Vps20, Vps4	*Mycobacterium Tuberculosis*	The ESCRT machinery directly acts on the bacterial phagosome	(Philips et al., 2008)
ESCRT-I/III, associated	Tsg101, CHMPs, AIP1/Alix	RNA virus infections (e.g. HIV-1, Ebola, EIAV, Rabies, Lassa)	Hijacking the ESCRT machinery via L-domain motifs, virus budding via MVE pathway	(Morita and Sundquist, 2004)
Other				
ESCRT-III	CHMP4B (Snf7-2)	Autosomal dominant cataract	Gain-of-function defects CHMP4B triggers loss in lens transparency	(Shiels et al., 2007)

Table 2   ESCRTs and disease

Neurodegeneration and autophagic clearance

Endocytosis is crucial for signaling, trafficking, metabolism and the integration of inter-cellular communication in the majority of mammalian cells. Neurons in particular have extensive and specialized needs for endocytosis. Firstly, their extreme polar shape places the sites most active in signal transduction, the axons and dendrites, at great distance from the soma where many targets and effectors of these signaling effects are located. This spatial burden also affects structural maintenance of axons, dendrites and synapses and complicates the transport and recycling of membrane components back and forth from these distant regions (Parton et al., 1992). Secondly, synaptic transmission itself relies to a great extent on rapid endocytic processes which i) ensure the maintenance of adequate numbers of synaptic vesicles and ii) control the recycling and degradation of pre- and post-synaptic membrane proteins. With endocytosis playing such prominent roles in neurons it is not surprising that even subtle aberrations in its mechanisms can potentially lead to severe symptoms derived from dysfunction of the brain or central nervous system. In particular, endocytosis and lysosomal degradation appear to be important factors in the protection against neurodegeneration and were recently reported to be dysfunctional in a number of neurodegenerative diseases, e.g. Alzheimer's diseases, Parkinson's diseases and Niemann-Pick type C disease (Nixon, 2005).

Besides the endocytic pathway, a second pathway that plays an important role in the protection against neurodegeneration, as for instance was demonstrated in human cell models and in *Drosophila* and mouse models for Huntington's disease (HD), is autophagy (Ravikumar et al., 2004; Yamamoto et al., 2006). Autophagy is the major pathway for clearance of potentially toxic aggregate-prone proteins and the only known pathway to degrade damaged organelles (Box 2: Autophagy: a short introduction). Basal autophagy is required for preventing the accumulation of abnormal proteins which can ultimately lead to neurodegeneration, even in the absence of disease-associated mutant proteins (Hara et al., 2006; Komatsu et al., 2006). A number of neurodegenerative disorders are characterized by the accumulation of cytosolic protein-aggregates and dysfunctional autophagic compartments, which raises the possibility that inefficient removal of aggregated proteins is at least contributory to the disease (Rubinsztein, 2006).

Box 2   Autophagy: a short introduction

Autophagy is a strictly regulated lysosomal pathway that degrades cytoplasmic material, long-lived proteins and organelles and is essential for survival, differentiation, development and homeostasis. A basal level of autophagy is important for cell homeostasis and the pathway is upregulated under conditions of stress caused by for instance amino acid starvation, unfolded protein response or viral infection.

Four different autophagic routes are distinguished: (1) macroautophagy (hereafter referred to as autophagy) in which a portion of cytoplasm is engulfed by a specialized organelle, the autophagosomes, which subsequently fuses with the lysosome and delivers the cytoplasm for degradation (Mortimore et al., 1983); (2) microautophagy, in which the lysosomal membrane directly sequesters a portion of the cytoplasm (Ahlberg et al., 1982); (3) chaperone-mediated autophagy, in which proteins are directly translocated across the lysosomal membrane (Cuervo and Dice, 1996), and (4) organelle specific autophagy, for instance; crinophagy in which secretory vesicles fuse directly with lysosomes, and mitophagy, in which mitochondria fuse directly with lysosomes

The control of autophagy is nutritionally, hormonally and developmentally regulated through multiple signalling pathways (Levine and Klionsky, 2004). The induction by a stress-signal leads to the elongation of a flat membrane cistern, the phagophore or isolation membrane, which engulfs a portion of cytoplasm, thereby forming an autophagosome. The elongation process requires an elaborate molecular machinery in which autophagy related genes (Atg) play a crucial role. Two protein conjugation systems are known to be required for autophagosomes formation, the Atg12-Atg5 conjugation and Atg8/LC3-phosphatidylethanolamine conjugation systems (Ohsumi, 2001), that are both activated by Atg7 (Mizushima et al., 2003). Upon activation, Atg12 is transferred to Atg5 whereas Atg8/LC3 is conjugated to phosphatidylethanolamine and by this means to the membrane. Atg8/LC3 localizes to the limiting membranes of autophagosomes (Kabeya et al., 2000) and mediates membrane tethering and elongation of the phagophore (Nakatogawa et al., 2007). Ultimately, the autophagosome fuses with a lysosome or with an endosome, forming an autolysosome or amphisome, respectively (Berg et al., 1998; Gordon and Seglen, 1988), to deliver the engulfed cytoplasm for degradation. The degradation products are transported back to the cytoplasm for reuse (see figure).

Autophagy is an adaptive event and serves to protect organisms against diverse pathologies, including infection, cancer, neurodegeneration, aging and heart disease.
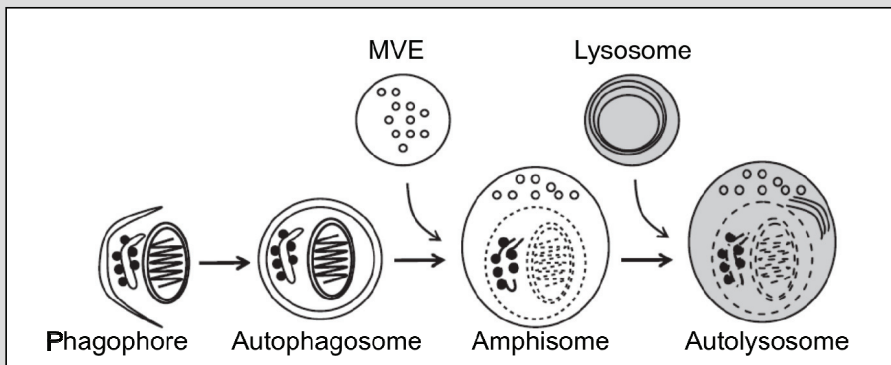
Adapted from: (Eskelinen, 2008)

Recently, mutations in the ESCRT-III subunit CHMP2B/Vps2 were associated with a rare form of autosomal dominant frontotemporal dementia (FTD) (Skibinski et al., 2005) and amyotrophic lateral sclerosis (ALS) (Parkinson et al., 2006), two neurodegenerative diseases that present with ubiquitin- and p62/Sequestosome-1 positive inclusions in affected neurons (Holm et al., 2007). p62/Sequestosome-1 is a common component of protein inclusions associated with neurodegenerative disease (Talbot and Ansorge, 2006) and owing to its interaction with the autophagic protein LC3/Atg8 as well as its aptitude to bind polyubiquitin through its UBA domain, p62 provides a possible link between protein aggregation and autophagic clearance (Bjørkøy et al., 2005; Pankiv et al., 2007; Vadlamudi et al., 1996). The role of ESCRTs with regard to autophagy, on the other hand, was relatively unexplored until recently. In **PAPER III** we demonstrate that depletion of ESCRT subunits in mammalian cells causes accumulation of autophagosomes and ubiquitin-, p62-, LC3 positive protein-aggregates. The inhibition of autolysosome formation in ESCRT-depleted cells and in cells expressing CHMP2B mutants indicates a role for ESCRTs in the efficient fusion between autophagosomes and lysosomes. How ESCRTs mediate fusion is not yet clarified. However, failed recruitment of the class C vps/HOPS complex was previously demonstrated to be required for the fusion between autophagosomes/amphisomes and endo-lysosomal compartments (Lindmo et al., 2006; Pulipparacharuvil et al., 2005). Interestingly, Vps18, a subunit of the class C Vps/HOPS complex, is known to interact with Hrs/ESCRT-0 and Tsg101/ESCRT-I (Kim and Akazawa, 2007). In the context of membrane fusion it is worth mentioning that a syntaxin N-terminus domain is predicted in Tsg101 (NP_006283, NCBI protein database). A member of the syntaxin family, syntaxin 1, is known to be one of three crucial soluble N-ethylmaleimide-sensitive factor attached protein receptors (SNAREs) implicated in membrane fusion (Ungermann and Langosch, 2005). Nevertheless, the dramatic alterations of endosome morphology and the formation of membranous stacks after Tsg101 depletion, suggest a specific role of Tsg101 in membrane dynamics, possibly by regulating fission and fusion of membranes. Furthermore, proper maturation of endosomes and accurate performance of endocytic traffic may be required in order to provide membrane proteins and lipids necessary for either tethering or fusion between compartments of the endocytic pathway and the autophagic pathway. Even though the possibility that non-MVE related ESCRT functions affect autophagic clearance can not be excluded, our data, strengthened by studies in *Drosophila* (Lee et al., 2007; Rusten et al., 2007), strongly indicate that dysfunctional

MVEs inhibit autophagic degradation and lead to the formation of cytosolic protein inclusion that ultimately may cause neurodegenerative disease.

TAR-DNA-binding protein 43 (TDP-43) is identified to be the major ubiquitinated component of protein inclusions found in affected cells of patients with the most common type of FTD, frontotemporal lobar degenerations with ubiquitin deposits (FTLD-U), and ALS and is the common pathological substrate linking these neurodegenerative disorders (Neumann et al., 2007). In **PAPER III** we demonstrate that TDP-43 accumulates in the p62- and ubiquitin-positive aggregates in cells depleted of ESCRT-subunits, suggesting a link between TDP-43-positive inclusions and proteins required for MVE formation and autophagic degradation. However, in contrast to other cases of FTLD-U, protein inclusion in cells from patients with the CMMP2B mutation are TDP-43 negative (Holm et al., 2007) and this may suggest for MVEs to have distinctive effects in TDP-43-positive and TDP-43-negative FTLD-U. It is worth noting that depletion of ESCRT subunits in cellular or *Drosophila* models for HD inhibits clearance of expanded polyglutamine aggregates (**PAPER III**, and (Rusten et al., 2007)). This suggests a wide-ranging functional role for ESCRTs in mechanisms for autophagic clearance.

In addition to CHMP2B, a number of indirect links between the ESCRT machinery and neurodegeneration were recently found. For instance, a null mutation of the Tsg101 ubiquitinating E3 ubiquitin ligase Mahogunin was reported to cause spongiform neurodegeneration, a hallmark of prion-like disease, in mice (Kim et al., 2007). SIMPLE, another Tsg101 interacting protein, is mutated in Charcot-Marie-Tooth type 1C, a hereditary disease causing demyelinating peripheral neuropathy in humans (Shirk et al., 2005). Furthermore, the putative ESCRT-III interacting proteins spastin and spartin are mutated in hereditary spastic paraplegia, a group of diseases characterized by length-dependent degeneration of the distal end of long axons (Patel et al., 2002; Reid et al., 2005).

Cancer
The dysregulation of growth factor receptor signaling by constitutive overexpression, overstimulation or mutational activation is known to be strongly contributory to the pathogenesis of various types of cancer (Yarden and Sliwkowski, 2001). Owing to its

important role in the down-regulation and signal attenuation of several growth factor receptors (**PAPER I** and **II**), the ESCRT machinery is a critical factor in the control of cell growth and proliferation and is consequently associated with carcinogenesis. Interestingly, the mammalian ESCRT-I subunits Tsg101 and Vps37A/HCRP1 are located to chromosomal regions that frequently are either deleted or mutated in cancers, and were proposed to function as tumour suppressors accordingly (Li and Cohen, 1996; Xu et al., 2003). Downregulation of TSG101 in mouse fibroblasts is found to initiate cell tranformations, and injection of these cells into nude mice results in metastatic tumour growth (Li and Cohen, 1996). Contradictory, however, conditional knock out of TSG101 in mice is demonstrated to result in increased apoptosis, but does not lead to neoplastic transformation or tumourigenesis (Krempler et al., 2002; Wagner et al., 2003). Conversely, Tsg101 is reported to be upregulated in a variety of malignant tumours, i.e. papillary thyroid carcinomas, gastrointestinal stromal tumors, ovarian carcinomas and a subset of mammary carcinomas, and is suggested to serve as a prognostic indicator (Koon et al., 2004; Liu et al., 2002; Oh et al., 2007; Young et al., 2007a; Young et al., 2007b). Besides its functions in endocytosis as a component of the ESCRT machinery, Tsg101 is known to be critical for normal proliferation and growth by regulating the levels of proteins that are involved in controlling the cell cycle (Carstens et al., 2004; Krempler et al., 2002; Li et al., 2001; Oh et al., 1998). Through which mechanisms and interactions exactly Tsg101 affects proliferation and carcinogenesis remains to be elucidated. However, Tsg101 is highly conserved among species, maintained at steady-state levels and is found to interact with a considerable number of diverse proteins through its variety of binding domains. This strongly indicates Tsg101 to be a protein of versatility and of great importance to cell function. The requirement of Tsg101 for autophagic degradation (**PAPER III)** in combination with the role of autophagy in carcinogenesis (Levine, 2007) provides another link between Tsg101 and cancer and allows us to draw an even more complex, yet more interesting picture.

Expression of the other ESCRT-I subunit associated with tumor suppression, Vps37A/HCRP1, was found to be strongly reduced in hepatocellular carcinomas. Even though the exact pathogenesis remains unclear, data suggest that Vps37A/HCRP1 plays a role in suppressing proliferation and malignant transformation in addition to preventing cell invasion and tumor metastasis (Xu et al., 2003; Yokota et al., 1999). Also subunits from other ESCRTs and ESCRT-associated proteins are implicated in tumor growth, either directly or indirectly. For instance, the ESCRT-0 subunit Hrs is involved in the development

of benign brain tumors (e.g. Schwannomas, meningiomas, ependymomas), through interaction with the neurofibromatosis 2 tumor suppressor protein Schwannomin and regulation of STAT signaling (Gutmann et al., 2001; Scoles et al., 2002). Similarly, the ESCRT-III-subunit Vps24/CHMP3 is, in concert with insulin growth factor-binding protein related protein 1, found to induce neuroendocrine cell differentiation resulting in progressive cancers, i.e. prostate cancer and non-small cell lung cancer, with poor prognosis (Walker et al., 2006; Wilson et al., 2001). Finally, CHMP1A, which encodes an ESCRT-III-associated protein, was recently reported to function as a novel tumour suppressor gene in ductal pancreatic tumour cells (Li et al., 2008).

The role of ESCRTs in malignant tumour growth has been strengthened by data from studies in the model organism *Drosophila melanogaster*. In this organism, clonal loss of Tsg101 in epithelia is demonstrated to cause massive hyperplasia of surrounding wild type tissue even though the mutant cells undergo apoptosis (Moberg et al., 2005). A similar phenotype is found upon loss of ESCRT-II subunit Vps25, whereas loss of the ESCRT-0 subunit Hrs has no such effect (Thompson et al., 2005; Vaccari and Bilder, 2005). The tumour growth is most likely a result of hyperactivation of Notch and growth factors which accumulate in aberrant endosomes, in combination with the loss of epithelial polarity in mutant cells. The discrepancy of data found in flies and in mammals is not apparent but could be related to the more complex cellular mechanism in higher eukaryotes. Nevertheless, these data support the idea that ESCRT proteins, either individually or in complex, are involved in carcinogenesis in humans. However, additional studies are required to unravel their specific functional mechanisms. It is worth mentioning that ESCRTs are known to play a role in cytokinesis (Carlton and Martin-Serrano, 2007; Morita et al., 2007). Cytokinesis is the process by which cells separate after the duplication and spatial segregation of their genetic material (Barr and Gruneberg, 2007), and incomplete cytokinesis can result in aneuploid cells, which is a characteristic of cancer.

Infections

The relation between ESCRTs and infection is rather double-sided. On one hand, endocytic and autophagic pathways are important actors in innate immunity and the ESCRT machinery appears to be crucial for the elimination of certain bacteria by lysosomal

degradation. For instance, Mycobacteria have the capacity to prevent fusion of the macrophagic phagosomes in which they reside with lysosomes and are hence able to survive and replicate intracellularly. Several ESCRT components were recently identified to restrict microbial replication and impediment of ESCRT function was found in cells prone to mycobacterial infections (Philips et al., 2008; Vieira et al., 2004). How ESCRTs intervene with mycobacterial replication is not yet known, but is likely related to phagosome formation and/or phagosome-lysosome fusion and would be highly consistent with the proposed function of ESCRTs in the fusion reaction of autophagosomes with lysosomes (**PAPER III**).

On the other hand, however, enveloped RNA viruses (e.g. HIV-1, Ebola, rabies virus) have the ability to exploit the ESCRT machinery in favor to their budding (Morita and Sundquist, 2004). Their structural proteins contain one or two of the three different classes of "late" (L) domains, i.e. P(S/T)AP, PPXY or YP(X)nL, through which they interact with their target protein. For instance, the P(S/T)AP motif of HIV-1 Gag binds directly to the N-terminal UEV domain of the ESCRT-I subunit Tsg101 and this interaction is required to facilitate HIV-budding (Pornillos et al., 2002). Interestingly, Hrs also contains a PSAP motif and recruits Tsg101 in the same manner (Bache et al., 2003a). However, HIV-1 Gag was demonstrated to have a sevenfold higher affinity than Hrs for Tsg101 (Pornillos et al., 2003). Moreover, HIV-1 Gag recruits the ESCRT-III associated protein AIP1/Alix through interaction with its YP(X)nL L-domain (Strack et al., 2003). Like Tsg101, AIP1/Alix is demonstrated to be involved in MVE biogenesis and inward budding (Falguieres et al., 2008; Odorizzi et al., 2003). Current data suggests that enveloped RNA viruses ultimately recruit ESCRT-III and Vps4 in order to initiate final abscission of virions from the plasma membrane (Martin-Serrano, 2007). The exact functions of ESCRT-III and Vps4 in this process are yet to be clarified. However, clear similarities with the inward budding of vesicles into MVEs are brought to mind and point out the great value of models and virus studies in the elucidation of molecular cell mechanisms.

The double-sided influence of ESCRTs on the host's immunity and the potential carcinogenicity of ESCRT disruption bring about challenges in the development of antiviral drugs targeting the ESCRT machinery. Nevertheless, bearing in mind the relatively recent discovery of the ESCRT machinery (Katzmann et al., 2001) and the astonishing progress that has been made during the past decade in acquiring knowledge of the biochemical and

physiological properties of the ESCRT machinery, we should nurture our ambitions and be nothing but optimistic for the future.

Methodological considerations

The majority of methods used in this work are well-established and well-described in textbooks and scientific publication. This section will therefore not contain a detailed description of these methods, but will rather be used to discuss a number of aspects and pitfalls worth noting.

Electron microscopy vs. Immunofluorescence Confocal Microscopy?

The electron microscope has profoundly influenced our understanding of the cell. Electron microscopes utilize the much smaller wavelength of the electron (i.e. < 0.005 nm (at 60kV) compared to a wavelength of 380 nm for violet light), thereby permitting not only another thousandfold increase in magnification but, importantly, also a parallel improvement in resolution capacity. This led to the visualization of viruses, DNA and smaller organelles for the first time and allowed biologists to both define and expand the world of light microscopy. Initially, in the field of biology, electron microscopy (EM) was used predominantly for the purpose of describing cell structures and components. However, in time many new techniques for sample preparation were developed, including several good immuno-EM methods that allowed the localization of molecules within their cellular environment, which led to the increased popularity of functional and analytical approaches (Slot and Geuze, 1985). During the nineties, however, with new and better techniques for light microscopy developing, interest in EM declined steadily. However, it should be stressed that, no matter how far the limits of light microscopy will be pushed, it will never be an adequate alternative for the use of EM approaches. The resolution that can currently be achieved by immunofluorescence (IF) microscopy is, under specialized circumstances, around 100 nm (Klar et al., 2000). In comparison to the down to 0.3 nm resolution provided by transmission electron microscopes (TEMs) used for applications in life sciences, it is obvious that these two methods are in separate leagues and are by no means meant to replace each other (see example in Figure 5).
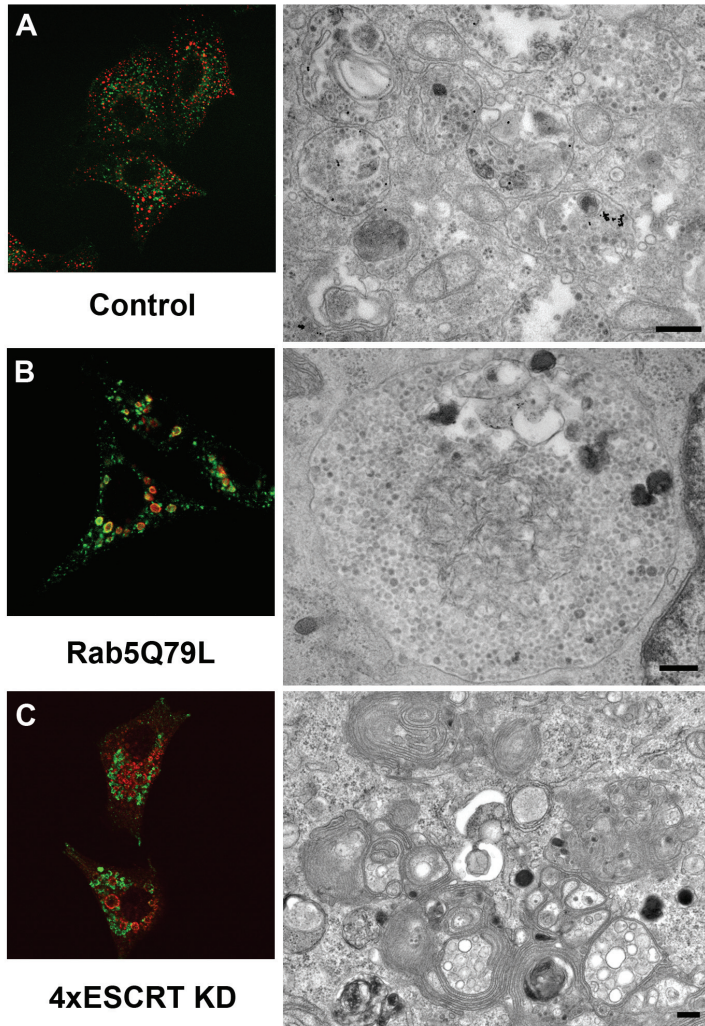
Figure 5 **(A)** IF confocal microscopy of control cells shows punctuate endosomal labelling to the early endosomal marker EEA1 (green) and the late endosomal marker CD63 (red) (left panel). By EM endosomal compartments are visible as individual compartments of normal size (300-400 nm) (right panel). In **(B)** endosomal labelling of Rab5Q79L transfected cells is shown. Overexpression of this constitutively active form of Rab5 leads to the enlargement of endosomes due to an increased fusion rate. By IF microscopy, the enlarged endosomes are clearly visible through labelling to EEA1 (green) and CD63 (red). There is some colocalization (yellow) between the two markers. By EM (right panel **B**) strongly enlarged endosomes are visible, representing the red and green labelled structures in the left panel IF image. In **(C)** the pattern of IF labelling (EEA1 is red, CD63 is green) is largely similar to the labelling pattern in **(B)**. There appears to be no colocalization between the two markers, but structures are strongly enlarged. However, the EM image in **(C)** shows a completely different morphology than in **(B)**, with large areas of membrane sheets and folds **(C)** in addition to enlarged endosomal structures (not shown) with a different morphology to the enlarged endosomes in **(B)**. This shows that even though one can get comparable images by IF confocal microscopy, one can observe very different morphological features by EM. Scale bars, 200 nm. (IF image **(B)** by C. Sem-Jacobsen).

Immunofluorescence confocal microscopy has developed into one of the most popular techniques used in the field of cell biology and adjoining fields during the past decade. Not only is nearly any research group keen to use the method, it has been increasingly difficult to publish in scientific journals without colorful IF images to support your data. In part this is understandable: IF microscopy is a powerful method which allows fully hydrated specimens to be visualized under natural environmental conditions. Even though the direct imaging of, for instance, protein molecules is impossible, those may be localized by use of probes with specific binding properties (e.g. antibodies, enzyme substrates, etc.). Importantly, IF microscopy can be used for life cell imaging, which allows the study of labeled protein molecules in a spatial and temporal matter under functionally changing circumstances. These methodological advantages in combination with the relatively easy preparation methods and quick data acquisition make IF confocal microscopy a widely used and highly appreciated method. However, the facts that the confocal microscope occupies such a prominent position in nearly every research facility and that the microscope is readily used by anyone with or without an understanding of the basic physics principles of the microscope lead to the acquisition of great errors instead of great images all too frequently (North, 2006).

Even though the vast amount of advantages, like in any other method used in life sciences, there are a few limitations to the use of EM. The biggest disadvantage of EM in life sciences is that direct monitoring of dynamic processes in samples is not possible. Since visualization by TEM has to be performed under vacuum conditions in order for the electron beam to travel through, the sample is either dehydrated or frozen, and so inactive. It is however a misconception that EM can only provide a static picture of events. Similar to other biochemical methods, kinetic data of a protein can readily be acquired by studying the protein at a few different time-points, as for instance in a pulse-chase experiment. In combination with immunogold labeling of cells and quantitative analyses, this approach is equally or possibly even more reliable than any other and is providing a large amount of (detailed) data. This, however, leads to two potential drawbacks of EM, i.e. i) it is more time-consuming than several other methods used in cell biology and biochemistry, and ii) the amount of data acquired by EM is huge and can potentially be overwhelming and confusing at first sight. Owing to stereology – the mathematically based approach for

quantifying structural parameters such as for instance cell surface areas, and length and volume of structures – the large amounts of data can be efficiently interpreted in an unbiased manner (Mayhew, 1991). It does nevertheless also highlight where EM differs from many other cell-biology approaches: EM is based largely on experience, it takes a well-trained eye to distil specific data from the quantity, and the training to acquire this experience takes years (Geuze, 1999; Griffiths, 2004).

In conclusion, novel approaches for both electron microscopy and IF confocal microscopy are under constant development, and are very likely to provide us with more exciting new data in the near future. The relatively recent introduction and nowadays more regular used method of 3D-EM-tomography, for instance, has already led to novel structural insights in the field of cell biology. In particular, in the first 3D reconstruction of the eukaryotic cell (of the yeast *Schizosaccharomyces Pombe*) it was shown that mitochondria form extended tubular networks (Hoog et al., 2007) and are no individual entities as was initially assumed. Moreover, ILVs in the MVEs of B-lymphocytes and dendritic cells were shown to be free vesicles and not stay attached to the limiting membrane (Murk et al., 2003). These are only two recent examples that are promising for further discoveries generated by the use of microscopy. Additionally, with the mapping of whole genomes, an enormous number of gene products require elucidation to which localization data often provides the first clue. It is therefore worth emphasizing that electron microscopy and IF confocal microscopy are complementary and irreplaceable methods and especially, that it is crucial for the research community to continue investments in EM facilities and the training of EM-experts.

Conclusions, Perspectives and Outstanding Questions

In this thesis we have focused on the role of ESCRT proteins and phosphoinositides in MVE biogenesis, endocytic traffic and pathogenesis. The included work has led to the following main conclusions:

I   The ESCRT-III subunit Vps24 is, similar to the other ESCRTs characterized so far, required for degradative trafficking of EGFR. However, in contrast to Tsg101/ESCRT-I, Vps24 does not play a role in the attenuation of receptor signaling.

II   ESCRT-II plays an important role in the degradative sorting of at least two important receptor families. Similar to Vps24/ESCRT-III and in contrast to ESCRT-0 and ESCRT-I, ESCRT-II is not involved in the silencing of EGF signaling from endosomes.

III   Depletion of ESCRT subunits or the overexpression of CHMP2B mutants inhibits autophagic degradation, leading to the accumulation of ubiquitin-positive aggregates that contain proteins associated with neurodegenerative disease.

IV   RILP is required for the trafficking of EGFR, and most likely other (ubiquitinated) receptors, for lysosomal degradation. RILP is involved in the biogenesis of MVEs, perhaps by coordinating this process with dynein-mediated endosome motility.

V   Depletion of the ESCRT machinery leads to a dramatic alteration in morphology of components of the endocytic pathway, yet, compartments stay clearly differentiated. MVEs can still be formed, indicating that ESCRT-independent mechanisms for the formation of ILVs and/or MVEs exist.

VI   Results demonstrate the usefulness of the dimerizer-inducable GFP-Fv-FYVE(Hrs) probe in time-resolved ultrastructural mapping of PtdIns(3)P but also caution that PtdIns(3)P sequestration may cause unexpected disturbance of endosomal functions.

During the past decade, parallel studies in yeast, *Drosophila* and mammalian cells have led to the elucidation of a great deal of the sophisticated machinery that is responsible for the degradative sorting of ubiquitinated receptors and, at least in part, the biogenesis of MVEs. Moreover, a number of additional functional roles of this machinery were discovered in, for instance, viral budding, cytokinesis and autophagy. With the accumulation of knowledge the complexity of cellular processes becomes apparent and brings along the challenge to fit all the acquired data, often from different model organisms, into one coherent model.

A recent comparative genomic analysis shows that ESCRT factors are well conserved across the eukaryotic lineage and complexes I, II, III and III-associated are almost completely retained, indicating an early evolutionary origin (Leung et al., 2008). Interestingly, homologues of eukaryotic ESCRT-III components and the ATPase Vps4 were found to play a crucial role in the process of cell division in the prokaryote *Sulfolobus acidocaldarius* of the domain of Archaea (S.D. Bell, ESCRT meeting Cambridge 2008, unpublished). This is proposed to be reflective of an ancesteral role for at least part of the ESCRT machinery in cell division and supports data found in eukaryotes that besides their role in the sorting of membrane proteins, ESCRT proteins play an important role in regulating membrane dynamics. Their regulation mechanisms are not yet clarified, however, and two crucial questions that remain unanswered are how ESCRTs promote inward vesiculation of the endosomal membrane and how sorting and signaling events and MVE biogenesis are coupled mechanistically.

In mammalian cells, MVEs play other pivotal roles besides their functions in the sorting of cargo for lysosomal degradation and growth regulation. In several cell types, for instance B cells and T cells, dendritic cells, neuronal cells and tumor cells, MVE are known to have the capability to fuse with the plasma membrane and secrete their ILVs, now called 'exosomes', into the extracellular environment (van Niel et al., 2006). The protein and lipid composition of exosomes reflects the specialized function of their original cell. For instance, B lymphocytes are known to secrete exosomes during exocytic fusion of the multivesicular MHC class II compartment with the plasma membrane, hereby mediating antigen presentation and activation of the immune response (Raposo et al., 1996). Additionally, the release of exosomes provides a mechanism of intercellular communication and can

potentially function as disease biomarkers and vaccine candidates (Schorey and Bhatnagar, 2008).

Given the complexity of the ESCRT machinery, it seems surprising that not more ESCRT subunits are found to be mutated in diseases to date. It is, however, important to emphasize that the ESCRT machinery is essential for viability (Komada and Soriano, 1999) and that dominant ESCRT mutations may cause prenatal death. The more recently established link between the molecular mechanisms of ESCRTs and pathogenesis highlights the importance of unraveling the biochemical properties of the ESCRT machinery and its role in physiology and pathogenesis. Ultimately, the ESCRT machinery may be a beneficial marker in diagnostics and an advantageous target for therapy within cancer, neurodegeneration and viral infection.

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