Manipulating endosomal systems: the molecular mechanisms of transport decisions and Salmonella-induced cancer

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Chapter 1

An introduction to endocytosis

In order to maintain homeostasis, the eukaryotic cell uses a multitude of ways to regulate its internal functions. Various organelles perform, sometimes cell-type specific, functions to facilitate processes such as cell growth, proliferation, differentiation, energy production and migration. A key process that connects the workings of all of these organelles is endocytosis (*Schmid et al., 2014*). During endocytosis cargo molecules are engulfed by a lipid bilayer membrane, forming an endosome, that then mediates transport of cargo in the cells. Through this system, the cell is able to take up molecules including activated receptors and nutrients from the plasma membrane, exchange cargo between organelles or introduce newly synthesized molecules for transport towards their site of function. An opposite mechanism, called exocytosis, also takes place where intracellular cargo is released in the surrounding space after vesicle fusion with the plasma membrane. By organizing transport, a major role of the endosomal system lies in the degradation of cargo molecules. Endocytosis results in a gradual acidification of organelles and the acquisition of hydrolytic enzymes. As a result, various cargo undergo degradation in the later compartments of the endocytic track, ending up in lysosomal compartments. Through lysosomal degradation the cell is able to acquire building blocks for new protein, lipids and carbohydrate synthesis, acquire other essential cellular compounds, regulate the availability of nutrients during starvation, as well as control the activity of enzymes and proteins entering the endosomal system. In addition, the acidic lysosomes can help in the control of potentially harmful entities such as pathogens and may eliminate unnecessary or dysfunctional molecules and protein aggregates by the process of autophagy (*Di Fiore and von Zastrow, 2014; Lim and Zoncu, 2016*). Altogether, the endosomal system functions as a highly diverse cellular system that consist of numerous vesicle subtypes, all of which work together to perform a wide and complex array of functions essential for the broad biology of cells.

The molecular players that regulate endocytosis

The endosomal system consists of numerous vesicles occupying various stages of maturation, which can be discriminated based on the different molecular landscapes present on their membranes. Most notable, at different stages of endosomal maturation, various members belonging to the Rab, Arf and Arf-like (Arl) families of small GTPases are associated with specific endosomal membranes (*Stenmark, 2009; Numrich and Ungermann, 2014*). These GTPases are switchable molecules that alternate between an active GTP-bound and inactive GDP-bound state. Inactive GTPases are mainly cytosolic and without function. However, when activated, they become competent to associate with select endosomal membranes and recruit specific effector molecules to mediate key endosomal processes, including transport and fusion. In order to facilitate timely recruitment of GTPases to specific membranes, two types of molecules regulate the GTP/GDP switch. *Guanine Exchange Factors* (GEFs) facilitate the exchange of GDP for GTP, thereby activating the GTPases. In an opposing fashion, *GTPase Activating Proteins* (GAPs) promote hydrolysis of GTP to GDP, which renders the GTPase inactive and results in its release from membranes (*Bos et al., 2007; Fukuda, 2011*). By orchestrating timely recruitment of particular

GTPases to spatially and temporally discrete segments of the endosomal system, these molecules play a vital role in regulation of endosomal transport, fusion and maturation (*Stenmark, 2009*).

After uptake from the plasma membrane, newly formed *early endosomes* (EE) become marked by the GTPase Rab5 (*Woodman, 2000*). At this stage of endocytosis, extensive recycling back towards the plasma membrane can occur (*Goldenring, 2015*). By balancing internalization and recycling of proteins residing on the plasma membrane, the cell is able to regulate their surface levels and thus their interactions with the outside world. Later in the endocytic pathway, internalized vesicles undergo step-wise maturation initiated when these nascent endosomes fuse through the help of the tethering factor *Early Endosome Antigen 1* (EEA1) (*Simonsen et al., 1998*). At this stage, the cell has to decide whether to recycle cargo or send it further down the endocytic route towards degradation in lysosomes. In the latter case, a new GTPase termed Rab7 is recruited to the maturing endosomal membrane through a GTPase hand-over mechanism, which at the same time removes Rab5, resulting in a Rab7 positive *late endosome* (LE) (*Poteryaev et al., 2010; Nordmann et al., 2010; Huotari and Helenius, 2011*).

Characteristic to the divide between the early and late endosomal populations is their cellular localization. Upon maturation, endosomes move away from the periphery of the cell and towards the perinuclear region through a process that relies on GTPase function (*Wandinger-Ness and Zerial, 2014; Neefjes, 2017*). When activated, GTPases interact with effectors that bind specialized motor proteins (Figure 1, box 1). These motors can ‘walk’ over the microtubule cytoskeleton, thereby dragging their associated vesicles along. In order to move late endosomes towards the perinuclear area, Rab7, in combination with its effector molecule *Rab-interacting Lysosomal Protein* (RILP), recruits the Dynein motor (*Jordens et al., 2001*). Since motor proteins can only move in one direction over the polar-organized microtubules, Rab7-RILP-Dynein activity directs vesicles towards a region called the *Microtubule Organizing Center* (MTOC), close to the nucleus. In order to move late endosomes in the opposite direction towards the plasma membrane, Rab7 is able to recruit a different effector called *FYVE and coiled-coil domain containing 1* (FYCO1), which mobilizes Kinesin-1 (*Pankiv et al., 2010*). This member of the kinesin motor family transports organelles to the plus end of microtubules and therefore takes endosomes away from the MTOC towards the cell’s periphery. This enables transport of cargoes that pass through the late endosomal compartment on their way to the plasma membrane, as illustrated by the immune receptor *Major Histocompatibility Complex II* (MHCII). Here, in the acidic late endosomal environment, MHCII acquires peptides to be presented at the cell surface to the immune system. Once loaded with a cognate peptide, MHCII needs to be transported to the plasma membrane in order to instigate the immune response (*Neefjes et al., 1990; Neefjes et al., 2011*). Although transport events, such as described for MHCII, move cargo in a net outward direction, in reality, fast loading and unloading of transport complexes ensures endosomal transport to occur fast and in a bi-directional and stop-and-go fashion. This high mobility increases the chance of interactions between endosomes themselves and between endosomes and other

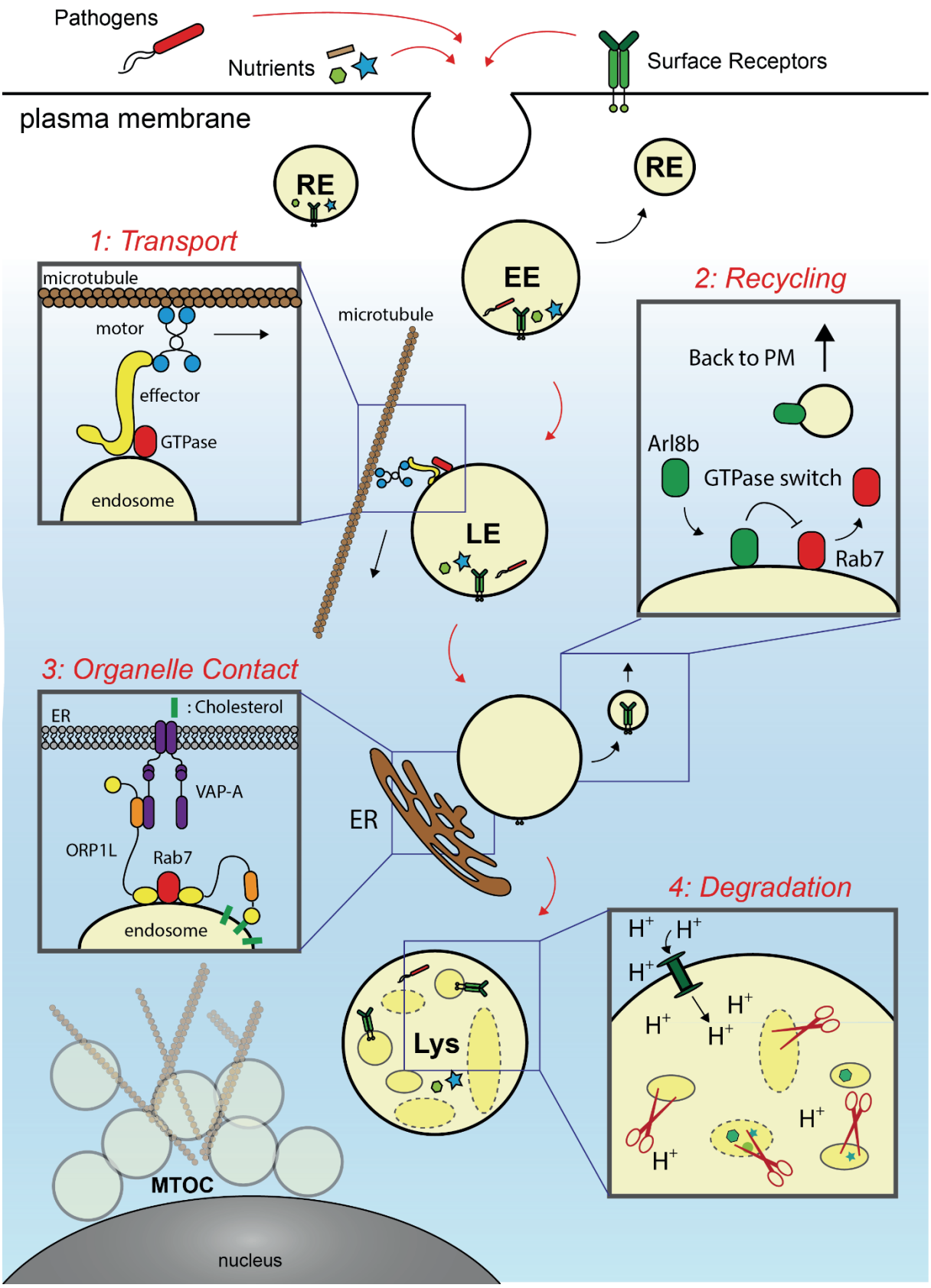

Figure 1: The endocytic route in a nutshell
At the plasma membrane a plethora of cargo, including pathogens, nutrients and activated receptors, are targeted for internalization in the endocytic track. Upon internalization through (***Legend continues on next page***)

organelles, allowing high levels of regulation and increased endosomal functioning (*Bonifacino and Neefjes, 2017*).

Deciding the fate of endocytosed cargo: degradation or recycling?

The final stage of minus end-directed cargo transport is its entry into the lysosomal compartment (Lys). Not only cargo retrieved from the plasma membrane, but also cytoplasmic material taken up through the process of autophagy is delivered to this compartment for degradation (Figure 1, box 4) (*Luzio et al., 2007*). In the first step towards degradation, membrane-bound molecules are internalized from the limiting endosomal membrane into intraluminal vesicles (ILVs) in a sequence of events mediated by proteins belonging to the *endosomal sorting complexes required for transport* (ESCRT) machinery (*Henne et al., 2011*). This process is initiated by the recognition of ubiquitin-tagged cargoes on the endosomal membrane by specific ESCRT molecules, resulting in the recruitment of the full ESCRT machinery complex. This complex then initiates the uptake of membrane cargoes into ILVs, resulting into the formation of so-called Multi-vesicular Bodies (MVBs). This process halts protein Once removed from the cytoplasm and taken up in the LE/Lys compartment, cargo is

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membrane budding and scission from the membrane the cargo enters the early endosomal compartment (EE). At this stage, extensive recycling back to the plasma membrane takes place. When not selected for fast recycling, cargo can be sent further down the endosomal pathway where maturation results in the formation of the late endosomal (LE) and lysosomal (lys) compartments. Upon maturation, endosomes move away from the peripheral region where EEs reside towards the perinuclear region. In order to move across the microtubule cytoskeleton, endosomes recruit effector molecules through their activated GTPases on the cytosolic site of the vesicular membrane. Effector molecules subsequently recruit various proteins including motor proteins, which allow the endosomes to move along actin or microtubules (box 1). A net recruitment of inward directed transport complexes results in the formation of the perinuclear cloud of late endosomes close to the microtubule organizing center (MTOC). Although inward transport moves endosomes and their cargo in the direction of lysosomal degradation, cargoes can be extracted from the endosomes for recycling. In order to avoid further degradative processing, newly formed vesicles are loaded with other regulatory protein complexes. New GTPases are recruited sometimes at the cost of existing GTPases (box 2). These newly formed vesicles are able to recycle cargo back towards the plasma membrane and avoid endosomal regulation that primes them towards degradation.

During endosomal maturation, the number of contacts with other endosomes increases, which further stimulates maturation through endosomal tethering and fusion. Also, contacts with other cellular organelles occurs, most notable the endoplasmic reticulum (ER). The ER membrane harbors many regulatory proteins that control positioning and movement of LEs. Through the Rab7 associated cholesterol sensor ORP1L ER-contact sites are formed dependent on the levels of cholesterol in the endosomal membrane (box 3). When levels of cholesterol are high, the sensor domain of ORP1L binds to cholesterol, resulting in a protein formation that allows recruitment of the transport complexes. When levels of cholesterol are low, the conformation of ORP1L changes, which allows its FFAT domain to interact with the ER resident molecule VAP-A. The resulting ER tethering stops endosomal movement and allows for ER mediated endosomal regulation.

Finally, endosomal cargo reaches the lysosomal compartment (box 4). Due to import of protons, the endosomes acidify. This promotes the activity of endosomal degradative enzymes, which break down endosomal cargo, as a final step in endosomal transport.

set for degradation. Through proton pumps and chloride ion channels, protons (H+) are imported inside the LE/Lys lumen. The resulting low pH (4,5-5,0) is ideal for activation of lysosomal enzymes responsible for proteolysis (*Hu et al., 2015*). Following degradation, cells either clear themselves from the remnants or use them as crucial nutrients for cell maintenance during starvation.
Not all molecules entering the endosomal system are targeted for degradation. These obviously include the lysosomal enzymes. To avoid degradation through bulk flow towards the lysosomal compartment, cells possess mechanisms to salvage specific cargoes throughout the endocytic pathway through a process generally termed recycling (Figure 1, box 2) (*Taguchi, 2013; Goldenring, 2015*). To this end, selected cargoes are sorted inside vesicles budding from the maturing endosome. These buds or tubules recruit transport motor machinery for transport away from the mother endosome, often towards the cell periphery and/or the plasma membrane. In order to avoid the Rab5-to-Rab7 conversion poised towards degradation, recycling GTPases must take over. During the early stages of endocytosis extensive recycling takes place through recruitment of Rab11 and Rab35 amongst others (*Ullrich et al., 1996; Chaineau et al., 2013*). However, recycling GTPases, such as *ADP-ribosylation factor-like protein 8b* (Arl8b), can mediate cargo transport away from the LE/Ly compartment and thereby provide the possibility to salvage materials just prior to the final stage of endocytosis (*Hofmann and Munro 2006; Rosa-Ferreira and Munro, 2011*). How acquisition of the LE/Lys recycling GTPase Arl8b occurs, along with the concomitant removal of the previous GTPase Rab7 is described in *Chapter 3* of this thesis.

Lipids: intrinsic timers of endocytosis
In order to regulate all steps of endocytosis, it is important for cells to control the molecular landscape on endosomal membranes. This is accomplished through recruitment of GTPases, and by extension their effector molecules, at the right place and time along the endocytic route. Crucial to this regulation is the lipid content of the endosomal membrane. The membrane itself is formed by a bilayer of phospholipids, which consist of a hydrophilic ‘head’ and one or two hydrophobic alkyl ‘tails’. During endosomal maturation, the lipid content of this membrane bilayer continuously changes and functions as a molecular ‘clock’ to determine which complexes will be preferentially recruited at any given time. A key membrane lipid involved in this temporal control is phosphatidylinositol (PI) (*Jean and Kiger, 2012; Schink et al., 2016*). This molecule can be phosphorylated by PI kinases on one -or multiple- of the 3-, 4- and 5-hydroxyl groups of the inositol head group, resulting in 7 possible phosphorylation combinations. These PI forms are present at different stages of the endosomal track, where they mediate direct and indirect recruitment of key proteins to endosomal membranes. Exchange of the PI content in the membrane provides the basis for the cascade of regulatory proteins involved in membrane dynamics throughout endocytosis. For example, early in endocytosis PtdIns(4,5)P2 promotes vesicle internalization through direct recruitment of the μ2 subunit of the AP2 adaptor complex, which mobilizes the clathrin coat for clathrin-mediated endocytosis (*Rohde et al., 2002; Schink et al., 2016*). Upon separation of the endocytic vesicle from the

plasma membrane, PtdIns(4,5)P2 is dephosphorylated to PtdIns4P, resulting in the release of AP2 and uncoating of the vesicle (*Cremona et al., 1999*). Further down the endocytic pathway, rising levels of PtdIns3P on the endosomal membrane attract factors that instigate recruitment of Rab7 and subsequent removal of Rab5, effectively targeting endosomes for inward transport and increasing maturation (*Zoncu et al., 2009; Poteryaev et al., 2010*). Changing PI content on the membrane has also been found to facilitate other endosomal processes, such as recycling and exocytosis, by targeting specific protein machinery to the appropriate endosomal membranes at the right time (*Schink et al., 2016*).
In addition to PI, cholesterol (another membrane lipid) is also involved in the regulation of endosomal trafficking. Through the Rab7-associated cholesterol sensor *Oxysterol-Binding Protein-Related Protein 1L* (ORP1L), late endosomes are able to respond to varying levels of cholesterol on endosomes (Figure 1, box 3). When cholesterol levels are high, the sensory ORD domain in ORP1L recognizes and binds to cholesterol in the endosomal membrane. The resulting configuration allows Rab7 to attract the machinery for minus-end microtubule-based transport consisting of the multi-subunit *Homotypic fusion and vacuole Protein sorting* (HOPS) tethering complex and the $p150^{glued}$ subunit of the dynein motor (*van der Kant., 2013*). Conversely, in the absence of cholesterol, the ORD domain remains untethered to the endosomal membrane, exposing its FFAT motif to bind VAP tethering molecules on the *endoplasmic reticulum* (ER). This leads to the release of the minus end-directed transport machinery and formation of a *membrane contact site* (MCS) between the endosome and ER (*Rocha et al., 2009; van der Kant et al., 2013; Wijdeven et al, 2016*). Due to increasing levels of cholesterol during endosomal maturation, ORP1L functions as a timer for endosomal transport and fusion towards the endo-lysosomal system. Hypothetically, this also results in the retention of low cholesterol, ER tethered vesicles and their cargo keeping them available for further regulation and potential recycling. Recent advancements in our understanding of the interactions between endosomes and the ER have shown that the ER plays a much larger role in regulating endosomal transport than previously thought. Importantly, the positioning within the cell dictates the behavior of endosomes. While the peripheral pool of endosomes is highly mobile, the endosomal ‘cloud’ located closer to the nucleus is far more stationary, a situation which favors tethering, fusion and maturation. The ER plays a pivotal role in keeping this organization in place. By regulating the ubiquitination landscape on the late endosome through the ER-located ubiquitin ligase RNF26, the ER delegates retention, but also the recruitment of multiple regulatory transport complexes towards the endosomal membrane (*Jongsma et al, 2016; Neefjes et al., 2017*). Combined, this indicates that based on lipid content, especially by cholesterol, endosomal positioning, fusion and movement are placed under control of regulatory systems such as the ER.

Endocytosis and disease
Endocytosis plays a critical role in many vital cellular processes, ranging from protein homeostasis and control of nutrient availability to regulation of signaling cascades. Consequently, failure to regulate endocytosis could result in numerous diseases

associated with these processes (*Maxfield, 2014*). Degradation of cargo molecules is an important function of the endocytic pathway, and when flaws occur in this process, stacking of unwanted proteins or (glyco)lipids can take place in the lysosomal compartment. The resulting lysosomal storage diseases can have a severe impact on the health of the cell (*Ferreira and Gahl, 2017*). This is best exemplified by the diseases associated with erroneous processing of cholesterol, in itself a regulator of endocytosis. In mammalian cells, intracellular cholesterol can be derived either through synthesis in the ER or uptake from LDL/HDL particles via *Low-Density Lipoprotein* (LDL) receptors (*Goldstein and Brown, 2009*). Due to increasing levels of fat and cholesterol in the Western diet, endosomal biology is also affected by large amounts of cholesterol. Various diseases affecting endosomal lipid handling (including Gaucher and Niemann Pick disease) all result in neurological diseases and early mortality. Increased cholesterol levels and their associated cardiovascular disease, make the failure to maintain a healthy cholesterol-balance one of the leading causes of death worldwide (*Röhrl and Stangl, 2013; Maxfield, 2014*).

Intracellular handling of cholesterol is affected not only through imbalanced dietary intake, but can also be deregulated in genetic diseases. An extensively studied example of such lysosomal storage diseases is Niemann Pick disease C. Luminal endosomal proteins *Niemann Pick disease protein 1 and 2* (NPC1 and NPC2) work together to transfer the cholesterol residing in the LE to the ER. Mutations in *NPC1* and *NPC2* genes then results in elevated cholesterol storage in the lysosomal compartment. This phenotype clinically manifests itself through a wide spectrum of symptoms, depending on the cell types and tissues affected (*Xu et al., 2007; Gelsthorpe et al., 2008; Neefjes and van der Kant, 2014*).

Although cholesterol is not able to pass the blood-brain barrier, nerve cells can also succumb to endocytosis related diseases (*Perret et al., 2015; Solé-Domènech et al., 2016*). One major example is Alzheimer's disease (AD), which is caused by plaque formation upon mishandling of (amongst others) the *Amyloid Precursor Protein* (APP), resulting in neurodegeneration and loss of nerve functioning and viability (*Hu et al., 2015*). Newly synthesized APP passes through the ER and Golgi and is transported through the endosomal system to the plasma membrane where it is reportedly involved in synapse formation and stability (*Montagna et al., 2017*). At various stages of endocytosis and at the plasma membrane, specific secretases cleave APP, generating Aβ peptides that are prone to aggregation and constitute the main component of AD-related plaques (*Toh and Gleeson, 2016*). Recent studies have shown the role the endosomal system in the handling of APP and its contribution to Alzheimer's disease (*Woodruff et al., 2016; Kimura and Yanagisawa, 2017*).

Besides affecting storage and processing related processes, defects in endocytosis have also been extensively reported in oncogenesis (*Mellman and Yarden, 2013)*. Like healthy cells, tumor cells rely on the endosomal system to maintain homeostasis. Additionally, tumor cells require a functional degradation system, which enables them to cope with stress and nutrient starvation through the process of autophagy (*Mathew et al., 2007*). Besides its function in cell maintenance, endocytosis also plays a more direct role in oncogenesis. This is best illustrated by the role of endosomes in the

turnover of signaling receptors. In order to keep proliferative signaling in check, activated receptors are endocytosed and targeted for lysosomal degradation to allow temporal signaling. The balance between receptor activation and endosomal degradation tunes the cellular response to proliferating stimuli. When this balance shifts away from the latter, oncogenic signaling pathways, including RAS, *mitogen-activated protein kinase* (MAPK), *mammalian target of rapamycin* (mTOR) and *epidermal growth factor receptor* (EGFR), can be impacted to stimulate tumor formation through increased growth and differentiation signaling (*Sorkin and von Zastrow, 2009; Schmid, 2017; Bakker et al., 2017*).

Concluding remarks

The endocytic system is a highly complex and dynamic system that allows the cells to regulate a wide variety of processes. By transport of cargo, it affects the activity of catalytic molecules or mediates their degradation. It also allows the cells to respond to extracellular cues through handling of activated receptors and to cope with stress and nutrient deprivation. Although the molecular players are known of many endosomal processes, how these systems are activated at the right place and time is poorly understood. Chapter 2 of this thesis will further dissect the function of the endocytic pathway in relation to the EGFR, the primary transmembrane tyrosine kinase signaling molecule that is processed by endosomal uptake after ligand binding to terminate signaling. In Chapter 3, I will discuss in detail a newly identified mechanism for cargo retrieval from the LE/Lys compartment for recycling to the periphery. These chapters illustrate the complex temporal and spatial control of endosomal processes in cells.

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