Chapter 1: Introduction
Introduction

Yeasts are unicellular eukaryotic organisms that mankind has used for biochemical processes for millennia. Fermentation, a metabolic process in which a carbon source is converted into a product in the absence of exogenous electron acceptor, e.g. the conversion of glucose into ethanol, was already used to produce beer in ancient Egypt. A Dutch scientist, Antonie Philips van Leeuwenhoek (1632-1723) first saw yeast cells under the microscope in 1680. He watched globules floating through beer. The globules of yeast were formed by 6 smaller globules, which were “of the same size and fabric as the globules of our blood”. He observed single globules, and smaller aggregates, but he assumed the globule formed of 6 smaller globules to be “a perfect globule of yeast” and all singlets tend to this state. Van Leeuwenhoek thought that yeast is formed from flour by dehydration and rehydration; he made the observations 79 years before the cell theory was formulated. The first impact of science on the brewing process can be traced to Emil Christian Hansen (1842-1909), who worked at Carlsberg Laboratory in Copenhagen, Denmark. Hansen realized in 1883 that the yeast used for brewing consisted of a mixture of different strains. Hansen then succeeded in the isolation of strains and made the brewing of beer more consistent by using single strains. The word enzyme, first used by Wilhelm Kühne (1837-1900), comes from Greek ἔνζυμον, which means “in yeast”. Yeast extracts were used by Eduard Buchner (1860-1917) to show that living cells are not required for fermentation, for which he was awarded the Nobel Prize in 1907. *Saccharomyces cerevisiae*, a budding yeast, was the first eukaryote of which the genome was fully sequenced. *S. cerevisiae* is an often-used model organism for biochemical and cell biological research. It is an eukaryote, which is relatively easy to work with. In many aspects it serves as excellent model of more complex eukaryotic organisms. On top of that, yeasts are heavily used in the biotechnological industry, and it is difficult to name all the processes in which yeast is applied. A report by Business Communications Company Research stated that the global market for yeast products reached $7.1 billion in 2016, and the predictions are that in 2022 the number will have grown to $10.7 billion. Yeasts produce a lot of different chemicals and proteins, but purification of biochemicals from the cells is still costly. For biotechnological applications it is generally beneficial if the cells can excrete the commodities. To make that possible, we need deeper understanding of the yeast plasma membrane and plasma membrane proteins, especially the delivery, stability (turnover), and mobility of the proteins.

In my PhD thesis I have used *S. cerevisiae* to study the dynamics and localization of the proteins in the plasma membrane (Chapters 3 and 4). A brief introduction on the traffic of proteins to and from the plasma membrane is given below. An overview of the diffusion of proteins in biological membranes is given in the
introduction to Chapter 4. Part of my thesis work required improving the surface immobilization of cells to allow for measurements of protein localization and dynamics with higher precision; the newly developed immobilization method is presented in Chapter 2.

**Transport of proteins to the plasma membrane**

In eukaryotes, ribosomes bound to mRNA for plasma membrane proteins are associated with the endoplasmic reticulum (ER). Most plasma membrane proteins are inserted into the ER membrane during the translation process. Subsequently, the proteins are transported via the Golgi to the desired compartment, while being modified during the trafficking. ER to Golgi transport in yeast is dependent on a cytosolic GTP-binding protein Sar1p, which initiates this trafficking process. When Sar1p is placed under control of the *GAL1* promoter, cells can grow on galactose (the Sar1p protein is produced in these cells), but the cells die upon transfer to glucose medium, in which the expression of *SAR1* is repressed. Dead cells accumulate proteins in core-glycosylated form, as they remain in the ER before further processing can occur. There are special regions in the ER named ER exit sites (ERES), or transitional ER in higher eukaryotes, where formation of vesicles for travel towards the Golgi takes place. The transport between ER and Golgi happens via COPII vesicles, which are formed *in vitro* when Sar1p, Sec13p and Sec23p complexes are present. Sar1p initiates the process of formation of COPII vesicles when it is in the GTP-bound state. Subsequently, the Sec23p complex is recruited, which in turn recruits the Sec13p complex. These proteins form a COPII coat, which causes deformation of the membrane and release of the vesicle. The hydrolysis of GTP is followed by release of Sar1p and disassembly of the COPII coat, which is necessary for the fusion of the transport vesicles with the Golgi membrane. The recruitment of ER proteins to COPII vesicles is dependent on an ER export signal, but many of the recruited proteins have none of the known localization signals.

The Golgi is made of multiple compartments called cisternae. ER transport vesicles fuse with the cisternae (early, or *cis* Golgi), which are different from the cisternae from which proteins are transported out of the Golgi (late, or *trans* Golgi). Proteins travel from the *trans* Golgi to specific membranes, such as the plasma membrane, via exocytotic vesicles. Maturation of the proteins occurs while they progress from the *cis* to *trans* Golgi. The *cis*-Golgi markers Rer1p, Sed5p, or Vrg4p have been labeled fluorescently as have the *trans*-Golgi proteins Gosp1 or Sec7p. Tracking cisternae over time was used to determine whether cisternae are stable compartments between which proteins are transported, or whether they mature together with the cargo. None of the observed cisternae showed markers exclusively present in the *cis-* or *trans*-Golgi for the lifetime of the compartment. Instead, in multiple cisternae *cis*-Golgi markers were replaced by *trans*-Golgi
markers, while none of the cisternae changed from trans- to cis-Golgi. Thus, the compartments of the Golgi are not stable structures and the cisternae mature with the transported proteins.

In the trans-Golgi proteins are sorted and loaded into different vesicles depending on their target compartment. Plasma membrane proteins are transported via the secretory pathway. Randy Schekman was awarded the Nobel Prize in 2013 for his research on vesicular trafficking in yeast, in which he used temperature-sensitive mutants\textsuperscript{16,17}. The plasma membrane proteins, together with secretory proteins, are transported from the Golgi to the plasma membrane in two types of vesicles: One type carries mostly proteins that are to be secreted and are delivered to the plasma membrane in less than 5 minutes\textsuperscript{17}. The other type is more abundant, and probably targeted to specific places on the plasma membrane due to the presence of Snc1p, one of two SNARE proteins suspected to be in post-Golgi vesicles\textsuperscript{18}. SNARE proteins are present in the membrane of vesicles as well as in the target membrane. Physical interactions between vesicle- and membrane-bound SNARE proteins, followed by conformational changes, bring the membranes together to allow fusion. The delivery of the H\textsuperscript{+}-ATPase, Pma1p, to the plasma membrane, in the second type of vesicles takes more than 30 minutes\textsuperscript{19}. The reason behind the difference in time necessary for the delivery is not known. Most probably it is due to localized exocytosis via Snc1p containing vesicles, or to a different way of transporting vesicles inside the cell. The minimal complex of proteins that allows exocytosis, the exocyst, has been defined as a structure containing seven proteins: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and Exo70p\textsuperscript{20}. The exocyst localizes itself at the inner leaflet of the plasma membrane where the exocytosis will occur. It assists in the fusion of the vesicle with the membrane\textsuperscript{21}. The exocytosis of membrane proteins occurs with a preference for growing buds.

**Endocytosis**

Proteins that are no longer needed (and may become harmful to cells) are removed from the plasma membrane by endocytosis. Two different types of endocytosis are generally found in eukaryotic cells: (i) fluid-phase endocytosis, which is non-specific and happens continuously; and (ii) inducible endocytosis, which is triggered by proteins in the plasma membrane\textsuperscript{22}. Although a lot is known of exo- and endocytosis in yeast, there is to the best of my knowledge no specific information on the turnover of membrane lipids\textsuperscript{23–28}. However, in the fluid-phase endocytosis M\textsuperscript{ϕ} and L-cell fibroblasts internalize 3.1\% and 0.9\% of their surface every minute. This results in endocytosis of the entire surface area of the cell within 33 min and 2 h, respectively\textsuperscript{29}. In the yeast plasma membrane, which is more rigid and thick than a mammalian plasma membrane due to its high sterol content, an actin skeleton is
required for endocytosis. The actin skeleton provides the mechanical force needed to deform the PM and produce the vesicles\textsuperscript{24}. Wild type yeast cells do not have any actin skeleton-independent endocytosis pathways\textsuperscript{30}.

The minimal clathrin-coated vesicles are formed \textit{in vitro} from liposomes containing a nickel salt of 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (Ni\textsuperscript{2+}-NTA-DOGS), using polyhistidine-tagged epsin 1 fragment as an adaptor protein, clathrin and dynamin\textsuperscript{31}.

\textbf{Figure 1. Schematic representation of clathrin-dependent endocytosis.} Protein modules driving the different steps of the endocytic process are assembled sequentially from soluble cytosolic protein pools. After initiation of the endocytic site, cargo is recruited to this site and the membrane is shaped into an invagination, which is finally separated from the plasma membrane by scission. Uncoating releases the proteins of the endocytic machinery back to the cytosolic pool and releases a vesicle that can participate in intracellular membrane trafficking events. Source: Kaksonen and Roux (2018)\textsuperscript{32}.

Endocytosis of membrane proteins proceeds similarly in yeast and mammalian cells. During the initiation the dynamin is rapidly recruited after the clathrin and the adaptor protein 2 (AP2) complex are accumulated to their maximal level\textsuperscript{33}. After recruitment of dynamin the actin\textsuperscript{34}, N-WASP (Las17p in yeast\textsuperscript{35}), Arp2/3\textsuperscript{36} proteins, and Hip1R-cortactin complex\textsuperscript{37} arrive at the endocytic spot. A Hip1/R (Sla2p in yeast\textsuperscript{38}) accessory protein connects the clathrin with the actin skeleton, which starts pulling the clathrin-coated invagination away from the plasma membrane. Myosin VI has been reported to interact with the Dab 2 accessory protein and phosphatidylinositol 4,5-biphosphatate (PI(4,5)P\textsubscript{2})\textsuperscript{39}, whereas myosin 1E (Myo3/5p in yeast\textsuperscript{40}) binds a SH3 domain of the dynamin\textsuperscript{41}. Using the energy from GTP hydrolysis the dynamin contracts and at the same time makes a twisting movement, which results in supercoiling of membrane tubules. It is speculated that the myosin 1E pulls the dynamin towards the plasma membrane when the myosin VI pulls the clathrin-coated pit towards the cytosol, thereby separating it and creating a vesicle\textsuperscript{42}. The vesicle then fuses with a sorting endosome, a compartment with slightly acidic lumen\textsuperscript{43}. The lipid composition of the endosome membrane is different from that of the plasma membrane and together with the low internal pH accommodates dissociation of protein complexes. For example, a Toll-Like receptor
4 (TLR4) interacts with toll interleukin receptor adaptor protein MyD88 in a PI(4,5)P₂-dependent way in the plasma membrane. In early endosomes this complex is dissociated due to a lower concentration of PI(4,5)P₂⁴⁴.

Inducible endocytosis occurs when proteins are marked for internalization. Known endocytic signals can be grouped into three different classes: linear motifs, conformational determinants, and covalent modifications. The most studied linear motifs are YXXØ₄⁵, [FY]XNPX[YF]₄⁶, and [DE]XXXL[LI]₄⁷. Where X signifies any amino acid, Ø signifies a bulky, hydrophobic amino acid, and the square brackets list possible residues for that position. Unlike the linear motifs, the conformational determinants are unique to specific proteins such as R-SNARE or the Dvl2:μ2 complex⁴⁸–⁵⁰. Finally, mono-, or polyubiquitynation of the proteins⁵¹, or phosphorylation of hydroxyl amino acids⁵², can lead to internalization of membrane proteins. Ubiquitynation sites are different for each protein⁵³. The ε-amine group of lysine residues are ubiquitynated most commonly⁵¹,⁵⁴,⁵⁵, however the amine group at the N-terminus of a protein can also undergo this modification⁵⁶–⁵⁹, as well as cysteine residues⁶⁰,⁶¹. Phosphorylation in yeast is a protein-specific process in which (most commonly) serine, threonine, or tyrosine are modified with a phosphate group. More than 70% of the proteins in a large-scale study were phosphorylated by less than 3 out of 82 kinases tested. A number of phosphorylation motifs were identified with the most common being SXXD, where X signifies any amino acid⁶². It is not clear, however, to what extent these motifs and specific kinases induce endocytosis of the proteins. It is possible that each protein responds differently to phosphorylation. The internalized proteins can be recycled back to the plasma membrane, or degraded in the vacuole. Recycling of the proteins is discussed in the next section.

Recycling of membrane proteins

The membrane proteins without degradation signals are removed from the sorting endosome by creating lipid tubules with a diameter of about 50 nm. The high surface to volume ratio of the tubules separates the plasma membrane proteins from the soluble cargo⁶³. A hepatocyte-growth-factor-regulated tyrosine kinase substrate protein (HRS) binds polyubiquitynated proteins and clathrin⁶⁴. That interaction may prevent the pinching off of tubules in which ubiquitynated proteins are located, thereby keeping them in the endosomes. In yeast the HRS is part of an endosomal-sorting complex required for transport protein complex-0 (ESCRT-0). This complex can cluster on the membranes, which is enhanced by the presence of ubiquitynated proteins⁶⁵.

Some proteins are recycled back to the plasma membrane in a fast process that is dependent on a Rab4 GTPase⁶⁶. However, most endocytosed proteins are
transported from the sorting endosomes to the endocytic recycling compartment (ERC), which is morphologically and functionally distinct from the sorting endosomes. Transferrin receptors following this pathway return to the plasma membrane with a $t_{1/2}$ of 10 minutes. From the ERC the plasma membrane proteins are transported either to the plasma membrane or to the trans-Golgi for further sorting. Proteins not removed from the endosome for recycling are degraded in the vacuole.

In *S. cerevisiae* ubiquitynation acts as the main endocytic signal. When cells are grown in nitrogen-rich conditions, Gap1p, a general amino acid permease and a homologue of Lyp1p and Can1p, is (poly) ubiquinated, endocytosed, and degraded in the vacuole. Gap1p is not degraded when it is only mono-ubiquitynated, but then it is recycled back to the plasma membrane. Proteins that are ubiquitinated can stimulate formation of the clathrin-coated pits in *S. cerevisiae*, making the modification extremely important for regulating the protein composition of the plasma membrane.
Figure 2. Schematic representation of recycling of plasma membrane proteins. The model shows the post-endocytic itineraries of several molecules. The transferrin receptor binds its ligand, diferric transferrin; the low-density-lipoprotein receptor (LDLR) binds low-density lipoprotein (LDL); and the cation-independent mannose-6-phosphate receptor (CI-MPR) binds lysosomal enzymes. All of these membrane proteins concentrate into clathrin-coated pits, and their initial delivery site is sorting endosomes. The transmembrane proteins furin and trans-Golgi network (TGN)38 also enter through clathrin-coated pits. Most membrane proteins rapidly exit sorting endosomes and are either returned directly to the plasma membrane or are transported to the endocytic recycling compartment (ERC). Furin is retained in the sorting endosome as the sorting endosome begins to mature into a late endosome, and furin is delivered to the Golgi from late endosomes. From the ERC, essentially all of the LDLRs and transferrin receptors recycle to the cell surface. Transferrin, unlike most other ligands (for example, LDL), is not released from its receptor in the acidic environment of sorting endosomes. The two irons (Fe\(^3+)\) are released from diferric transferrin at the acidic pH and transported into the cytoplasm, but iron-free transferrin remains bound to its receptor until it is returned to the cell surface. At the neutral extracellular pH, iron-free transferrin is released from the receptor. About 80% of the internalized TGN38 and CI-MPR also returns to the cell surface, and the rest is delivered to the TGN. The CI-MPR can go from the TGN to late endosomes, where any ligand that is still bound can dissociate as a result of exposure to low pH. From the late endosomes, furin and free CI-MPR can move to the TGN, and molecules in the TGN can be delivered back to the cell surface. It is uncertain whether CI-MPR and furin are transported in the same or different vesicles between the TGN and late endosomes. The t1/2 values are approximate and cell-type dependent. Source: Maxfield and McGraw (2004)\(^67\).

MCC/eisosomes

The plasma membrane of \textit{S. cerevisiae} is not homogeneous. A complex microdomain has been described on the basis of optical and electron microscopy studies\(^70–72\). Many proteins are localized in distinct domains\(^71,73\). The H\(^+\)-ATPase, Pma1p, the most abundant protein in the \textit{S. cerevisiae} plasma membrane is excluded from membrane compartment of Can1p (MCC) and is present in the network around them, named membrane compartment of Pma1p (MCP)\(^71,73,74\). The MCC was first identified by fluorescence microscopy\(^73\) and its scaffold by so-called Bar-Amphiphysin-Rvs (BAR) domain proteins (Pil1p and Lsp1p). The scaffolding structure is known as the eisosome\(^75\) and stabilizes the furrow-like MCC structures\(^72\), which is necessary for localization of Can1p to MCC\(^76\). Hence, we term the structures MCC/eisosomes (Figure 3).
Figure 3. Schematic of the MCC/eisosomes. MCC (in blue) forms a discrete membrane structure that is stabilized by BAR proteins: Pil1p (purple) and Lsp1p (brown); they are essential for the formation of the invagination. Can1p (red) can enter the MCC/eisosomes. Sur7p (green) is one of the structural proteins of the MCC/eisosome. Pma1p (yellow) is excluded from the MCC and cortical ER (cER; gray) is excluded from the eisosomes. MCC, membrane compartment of Can1; MCP, membrane compartment of Pma1p.

In addition to Can1p, multiple transporter proteins have been shown to accumulate in MCC/eisosomes, including: Fur4p\(^77\), Tat2p\(^78\), Mup1p\(^79\), and Lyp1p\(^80\). The MCC/eisosomal proteins accumulate (partition) in the MCC/eisosomes in a substrate-dependent manner\(^78,79,81\). For Can1p mutants it has been shown that the protein is excluded from the MCC/eisosomes when it is in an inward open state\(^81\), that is under conditions that arginine is present in the medium. Furthermore, Can1p and Mup1p localization to the MCC/eisosomes depends on sphingolipids\(^79,81\).

The MCC/eisosome membrane scaffold is formed by two homologous proteins with BAR domains: Pil1p and Lsp1p. However, only Pil1p is necessary for the formation of the MCC/eisosomes\(^75\). In \textit{in vitro} Lsp1p and Pil1p assemble into helical structures\(^82\) and bind PI(4,5)P\(_2\) containing membranes forcing them to shape tubular structures\(^82,83\). Recruitment of Inp51p, a phosphatidylinositol phosphatase, might make the MCC/eisosome responsible for regulation of PI(4,5)P\(_2\) levels in the membrane\(^84\). Another protein that is found in MCC/eisosomes, independent of nutrition and cell cycle, is Sur7p, which function may be to tether other proteins to the MCC/eisosome. Sur7p is very stable and abundant tetranspanner protein\(^77\) and localizes exclusively to the boundary of the MCC/eisosomes\(^72\). Nce102p is the only transmembrane protein essential for the formation of the MCC/eisosomes. While Sur7p (and Pil1p) are localizing to the patches at the moment they emerge, Nce102p shows homogenous distribution in the plasma membrane until the bud reaches a diameter of around 1/3 of the mother cells suggesting that it is important at later stages of formation, or for stability of the eisosomes\(^85\). The MCC/eisosomes are stable for hours, which is longer than the cell cycle\(^71,73\) and the embedded proteins are protected from endocytosis\(^76,85,86\).
The goal of this thesis was to better understand amino acid transport in the plasma membrane of *S. cerevisiae* with the ultimate aim of engineering relevant transporters for amino acid export; specifically the efflux of lysine and arginine. Biochemical work to understand the energy coupling mechanism and biochemistry of the lysine and arginine transporters (Lyp1p and Can1p, respectively) was performed by other members of the group. Here, we report on the fate of the Lyp1p and Can1p proteins after they reach the plasma membrane. We used a combination of molecular biology and state-of-the-art optical microscopy methods to trace the fate of plasma membrane proteins in living yeast cells. There were indications that exocytosis and endocytic recycling are relatively fast compared to lateral diffusion of the proteins in the plasma membrane (see introduction to Chapter 4)\(^{87,88}\). This would imply that proteins may accumulate at certain sites in the membrane rather than distribute randomly, and this apparent polarity could have important physiological implications.

The slow mobility of proteins makes it technically difficult to quantify the diffusion coefficient. Experiments to measure diffusion usually take around 20 minutes, in which any movement of the cell introduces an error that cannot easily be corrected for. The super-resolution methods that we employed can calculate the localization of a protein with an accuracy of around 20 nm. This means that cells have to move less than 20 nm in 20 minutes and any treatment to immobilize cells should not affect their physiology. We discovered that the commonly used approach for immobilization of yeast produces background fluorescence, prompting us to search for a better method. In Chapter 2 we describe a new APTES-glutaraldehyde-based method to immobilize cells on glass surfaces that is suitable for high-resolution optical microscopy. Cells were immobilized for hours with negligible movement, while the cells stayed alive and divided. We show that the APTES-glutaraldehyde method can be used to immobilize various membranes as long as they contain primary amines accessible to the modified glass surface.

In Chapter 3 we determine the diffusion and localization of proteins in the yeast plasma membrane to find out whether the membrane is compartmentalized (e.g. due to the presence of fences and pickets, see introduction to Chapter 4) or whether the slow diffusion is an intrinsic property of the lipid composition (e.g. fluidity) of the membrane (Chapter 4). We measure slow diffusion of Can1p, Lyp1p and Nha1p, using Fluorescence Recovery After Photobleaching (FRAP), and observe that Lyp1p, like Can1p, localizes to the MCC/eisosomes in a substrate-dependent manner. Using the immobilization method described in Chapter 2, we pushed the limits of the microscopy and performed super-resolution measurements together with tracking of single particles. We show that the diffusion coefficients of Can1p, Nha1p, and
Pma1p are not affected by the proximity of the proteins to eisosomes, and did not find evidence for other compartments (confinement) that would explain the apparent slow diffusion. Can1p partitions in MCC/eisosomes, Nha1p can enter and leave MCC/eisosomes, whereas Pma1p is excluded from the structures. Moreover, we show that proteins are excluded from the MCC/eisosomes by steric hindrance, that is, when they have large cytosolic domains near the plasma membrane.

In Chapter 4, we introduce the history and current knowledge about lipid bilayers including the Fences and Pickets model. In our opinion, this widely accepted model for the plasma membrane of mammalian cells does not hold for yeast. In the experimental section of this chapter, we describe our attempts to understand the cause(s) for the slow diffusion of proteins in the yeast plasma membrane. The high fraction of saturated (sphingo-) lipids and ergosterol in the plasma membrane of yeast is most likely responsible for slow diffusion. We have tried to insert probes into the inner and outer leaflet of the membrane and determine whether both differ in their fluid properties. On top of that we measured the diffusion of Can1p-mNeonGreen protein as a function of temperature to further characterize the physical state of the yeast plasma membrane. Clearly, the yeast plasma membrane is in a more liquid-ordered state than that of bacteria or higher eukaryotes, but we have not yet found the ultimate answer for the cause of the slow diffusion.
References


