Summary
Living organisms are constituted by either one of two types of cells (smallest unit of self-reproducible life): prokaryotes and eukaryotes. Prokaryotic cells (bacteria), usually considered more primitive, are basically composed of a protective envelope and a membrane ("thin layer of lipids") that surround their cytoplasm (cellular space), which contains metabolites and their proteinaceous and genetic material. Eukaryotic cells have a more complex structure, because besides the membranes that surround them, these cells have additional membranes that enclose specific compartments in the cytoplasm called organelles ("small organs"). Examples of organelles are: nucleus, mitochondrion, vacuole, endoplasmatic reticulum and peroxisome.

Each one of these organelles has one -or sometimes more- specific function(s). For example, the nucleus acts as the storage compartment for most of the cell’s genetic material, while the mitochondria are the main place of energy generation ("gas station").

Among the organelles, peroxisomes draw attention due to their strong functional versatility. Despite the fact that their shape is very simple, the function of peroxisomes varies depending on the organism in which they occur, as well as on the growth stage of the cells or their environmental conditions. In fact, while peroxisomes of the human liver cells burn specific kinds of fat, peroxisomes of the filamentous fungus *Penicillium chrysogenum* are responsible for the production of the antibiotic penicillin. This versatility is due to the fact that peroxisomes in different cell types contain totally different sets of enzymes. Actually, we could say that the only common characteristic of peroxisomes is the ability to carry out oxidative reactions coupled to the production of harmful reactive oxygen species as byproducts (eg. hydrogen peroxide), which are, however, subsequently decomposed still inside the peroxisome. Nevertheless, the process of peroxisome formation (peroxisome biogenesis) is evolutionary conserved, meaning that despite the differences in their function, peroxisomes of different organisms contain very similar proteins in their membranes and are therefore, structurally similar.

The importance of the role of peroxisomes in human cells is best illustrated by the existence of several inherited human diseases that are due to a defect in the structure/composition of these organelles. These diseases, so far, cannot be cured and, in their extreme forms, result in the patients’ death during the first months or years of their lives.

*Yeast* species are small unicellular organisms, some of which are only slightly larger in size than normal bacteria. Strains of the genus *Saccharomyces* have been used for millenniums to make bread and wine. Yeast, as eukaryotes, resemble in many aspects and function similar to
higher evolved multi-cellular organisms like animals and plants. Because of this, the study of many biological processes in yeast contributes, in general, to our comprehension of these processes in higher organisms, even in man.

For the study of these small organisms in laboratories, billions of identical cells are grown at the same time, in solutions called *growth media* or on the top of a dish, which contains solid growth medium (due to the addition of agar, a substance with properties similar to gelatin). The composition of the growth medium is manipulated according to the aims of the scientist, but in general it is constituted by compounds needed for the cell growth, including: carbon source (glucose or another sugar, or alcohol like ethanol and methanol), nitrogen source, minerals and vitamins.

*H. polymorpha* is a yeast species that can be found in nature on decaying plant material (e.g. spoiled orange juice) and soil. During the growth of cells in medium containing glucose only one peroxisome is observed in each cell. However, when cells of this yeast grow on medium containing solely methanol as carbon source, the peroxisome multiplies so that at after 15-17 hours, numerous large peroxisomes can be observed in the cells (see scheme: figure 1A from chapter 1). During these growth conditions the *H. polymorpha* peroxisomes contain enzymes important for the utilization of this carbon source by the cells. Interestingly, when *H. polymorpha* cells grown on methanol are transferred to medium containing glucose, the production of these peroxisomes is aborted and the existing ones are destroyed by a very selective process. Firstly, membranes wrap around each peroxisomes sequentially to separate them from the *cytosol* (aqueous solution in which the cell organelles are dispersed), a process designated *sequestration*, and subsequently, the sequestered peroxisome fuses to another organelle called *vacuole*. The vacuole contains enzymes that are able to break down proteins and lipids and consequently, the peroxisome is degraded in the interior of the vacuole. In this way, simple molecules resulting from the destruction of the peroxisome can be re-utilized by the cell. In this view the vacuole can be considered a cellular “trash recycling unit”.

In the present study, *H. polymorpha* was used as model organism in peroxisome research. The main goal of this research was to find out the principles that govern the process of selective peroxisome degradation (also called *macropexophagy*) in *H. polymorpha*. As in *H. polymorpha* the peroxisomes induced by growth on methanol are relatively very large, the morphological and biochemical alterations that result from peroxisome degradation are very pronounced. Therefore, these alterations can then be easily monitored using specific types of microscopy and by quantification of the remaining amounts of a peroxisomal enzyme called *alcohol oxidase (AO)* few hours after the transfer of cells to medium containing glucose.
In order to carry out this research it was necessary to intervene in the \textit{H. polymorpha} cells at the level of the genetic material, the DNA. The genes of a cell dictate how proteins should be produced. The proteins determine the structure and the behavior of the cells. One way to understand how cells perform a biological process is to isolate and characterize the genes essential for this process. First, it is necessary to create \textit{mutants}. Mutants are cells that were subject to a type of treatment (chemical or other) that causes alterations in the DNA. In this way, genes suffer random perturbations (or \textit{mutations}) and as a consequence produce dysfunctional proteins or, eventually, no protein at all. Next, using specific assays, it is possible to select the cells that have mutations only in genes important for the process under study and to identify the affected gene. When the structure of the gene becomes known, it is possible, through DNA manipulating techniques, to modify the structure of a gene or even to delete it from the \textit{genome} (pool of genes of an organism) of the yeast. By comparing the behavior of normal cells (or \textit{wild type}) to the behavior of the mutant, or preferably of the strain deleted for the gene, it is possible to obtain information on the role that the mutated/deleted gene normally plays in the cell.

In the introduction of this thesis (chapter 1) a review on the current knowledge of peroxisome degradation processes in \textit{H. polymorpha} is presented.

In chapter 2 of this thesis we report the identification of the \textit{H. polymorpha MPP1} (methylotrophic peroxisomal protein regulator) gene. \textit{H. polymorpha} cells in which the \textit{MPP1} gene was deleted from the genome can not grow in media containing methanol as sole carbon source (a behavior called Mut-, from methanol non-utilizing). Using different techniques, we found that the \textit{MPP1} gene produces a protein responsible for the activation of many genes that are necessary for the development of peroxisomes that are normally induced by the methanol. Curiously, when the strain containing the deletion of the \textit{MPP1} gene (\textit{Δmpp1} cells) grows in media containing methanol and glycerol (an extra carbon source to support growth of \textit{mut-} cells), only one peroxisome is observed per cell, while in wild type control cells, grown under the same conditions, many peroxisomes are observed. This characteristic of \textit{Δmpp1} cells prompted us to test the already earlier developed hypothesis that (at least) one peroxisome of \textit{H. polymorpha} is not susceptible to degradation when the cells are transferred from media containing methanol to media containing glucose. We observed that, when \textit{Δmpp1} cells grown on media containing methanol and glycerol were shifted to glucose, the single peroxisomes present in these cells indeed were not affected and stayed intact. Experiments using wild type cells confirmed these results. Wild type cells were grown
for only 6 hours in media containing methanol. During this short period, the cells do not have time to multiply their peroxisomes and therefore, resemble \(\Delta mpp1\) cells in that only one peroxisome is observed in the vast majority of cells. Also in this case degradation of the single peroxisomes was not observed after the cells were shifted to glucose-containing medium. The mechanism that allows at least one peroxisome to be preserved is still unknown. Our current hypothesis to explain this is included in figure 3 of chapter 1 of this thesis. On the other hand, the physiological importance of this phenomenon is immediately recognized as it allows the cells to rapidly proliferate their peroxisomes in response to new environments that require new peroxisomal functions.

*H. polymorpha* mutants that have lost the ability to degrade peroxisomes are called *pdd* (an acronym for peroxisome degradation-deficient) cells. In chapters 3 and 4 of this thesis the identification of genes affected in mutants *pdd2* and *pdd15* is reported.

In the strain deleted for the *PDD2* gene occasionally sequestered peroxisomes were observed, however the sequestered organelles were not able to fuse with the vacuole. The *PDD2* gene is very similar to a gene of *Saccharomyces cerevisiae* (“baker’s yeast”) called *TUP1*. In *S. cerevisiae* the Tup1 protein localizes to the nucleus of the cell, where it functions in preventing a large variety of genes to produce their respective proteins. Nevertheless, at this moment the function of *PDD2* gene product in peroxisome degradation is only speculative.

The \(\Delta pdd15\) cells are only able to initiate the sequestration of the peroxisomes. Completely sequestered organelles were not observed. The Pdd15 protein shows similarity to three proteins of *S. cerevisiae*: Aut10p, Mai1p and Ygr223cp. Interestingly, Aut10p was reported to be essential for two transport routes to the vacuole, namely *autophagy* and *cytoplasm-to-vacuole targeting* (Cvt) pathway while Mai1p is essential only for the Cvt pathway and no role has been assigned to Ygr223cp yet.

*Autophagy* is a process that can be induced in various eukaryotic cells by limitation of the nitrogen compound from the environment. In mammalian and baker’s yeast cells autophagy takes place through a morphological process designated macroautophagy. When *S. cerevisiae* cells are transferred to growth medium lacking a nitrogen source, membranes wrap portions of the cytoplasm forming a specific kind of vesicles (*autophagosomes*) that can include various organelles and cytosol. Next, the autophagosomes fuse to the vacuole and are subsequently degraded together with their content. In this way the cell recycles dispensable cellular components during periods of nitrogen starvation.
The Cvt pathway is a process described for *S. cerevisiae* cells in which specific cargoes, two vacuolar enzymes called *aminopeptidase 1* and *alpha-mannosidase 1* (Ape1 and Ams1, respectively), are delivered to the vacuole where they function in hydrolysing molecules. Ape1 and Ams1 proteins are incorporated into vesicles called *Cvt-vesicles*. Subsequently the Cvt-vesicles fuse with the vacuole. The Cvt pathway is actually a process involved in the biogenesis of the vacuole, therefore quite different -in fact opposite to- autophagy and macropexophagy, which are degradative processes. Moreover, the Cvt pathway is not inducible but it happens constitutively ("all the time") during growth of the cells under nutrient-rich conditions.

Although the *H. polymorpha* Pdd15 protein seems to be more similar to Mai1p than to Aut10p, our results showed that the function of Pdd15p is also essential for degradation of peroxisomes upon induction of autophagy. However, autophagy in *H. polymorpha* happens in a different morphological way than macropexophagy or macroautophagy. In contrast to these two processes, the uptake of cytosol and organelles in *H. polymorpha* cells starved for nitrogen happens directly through engulfment of portions of the cytoplasm by the vacuolar membrane (see figure 2 of chapter 1). Sequestering membranes or autophagosomes are not observed. This mode of autophagy is called microautophagy.

The Pdd15 protein was localized at the vacuole and in the cytosol. The results described in chapter 4 corroborate to the conclusion that macropexophagy, Cvt pathway, macroautophagy and also microautophagy are related processes since they depend on the function of the same proteins. How these proteins are shared between processes that are so different in goals, induction factor and selectivity is unknown.

Recently, others in our group observed that selective peroxisome degradation and peroxisome biogenesis also share elements. Therefore, we sought to isolate novel mutants that were affected in these two oppositely directed processes (chapter 5). Selecting for cells with Mut and Pdd characteristics we found two mutants that were both affected in the same gene, namely *DAK*. This was quite surprising since the DAK protein is known to be an enzyme (dihydroxyacetone kinase), essential for the *methanol assimilation*, i.e., the formation of larger carbon-based structures (e.g. sugars and amino-acids) generated from methanol. Detailed analysis of the two *dak* mutants made clear that when grown on glycerol/methanol they display an aberrant peroxisome development pattern: only one very large peroxisome, eventually accompanied by a few very small ones. Another bizarre characteristic of the *dak* mutants is that during prolonged growth on medium containing glycerol and methanol, the peroxisomal enzymes (AO and others) were not anymore solely present inside the
peroxisomes but also in the cytosol. How the biochemical unbalance derived from the defect in methanol assimilation leads to all these peroxisomal disfuctions is topic for further research. Nevertheless, we were able to determine that the peroxisome degradation-deficient characteristic of the \emph{dak} strains suggested by our initial assays were most likely a side effect of both peroxisomal disfunctions. As explained before, our assays for the selection of \emph{pdd} mutants are based on the quantification of the remaining amounts of the peroxisomal enzyme AO a few hours after the induction of macropexophagy. However, the “last” (macropexophagy-resistant) peroxisomes of \emph{H. polymorpha} wild type cells (remember chapter 2!) are often smaller than the “last” (macropexophagy-resistant) peroxisomes of the \emph{dak} strains. Therefore, probably many of the “last” peroxisomes of \emph{dak} cells contain higher residual amounts of AO than the “last” peroxisomes of wild type cells. Moreover, the degradation of the AO upon induction of macropexophagy is dependent on its localization inside the peroxisomes (data already known from the literature). Hence, both the peroxisome proliferation defect and the mislocalization of AO to the cytosol possibly contributed to the initially observed relatively high AO levels after the shift of the \emph{dak} cells to peroxisome degradation conditions. In spite of that, the peroxisome degradation “machinery” of the \emph{dak} strains seems to function normally since, using microscopy, we observed that peroxisomes of these cells do fuse with the vacuoles upon induction of macropexophagy.