


Molecular Mechanisms of Autophagy in Yeast

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Science is a system of knowledge that is gradually accumulated over many years by society, but it is also an inherently human activity. I believe that every scientist is a product of the era in which they live. So I'd like to start from a brief introduction of my life, and then give a historical overview of my scientific work.

Early life

I was born in Fukuoka, southern Japan, in 1945, half a year before the end of World War II. This was a very challenging time in Japan, and everyone had difficulty getting basic daily necessities, including food. I myself suffered from severe malnutrition and was a very sickly child. Around this time my mother got tuberculosis and spent a long period bed-bound, but she was miraculously able to recover thanks to a gift of just-developed antibiotics from a family friend in Hawaii. When I was about 8 years old, my mother was finally able to resume a normal life. My brother, Kazuo, who is 12 and a half years my senior, left home to enter the Faculty of Literature at the University of Tokyo as I began elementary school. I was brought up through the efforts of my father and two sisters, Reiko and Junko, and many other people throughout what was a difficult time for our family.

My childhood home was surrounded by nature, with rice paddies, streams, hills and the sea all nearby. I spent a lot of time outdoors catching fish and picking plants. As an elementary school student, I was engrossed in collecting insects and watching the night sky. I would say that this intimacy with nature had a strong influence on me. My brother, every time he returned home from Tokyo during university holidays, would bring a book, such as carefully chosen works of George Gamow and Michael Faraday that broadened my awareness of the world beyond what we learned at school (Faraday, 1861; Gamow, 1940). Being a weak child, I had no talent for sports and was neither artistic nor gifted in literature. But I managed to achieve good results at school. At the time, I had a vague longing to be a scientist that was influenced by the expectations of my parents.

University & first experience of research

In high school, I joined the chemistry club, where I first experienced the wonder of chemical reactions. Partly thanks to this experience, I entered the University of Tokyo, where I initially hoped to become a chemist, but I had difficulty finding a field to specialise in. Thankfully, this was the time when the central dogma of molecular biology was just being established. Molecular biology immediately fascinated me, so I decided to join the lab of Kazutomo Imahori, who was one of the few scientists running a molecular biology laboratory in Japan at the time. Imahori's laboratory had adopted a physicochemical approach to assess interactions between proteins and nucleic acids. Under the direction of Akio Maeda, I began to study the role of ribosome subunits in protein synthesis. Although I was unable to produce particularly interesting results, my time in the Imahori lab was exciting as we participated in the work of a rapidly developing field and I got my first taste of the joy of experimental work. The experience also provided me with a strong sense of the continuous process of protein synthesis within cells.

From the second year of my doctoral studies, I decided to move to Kyoto University, where Maeda had joined the just-founded Department of Biophysics as an associate professor. Kyoto University provided a very free environment, and I was stimulated by talented new students and friends I met in the biochemistry lab of the Department of Chemistry. Throughout my graduate studies, the Summer Seminar for Young Biochemists also allowed me to make a large number of friends from all over Japan who would have a great impact on my life. In my research, I became interested in colicin E3, a toxic protein that is able to pass through the membrane of bacterial cells and instantly inhibits protein synthesis. I think I was also influenced by my growing interest in biological membranes during this period.

Post-doc life in New York

After I finished my doctoral studies, I enrolled in the lab of Gerald Edelman at The Rockefeller University, New York. I was asked was to establish an in vitro fertilisation system for mouse egg cells, which I reluctantly accepted. Experimentally it was not too difficult, but I had no idea how to examine such fascinating yet few early embryos. After some time, Mike Jazwinski joined our group from Arthur Kornberg's lab, where he had been studying phage DNA duplication. Taking inspiration from the elegant study of the cell cycle through the *cdc* mutants by Hartwell (Hartwell, 1974), a project to uncover the mechanism of DNA replication initiation in yeast began in Edelman's lab, which I joined in my final year. This was to be my first encounter with yeast as an experimental organism.

We had first planned to use isolated nuclei in this project, but it was difficult to obtain these organelles in an undamaged state. During our use of density-gradient centrifugation to purify nuclei, I noticed a white layer at the top of the centrifuge tube. Out of mere curiosity I looked at this white layer under the microscope, and realised that it was a highly-enriched vacuole fraction. The ease with which this organelle could be purified left a strong impression on me, and I think helped to decide the direction of my research.

Return to Japan and work on the vacuole

At the end of 1977 I returned to Japan as an assistant professor in Yasuhiro Anraku's lab at the University of Tokyo. Anraku's entire lab was tasked with studying the transporters and the respiratory chain in *E. coli*. In spite of this, Anraku allowed me to start a project on yeast, and in retrospect I really appreciate his decisiveness in affording me this opportunity, which resulted in the beginning of my nearly 40 years of work in yeast (Figure 1). In those days, the plasma membrane and its transport mechanisms were the focus of much research. But I have never been a competitive person, and prefer to work on a subject that few people are interested in. After a period of reflection, I decided instead to work on transport across the membrane of an intracellular organelle, the vacuole. While we now know that elaborate transport systems exist on the vacuolar membrane, this was by no means a fashionable topic.

At that time the vacuole was thought to be no more than a garbage dump in the cell, but I thought it must play yet unknown roles in cell physiology. First I established procedures for purifying vacuoles and making vacuolar membrane vesicles, and was able to show active transport systems of amino acids and calcium ions over the vacuolar membrane, providing evidence that the vacuole is

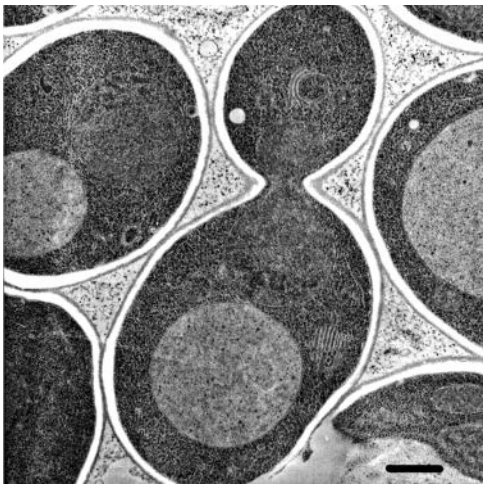


FIGURE 1. Thin-section electron micrograph of a yeast cell. The vacuole is visible as the large, pale region near the centre of each cell.

important for homeostasis of metabolites and ions (Ohsumi and Anraku, 1981; Ohsumi and Anraku, 1983). We also described a novel proton-pump on the vacuolar membrane, the V-type ATPase, which generates a proton gradient across the vacuolar membrane as a driving force of these transport systems (Kakinuma et al., 1981; Uchida et al., 1985) (Figure 2). These experiences played an important role in the development of my scientific interests, leading into my continued study of the vacuole and foreshadowing my research on autophagy. Since then,

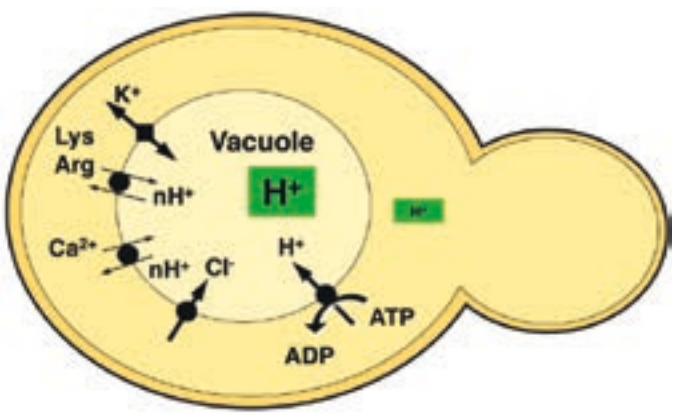


FIGURE 2. Vacuolar membrane transport systems in yeast. The V-type ATPase maintains the acidity of the vacuole by pumping H+ into the vacuole, while various active transporters modulate the flow of calcium, amino acids and ions between the cytoplasm and this organelle.

the structure and function of the V-type ATPase has become a major field of cell biology research in its own right.

STARTING MY OWN LAB AT TOKYO UNIVERSITY, KOMABA CAMPUS

In 1988 I was able to start my own lab—consisting of just myself—at the College of Arts and Sciences at the Komaba campus of the University of Tokyo. My lab here was modestly equipped, without any fancy instruments. I decided to devote myself to a related but completely novel task: the lytic function of the yeast vacuole. Since the vacuole is an acidic compartment, due to the function of V-type ATPase, and contains various hydrolytic enzymes including proteases, I thought it might be homologous to the lysosome of mammalian cells. However, I had no well-articulated idea how to test this hypothesis.

Thinking about the life of proteins

Before going into experimental details, I would like to briefly outline protein metabolism in cells. Proteins, which are a polymer of amino acids, are the key players in all biological processes. The central dogma of molecular biology states that protein is synthesised according to the genetic information encoded in DNA through RNA. As a consequence of this, many researchers throughout the latter half of the 20th century worked diligently to understand gene expression and its regulation, and the mechanisms of protein synthesis. Another important consideration that subsequently came to scrutiny was the compartmentalisation of the cell, as proteins are able only able to properly function after being delivered to the appropriate part of the cell. This spurred an effort to understand the localisation of each protein within the cell. Thanks to the efforts of researchers, we now know that each protein is intricately and precisely trafficked to the correct destination within the cell according to an intrinsic localisation signal (Figure 3).

However, one aspect that was missing from this picture when I started my own lab was the fate of proteins, or in other words the question of how intracellular protein degradation occurs. It was known that each protein has a distinct lifetime, ranging from a few minutes to several months. But for many years, the degradation of protein was thought to be a passive process that is not particularly important or interesting.

The significance of protein turnover

I used to begin biology class for first-year undergraduate students with this question: how many red blood cells are made within just one second in your body?

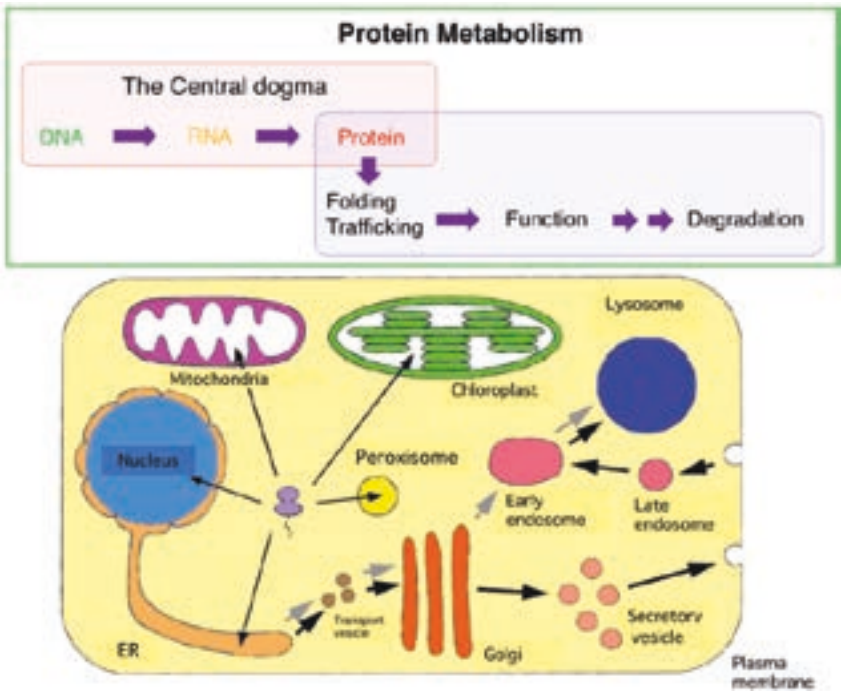


FIGURE 3. Overview of protein metabolism in cells. In addition to the emphasis on synthesis provided by the central dogma, the delivery, function and eventual degradation of proteins are important aspects of their life in the cell. The delivery of proteins to various cellular compartments following translation by the ribosome (purple) is represented at the bottom of the figure.

The calculation is quite simple and gives an answer of about three million cells per second. How about hemoglobin in the red blood cell? This number can also be easily derived, giving about $1x10^{15}$—one million billion—hemoglobin molecules per second. It follows that exactly the same amount of cells and proteins are degraded. The key message is that living things are extremely dynamically maintained.

The Japanese climate is characterised by four distinct seasons that influence our culture, and students at school learn that all things are in a cycle of continuous change. Plant leaves offer an excellent example of this concept. In spring, leaves develop and then actively synthesise starch by sunlight, but in autumn leaves become red or yellow and fall off (Figure 4). What we see as beautiful autumn colours is actually caused by the degradation of the leaf's photosynthetic machinery: green chloroplasts are completely degraded, and the resulting amino acids are transported to the trunk, leaving only the red or yellow-coloured

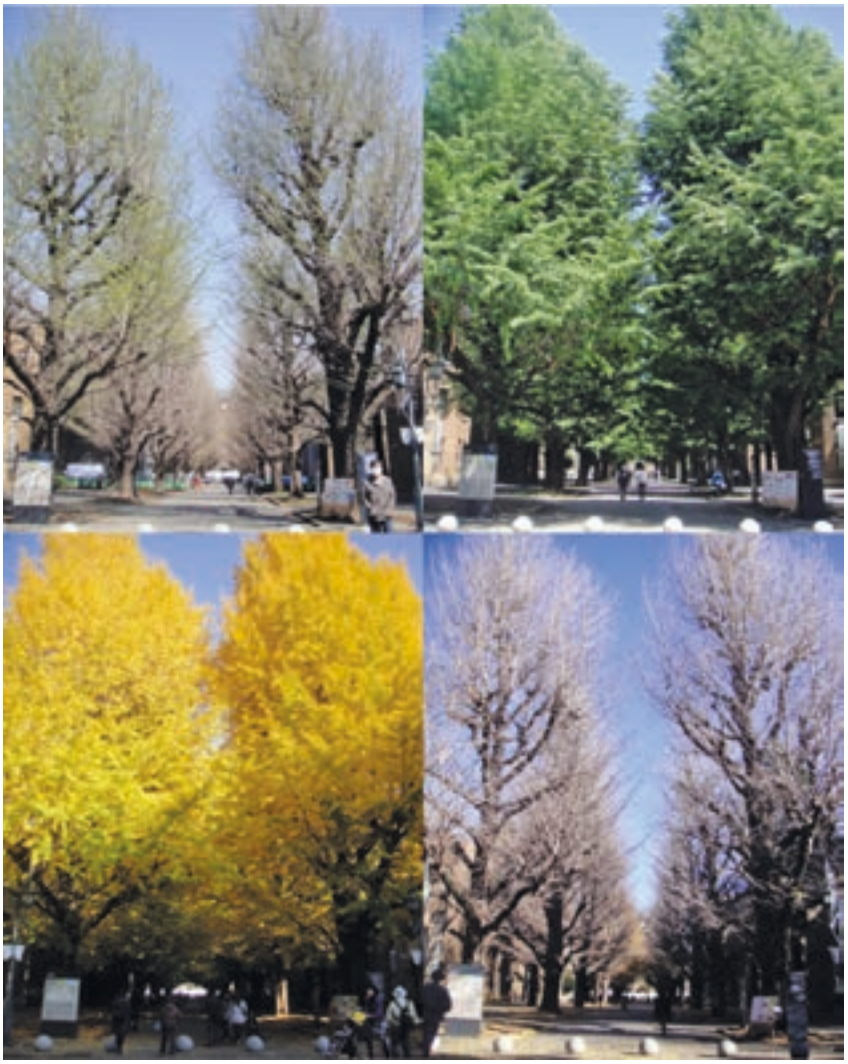


FIGURE 4. Ginkgo trees throughout the four seasons at the University of Tokyo. The removal of green chloroplasts in autumn, leaving golden accessory pigments in the leaves, is one striking example of the importance of degradation in the dynamic cycle of life.

accessory photosynthetic pigments in leaves. Similarly, rice leaves turn yellow at harvest time. All the protein in the leaves is degraded and transported to make proteins in rice grains for the next generation. These examples demonstrate that degradation is not an adverse process, but rather is essential for new construction or regeneration. Let's think about the human body. Our bodies make about 2–300 grams of protein every day, but we only intake about 70–80 grams of

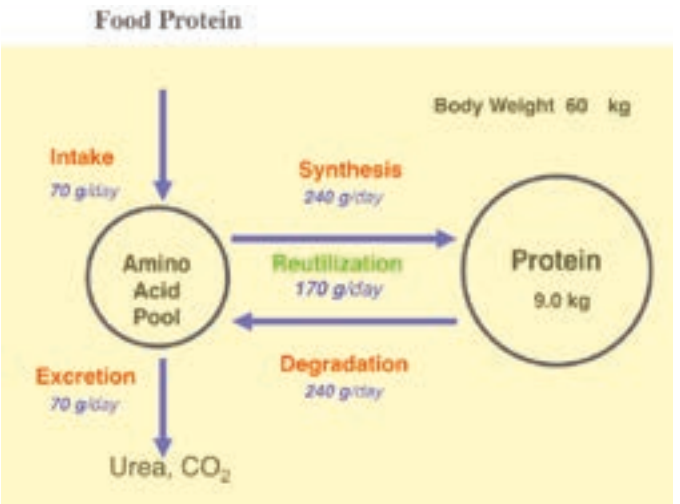
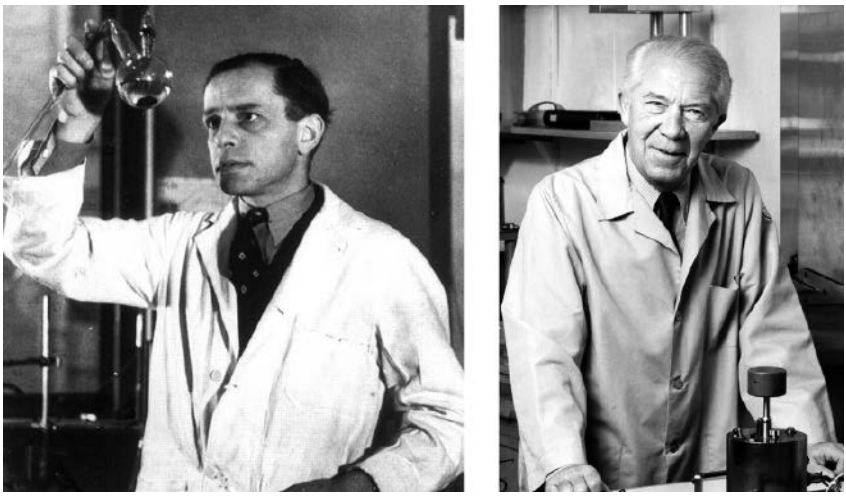


FIGURE 5. Protein dynamics in the human body. The bulk of amino acids required for synthesis are met not through dietary intake, but through the turnover of existing proteins in the cell.

protein from our diet (Figure 5). The amino acids required for protein synthesis mostly come from the degradation of the body's own proteins. Life is maintained by a tightly controlled balance between synthesis and degradation. Just two years ago I experienced this personally when I learned that my wife Mariko's mother had lived without any food and even water for 13 days. This was another shocking reminder that the recycling of cellular constituents is a potent force in the robust nature of life.

Historical perspectives on protein turnover

The establishment of the central role of proteins in biology was a very important development in modern biological science. In contrast to the process of synthesis emphasised by the central dogma, when I began my research the degradation of proteins was seen as a passive process that was not particularly important by many of my contemporaries. In this regard Rudolph Schoenheimer was a pioneer in the field, as he had proposed the concept of "protein turnover" in the 1930s following his development of isotopes to follow metabolic processes (Schoenheimer et al., 1939; Schoenheimer, 1942) (Figure 6A). Unfortunately, however, his contention was controversial and not widely accepted. After this, Christian de Duve, recipient of the Nobel Prize in 1974, used cell fractionation to discover the lysosome, an organelle containing a range of degradative enzymes (De Duve



(a) (b)

FIGURE 6. (a) Rudolph Schoenheimer and (b) Christian de Duve.

et al., 1955) (Figure 6B). Researchers at The Rockefeller University subsequently observed the delivery of extracellular material to the lysosome using electron microscopy (Novikoff et al., 1956). The delivery of intracellular material including organelles such as mitochondria to the lysosome was also observed. De Duve referred to these processes as 'heterophagy' (now known as endocytosis) and 'autophagy', respectively (de Duve, 1963). Autophagy, from the Greek for 'self-eating,' was described by The Rockefeller University researchers as having several hallmarks. Firstly, a membrane sac appears and enwraps a portion of the cytoplasm, forming a double-membrane structure, the autophagosome. Next, the outer membrane of the autophagosome fuses with the lysosome, and finally the inner membrane and its contents are degraded within the lysosome.

Autophagy research was continued by two key groups: one, led by Glenn Mortimore (Figure 7A), conducted detailed physiological and biochemical analyses of the phenomenon using liver perfusion in rats (Mortimore and Ward, 1976; Mortimore et al., 1983), while a second group, led by Per Seglen, examined the effect of nutrient starvation on autophagy and the regulation of autophagy, mainly using cultured cells (Seglen and Gordon, 1982; Seglen et al., 1980) (Figure 7B). However, as electron microscopy was the only means of assessing autophagy at the time and the lysosome is a dynamic organelle, most works focused on the analysis of membrane dynamics in autophagy, leaving the genes and proteins implicated in the autophagy mechanism unidentified for many years. A simpler model system was required to make further progress in the field.

(a) (b)

FIGURE 7. (a) Glenn Mortimore and (b) Per Seglen.

In the meantime, another intracellular degradation system, the ubiquitin/proteasome system, was discovered. As it became clear that this system plays important roles in the regulation of a variety of cellular processes, protein degradation suddenly attracted a huge amount of attention in biological research. This was ultimately recognized by the Nobel Prize in Chemistry in 2004, which was awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose, but the field of lysosomal degradation and autophagy remained quiet. Now it is thought that these two systems function together as the major means of cytoplasmic protein degradation.

Discovering autophagy in yeast by light microscopy

Assuming that the vacuole functions as a lytic compartment, the questions I had were when, what and how cytoplasmic constituents go across the vacuolar membrane and become accessible to vacuolar enzymes. First, I searched for a condition where massive protein degradation might occur in the yeast life cycle. Spore formation is a meiotic process that generates four spores and is induced by the depletion of nitrogen, the essential element of amino acids (Figure 8). I reasoned that this dramatic cellular remodelling should require the degradation of pre-existing proteins to make the proteins necessary for this cell differentiation.

I have to admit that I've always loved to observe yeast cells by light microscopy. The vacuole is relatively large, is the only clearly visible organelle in the cell and contains a salt-like solution that has a very low concentration of protein, so I knew that it would be easy to see structures if they were inside. I probably spent more time sitting at the light microscope than anyone else during my studies of

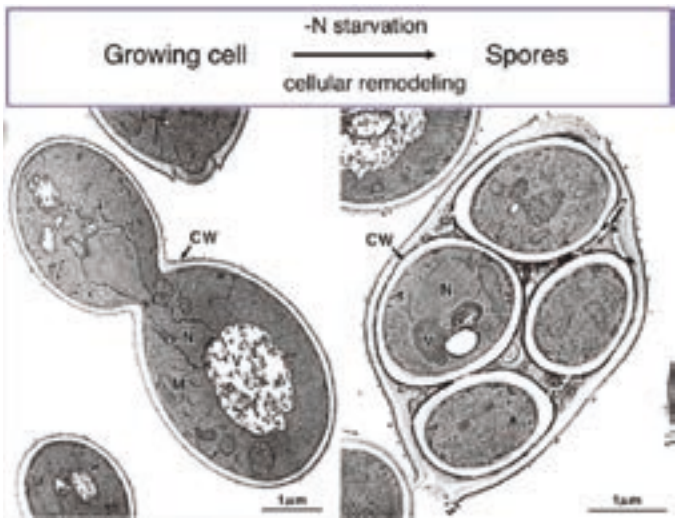


FIGURE 8. Morphological changes associated with sporulation in yeast. The onset of nitrogen starvation causes diploid cells to undergo meiosis and cell division, forming four spores in a process that requires vast remodelling of the protein component of the cell. N = nucleus, V = vacuole, CW = cell wall, M = mitochondrion.

the vacuole, looking for morphological changes that might provide hints about the function of this dynamic organelle.

I carefully observed the early stages of the sporulation process, but couldn't see any obvious changes in the vacuole. I thought that this may be due to the degradation of structures in the vacuole. I conceived that if I were to use a cell lacking vacuolar proteases, structures delivered to the vacuole for degradation would remain in this organelle and might be visible. Fortunately, the well-known yeast geneticist Elizabeth Jones had donated many proteinase mutants to the Yeast Genetic Stock Center at UC Berkeley, so I obtained these strains and shifted to nitrogen depleted medium.

Looking down the microscope, I saw many spherical structures vigorously moving around inside the vacuole. These structures appeared within 30 minutes and almost completely filled the vacuole after 3–4 hours (Figure 9). For me, this observation marked the exact starting point of more than 27 years of autophagy research. The reason that I was able to discover this phenomenon with only a common low magnification light microscope owes to the vigorous movement of these structures by Brownian motion. I was also fortunate that the structures, which we now call autophagic bodies, were large enough to be visible under a light microscope. In the otherwise tranquil yeast cell, this dramatic

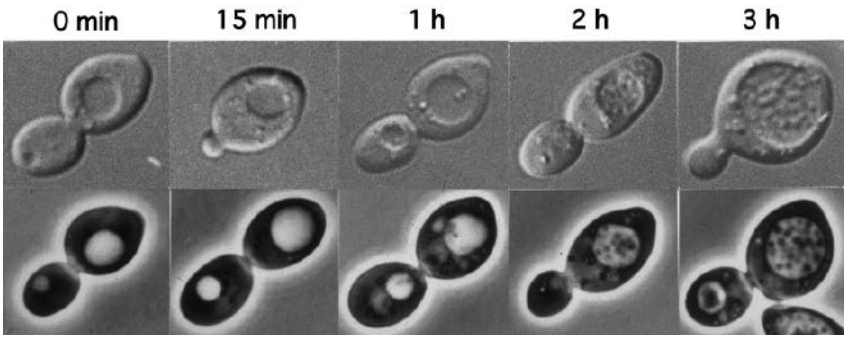


FIGURE 9. Accumulation of autophagic bodies within the vacuole under nitrogen starvation, as observed by bright-field (top) and phase contrast (bottom) light microscopy.

phenotypic change really struck me, and I remember staring down the microscope for some hours, unable to tear my gaze away. I knew that I had encountered an unknown and fascinating phenomenon; this was the starting point of my autophagy research.

Electron microscopy and the morphology of autophagy

I next called on the excellent electron microscopists Misuzu Baba and Masako Osumi to capture the morphology of this phenomenon by electron microscopy. Their efforts produced beautiful images that provided irrefutable evidence of the topological features of the entire autophagy process (Baba et al., 1994), as shown in Figure 10.

A thin section image of a nitrogen starved cell is shown in Figure 10A. Spherical structures can be seen within vacuole, and the high-magnification image presented in Figure 10B shows that these structures are bound by a single membrane. We can see that their contents are exactly the same as the cytoplasm, containing the same density of ribosomes and various cytoplasmic structures, such as the rough ER observed in Figure 10C, suggesting nonselective sequestration of cytoplasmic components. A membrane sac next to the vacuole, the isolation membrane, is also visible in Figure 10C enwrapping a portion of the cytoplasm. After completion of membrane expansion and sealing, a double membrane structure, the autophagosome, is formed (Figure 10D). Freeze-fracture imaging (Figure 10E) shows the fusion event of an autophagosome with the vacuolar membrane, where the outer membrane of the autophagosome is continuous with the vacuolar membrane (Baba et al., 1995), and the inner membrane structures that we named autophagic bodies are also present. Autophagic bodies

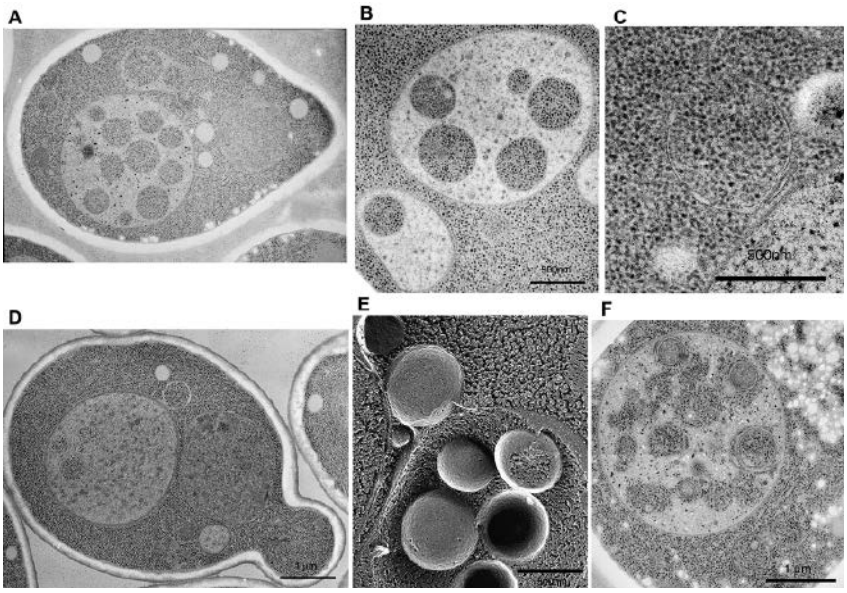


FIGURE 10. Electron micrographs showing topological phenomena associated with nitrogen starvation-induced autophagy. (A) Autophagic bodies within the vacuole, (B) high-magnification image of autophagic bodies, (C) cup-shaped isolation membrane adjacent to the vacuole, (D) an autophagosome adjacent to the vacuole, (E) freeze-fracture image of an autophagosome fusing with the vacuole, (F) mitochondria within autophagic bodies delivered to the vacuole.

occasionally contain mitochondria, as can be seen in Figure 10F, indicating that autophagy can degrade not only cytosolic protein but also supramolecular structures, such as ribosomes and even entire organelles, which is a characteristic feature of autophagy. This is in contrast to the specific recognition of smaller, individual targets by the ubiquitin/protease system. Autophagosomes are transient, ephemeral structures, and autophagic bodies are degraded immediately in the vacuole in wild-type cells, features that prevented their identification for many years.

A scheme of the autophagic process in yeast is presented in Figure 11. When cells are faced with starvation conditions, a small membrane sac appears next to the vacuole and expands to enwrap a portion of cytoplasm. Then, a double membrane structure, the autophagosome, targets the vacuole and fuses with the vacuolar membrane, releasing the inner membrane structure into the vacuole. In wild type cells these structures are immediately disrupted by vacuolar enzymes, and their contents are degraded for reuse. The proteinase-deficient mutant allowed us to follow the progression of autophagy as the accumulation of autophagic bodies.

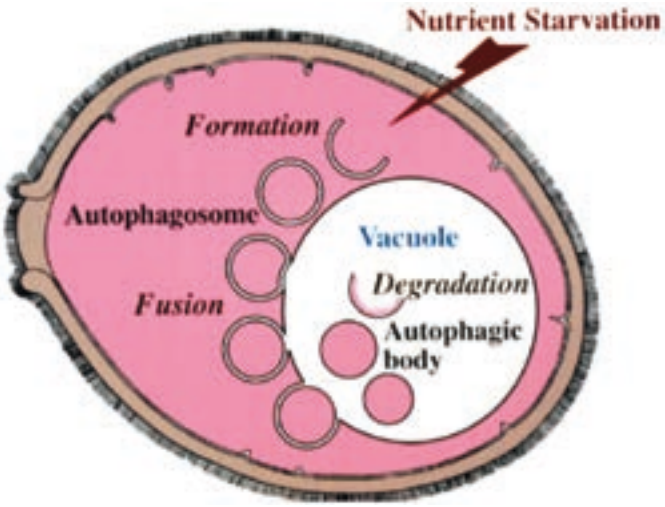


FIGURE 11. Schematic diagram of nitrogen starvation-induced autophagy in yeast.

Importantly, while the vacuole is a vastly larger organelle than the mammalian lysosome, the entire process that we observed was topologically the same as macroautophagy in mammalian cells, confirming that yeast could be used as a model organism in the study of autophagy (Baba et al., 1994). Kazuhiko Takeshige was also able to show early on that this phenomenon is not restricted to sporulation, but is a general cellular response to a range of nutrient starvation conditions (Takeshige et al., 1992). These factors gave the first indication that autophagy is a general process that is induced in diverse organisms in response to a range of stimuli, further convincing me that we had discovered an important cellular process.

Identifying autophagy-related genes in yeast

Yeast is a very good organism for the dissection of complicated biological phenomena through genetic analyses. In this regard I was inspired by the beautiful works of Lee Hartwell, whose group uncovered details of the cell cycle through their analysis of the *cdc* mutants (Johnston et al., 1977), and also by the intricate analysis of the secretory pathway using *sec* mutants by Randy Schekman's group (Novick et al., 1980). These contributions were recognized with the Nobel Prize in Physiology or Medicine in 2001 and 2013, respectively.

As we knew nothing of the genes and proteins that are involved in the autophagy mechanism, I thought that the isolation of mutants defective in autophagy

was the first step in opening up the field of autophagy. However, we had no idea what an autophagy-defective phenotype would look like. At this point, we decided to use autophagic body accumulation in the vacuole as a morphological indicator of autophagy progression. My first Master's course student, Miki Tsukada, was central at this stage of our work and did an excellent job isolating autophagy defective mutants. We took mutagenised cells, individually subjected them to starvation and checked for the accumulation of autophagic bodies, aiming to isolate cells in which autophagic bodies were not observed. Using this approach, we identified the first autophagy mutant strain, which we called *apg1*, now referred to as *atg1* (Tsukada and Ohsumi, 1993). We then confirmed, by the failure to induce protein degradation in this strain, that the autophagic body is indeed an intermediate structure of the degradation process. We also found that, as expected, the homozygous diploid mutant was not able to sporulate. However, under standard growth conditions in the nutrient rich medium YEPD, no difference was observed between the *atg1* strain and wild-type cells, and there appeared to be no obvious defect in vacuolar function or secretion.

It seemed impossible that such an intricate pathway of delivery to the vacuole could be controlled by a single gene, and I was convinced that we should be able to identify many more. We found that *atg1* cells that were subjected to prolonged nitrogen starvation lose viability faster than their wild-type counterparts. Assuming this loss of viability phenotype was caused by the defect in autophagy, we next conducted a primary screen looking at the viability of mutant strains. By isolating strains in which viability was reduced and then performing a secondary screen examining these strains for vacuolar morphology defects, we were able to identify roughly 100 autophagy-defective strains at once (Tsukada and Ohsumi, 1993) (Figure 12). Further genetic analysis showed that 14 complementation groups were among these mutants. As we now know that in nitrogen starved cells 18 genes are essential for autophagy, I can say that this refined approach was extremely effective. We initially predicted that these mutant strains would be characterised by defects at various stages of the autophagy mechanism, but as it turned out all of these genes were essential for the formation of the autophagosome, the most important process in autophagy.

This was a morphological screen, based on the inability to accumulate autophagic bodies in the vacuole. We therefore eliminated mutants characterised by a severe growth defect, cells lacking essential genes and also strains with abnormal vacuolar morphology. In addition, as we used the decline in viability as a primary screen, which only appears with a complete block of autophagy function, we only identified autophagy null mutants, to the exclusion of partially defective mutants.

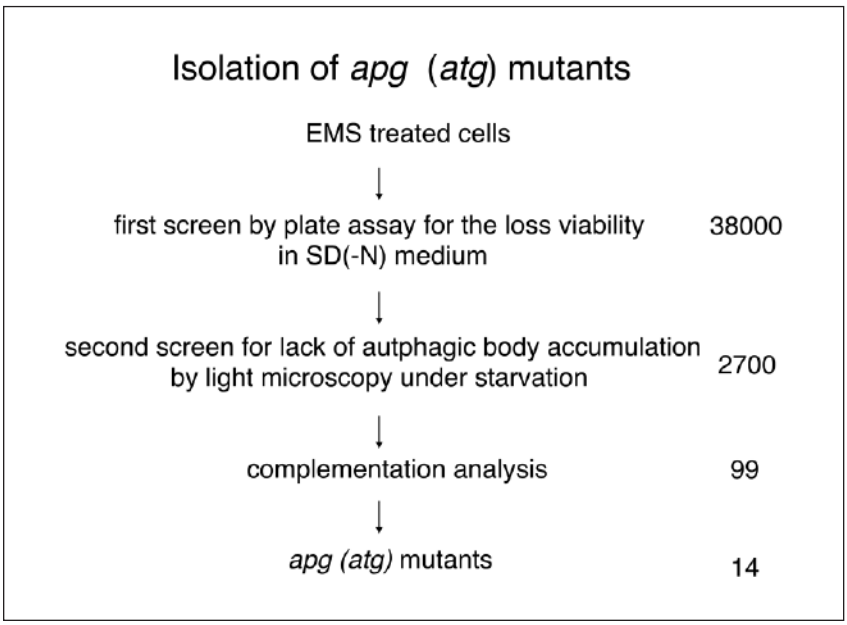


FIGURE 12. Overview of the experimental approach used to isolate autophagy-defective mutants. This screen allowed us to identify most of the *ATG* genes in a short period of time.

Next, we set out to clone and identify the genes implicated in the mutant phenotypes observed in the screen. Tsukada was the first to succeed, cloning the *ATG1* gene. Through the sequencing of *ATG1*, we learned that this gene encodes a protein kinase (Matsuura et al., 1997). However, as we identified the remaining genes, starting with Kametaka's work on *ATG5* (Kametaka et al., 1996) and *ATG6*, we found that all the other *ATG* genes encoded unidentified proteins that were otherwise not essential for growth in nutrient replete conditions and had no phenotypic implications for cells other than a defect in autophagy. Therefore, although we had identified nearly all the genes responsible for autophagy, we were unable to deduce anything about their function from their amino acid sequences and went through a difficult period where we were unable to officially announce our findings.

Yeast had been the subject of comprehensive genetic analysis throughout the world for many years at this point, and it was very surprising that autophagy genes had somehow escaped the attention of other researchers. I suspect that this is because researchers were interested in essential genes, and the conventional definition of an essential gene was the cell's inability to grow in nutrient rich medium when the gene in question was disrupted.

One important product of our time at the College of Arts and Sciences of Tokyo University that deserves mention was Takeshi Noda's establishment of an assay of autophagic activity (Noda and Ohsumi, 1998; Noda and Klionsky, 2008). Noda constructed a strain in which the precursor of the vacuolar alkaline phosphatase Pho8 lacks its N-terminal vacuolar targeting sequence, and expressed this precursor in the cytosol. Upon the induction of autophagy, a portion of the modified version of Pho8 would be taken up by the non-selective activity of autophagy and delivered to the vacuole. Here, the precursor is processed to yield the mature, enzymatically active form of Pho8. Therefore, we were able to quantitatively measure the extent of autophagy by assessing Pho8 activity. This assay, in which degradation can be quantitatively detected through the appearance of enzymatic activity, has been an excellent and widely-used tool in the analysis of autophagic activity that is still used today. I hope that this type of enzymatic assay can also be developed for use in other organisms, especially mammalian systems.

In this way, my career in autophagy research began in that small laboratory at the Komaba campus. The discovery of autophagy by light microscopy, its morphological analysis by electron microscopy, the isolation of mutant strains implicated in autophagy and the beginning of the identification of genes responsible for the process were all achieved within eight years.

A GOLDEN AGE OF AUTOPHAGY RESEARCH AT NIBB

In 1996, I became a professor at the National Institute for Basic Biology (NIBB) in Okazaki, which provided us with a very conducive research environment. I asked Tamotsu Yoshimori to join our lab as an associate professor to start work on autophagy in mammalian cells, and also Takeshi Noda and Yoshiaki Kamada to join us as assistant professors. The following year, Noboru Mizushima came to my lab as a postdoctoral researcher, and later as an assistant professor. Year by year, talented postdoctoral researchers and graduate students gathered to my lab. In 1997, just after we started at the NIBB, we also organised the first International Symposium on Autophagy (ISA) meeting, which we held at the institute. This forum for the discussion of autophagy research has proven to be very successful, and we will hold the 8th ISA in 2017, the largest yet.

Thanks to the NIBB, we were able to produce a truly unique research environment where our work in yeast was accompanied by studies in mammalian and plant cells. I was expecting that it would take a long time to identify all the genes required for autophagy, but thanks to our collaboration with Mariko's laboratory at the Teikyo University of Science, which helped with cloning, as well as the sequencing of the entire yeast genome in 1996, we were able to elucidate the

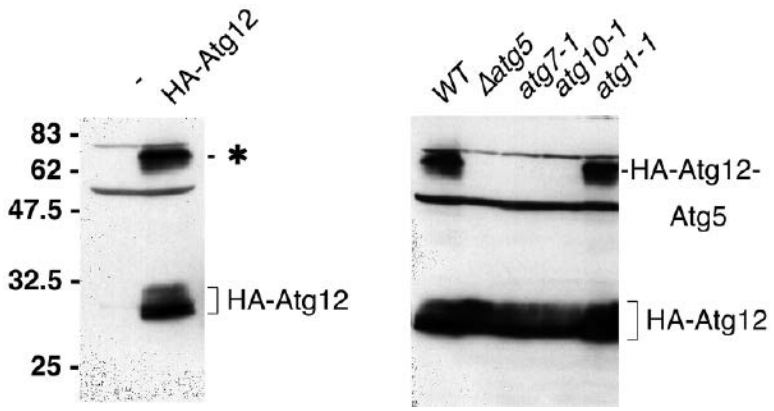


FIGURE 13. Immunoblot analysis of the Atg12 protein. (Left) The appearance of a band of much higher molecular weight than expected for Atg12 is indicated by an asterisk. (Right) The high molecular weight band disappears in cells lacking *ATG5*, *ATG7* or *ATG10*, but not *ATG1*.

genetic context of nearly all the *ATG* genes in a relatively short period. With our subsequent effort to identify interacting proteins of the characterised *ATG* gene products, we were able to confidently identify all 18 of the genes essential for autophagy under nitrogen starvation conditions. From this point, we focused on the characterisation of the Atg proteins and the mechanistic details of autophagy.

The Atg12 conjugation system

The first breakthrough was made by Mizushima when he examined the only just cloned Atg12 protein (Mizushima et al., 1998a). Using immunoblotting, he noticed two bands corresponding to Atg12, one of which indicated a much greater apparent molecular weight than that predicted from the protein sequence (Figure 13). In the absence of Atg5, Atg7 or Atg10, however, the high molecular weight band disappeared. This was the first indication we found of a relationship between Atg proteins, and within a short period we were able to demonstrate that these proteins together constituted a unique ubiquitin-like conjugation pathway. Although Atg12 is a vastly larger protein than ubiquitin, it ends with a glycine residue at its C-terminus and is expressed as a mature protein without any additional residues. Atg12 is then activated by Atg7, an E1 enzyme, to yield a thioester intermediate that is then transferred to the E2 enzyme Atg10, before it ultimately forms an isopeptide bond at a lysine residue at the centre of the Atg5 molecule. This Atg12-Atg5 conjugate then binds to a dimeric form of Atg16, yielding a functional dimer of Atg12-Atg5—Atg16 molecules (Figure 14A). A