THE EFFECTS OF AUTOPHAGY GENES ON AGEING

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ABSTRACT

THE EFFECTS OF AUTOPHAGY GENES ON AGEING

Autophagy is defined as a catabolic bulk degradation pathway. In *S.cerevisiae* autophagy is defined as a single cell's adaptation to starvation. Ageing can be defined as a gradual and progressive deterioration of the health by the time. *S.cerevisiae* is one of the most important model organism in the field of ageing studies. It has been demonstrated that in different model organisms cellular ageing requires inhibiton of particular nutrient sensing pathways leads to extension or shorten replicative and chronological life span that would be linked by regulation of autophagic pathway**.** In our study, we screened 29 deletion mutants of autophagy genes in order to determine relation between autophagy and ageing. We found out that 6 mutants showed reduced replicative life span. Furthermore, possible roles of boric acid, as an autophagy inducer was elucidated on replicative and chronological life span. Boric acid did not incerease replicative or chronological life span, in oppose, it shortened life span in a dose-dependent manner. Finally, to find out the link between autophagy and oxidative stress, we tested autophagy deficient cells for their hydrogen peroxide sensitivity. Absence of only five mutants rendered cells hydrogen peroxide sensitive. Overall, our results revealed that several mutants of autophagy related paths have shorter life spans compared to wild type cells and cells need to have an intact autophagy systems to benefit from life span extension.

ÖZET

OTOFAJİ GENLERİNİN YAŞLANMA ÜZERİNE ETKİLERİ

Otofaji, katabolik degredasyon yolağı olarak tanımlanmaktadır. Ancak *S.cerevisiae'*de hücrenin açlık koşullarına adaptasyonu sırasında işlev görmektedir. Yaşlanma ise sağlığın zamanla artan ve ilerleyen bir biçimde bozulması şeklinde tanımlanmaktadır. *S.cerevisiae* yaşlılık çalışmaları alanında kullanılan en önemli model organizmalardan birisidir. Farklı model organizmalarda yürütülmekte olan çalışmalar sonucunda hücresel bazı yolakların inhibisyonunun replikatif veya kronolojik yaşlanma süresini uzatabileceği veya kısaltabileceği ve bu yolakların otofaji ile ilişkili olabilleceği bildirilmiştir. Çalışmamızda yaşlanma ve otofaji arasındaki ilişkiyi belirlemek amacıyla 29 otofaji geninin delesyon mutantları analiz edilmiş ve 6 otofaji geninin replikatif yaşam süresinde azalmaya sebep olduğu belirlenmiştir. Bunun yanı sıra olası otofaji indükleyici ajan olarak borun replikatif ve kronolojik yaşlanma üzerine etkileri incelenmiş ve artan bor konsantrasyonu ile doğru orantılı olarak replikatif ve kronolojik yaşlanma sürelerinde azalma gözlemlenmiştir. Son olarak otofaji ve oksidatif stress arasındaki ilişkinin açıklanması amacıyla 29 delesyon mutantının hidrojen peroksit stresine karşı sergiledikleri fenotipler test edilmiş ve yalnızca beş mutantta hidrojen peroksite karşı hassasiyet gözlemlenmiştir. Çalışmamız sonucunda bazı otofaji genlerinin yokluğunun yaşam süresinde azalmaya sebep olabileceği ve ancak işlevsel otofajik mekanizmaya sahip hücrelerde yaşam süresinde uzama gözlenebileceği belirlenmiştir.

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PAS	Pre-Autophagosomal Assembly Site
PtdIns	Phosphatidylinositol
PE	Phosphatidylethanolamine
PtdIns(4,5)	Phosphatidylinositol 4,5-bisphosphate
PI 3-kinases or PI3Ks	Phosphatidylinositol 3-kinases
GFP	Green Fluorescent Protein
CMA	Chaperone Mediated Autophagy
S. pompe	*Schizosaccharomyces pombe*
S. cerevisiae	*Saccharomyces cerevisiae*
ROSs	Reactive Oxygen Species
CR	Caloric Restriction
CLS	Chronological Life Span
RLS	Replicative Life Span
µl	Microliter

CHAPTER 1

INTRODUCTION

1.1. Introduction to Autophagy

Autophagy was determined in the budding yeast *S. cerevisiae* by Takeshige and his colleagues in 1992. Under nitrogen, carbon and single amino acid depletion they observed formation of bodies within the vacuole and named them as an autophagic bodies (Takeshige, Baba et al., 1992). After a while, under the same conditions they showed that the budding yeast *S. cerevisiae* engulfs its own cytoplasmic components by forming autophagosomes which have double membrane and subsequently fuse with the vacuole to form autophagic body with a single membrane (Baba, Takeshige et al., 1994). However in single celled organisims such as yeast, autophagy is defined as a single cell's adaptation to starvation. In case of starvation conditions, autophagy is required in order to maintain free amino acid level which is needed for protein synthesis especially proteins that are up-regulated by starvation and decrease in protein synthesis is the result of autophagy deficiency (Onodera and Ohsumi 2005; Suzuki, Onodera et al., 2011). But in higher eukaryotes it has more complex roles. It's conserved nature from single celled organisms to higher eukaryotes has led to increased understanding of the autophagic process and its role in diverse biological processes.

1.2. Different Types of Autophagy

There are several different types of autophagy such as microautophagy, macroautophagy and CMA (Chaperone Mediated Autophagy). In micro and macro autophagy, large structures such as protein aggregates, organelles can be engulfed selectively or non-selectively, but in CMA only soluable proteins can be degraded in a selective manner. Furthermore special types of autophagic machineries are also discovered by various research groups such as ERphagy (Bernales, Schuck et al., 2007), mitophagy (Lemasters, 2005), nucleophagy (Roberts, Moshitch-Moshkovitz et al.,

2003), pexophagy (Sakai, Oku et al., 2006), xenophagy (Alexander and Leib, 2008), ribophagy (Kraft, Deplazes et al. 2008), aggrephagy (Beau, Esclatine et al., 2008).

In contrast to ubiquitin 26S proteasome system, autophagy is used for degradation of many intracellular proteins or organelles primarily by a non-selective manner (Nakatogawa, Suzuki et al., 2009).

1.3. Autophagic Machinery

Currently more than thirty autophagy genes are identified (Tsukada and Ohsumi, 1993; Thumm, Egner et al., 1994; Klionsky, Cregg et al., 2003). Among these genes eighteen of them (ATG1 - ATG10, ATG12 - ATG14, ATG16 - ATG18, ATG29 and ATG31) are essential for autophagosome formation in a conserved fashion (Klionsky, Cregg et al., 2003; Kawamata, Kamada et al., 2005; Kabeya, Kawamata et al., 2007) and they are divided into five subgroups: Atg1 Kinase and its regulators: ATG1, ATG13, ATG17, ATG29, ATG31 (Kamada, Funakoshi et al., 2000), Autophagy specific phosphatidylinositol 3-kinase complex: VPS34, VPS15, VPS30/ATG6, ATG14 (Kihara, Noda et al., 2001), Atg12 conjugation system: ATG12, ATG5, ATG7, ATG10, ATG16 (Mizushima, Noda et al., 1998), Atg8 conjugation system: ATG8, ATG3, ATG4, ATG7 (Ichimura, Kirisako et al., 2000), Atg2-Atg18 complex and Atg9: ATG2, ATG18, ATG9 (Noda, Kim et al., 2000; Barth, Meiling-Wesse et al., 2001; Shintani, Suzuki et al., 2001; Wang, Kim et al., 2001; Nakatogawa, Suzuki et al., 2009).

1.4. The Assembly of Autophagosome

The autophagosomes are formed at PAS (pre-autophagosomal assembly site) which is a dynamic place where many ATG proteins continuously assemble and disassemble. Under induction conditions ATG proteins are organized according to hierarchical relationships among the subgroups. The origin of pre-autophagosomal assembly site is not known.

1.4.1. Atg12 and Atg8 Conjugation System

Atg12p is a ubiquitin-like protein that covalently attached to the Atg5p and form Atg12p-Atg5p complex. Covalent attachment of Atg12p to Atg5p is mediated by two additional protein Atg7p and Atg10p. Subsequently Atg12p-Atg5p complex interacts with Atg16p and forms approximately 350kDa complex (Mizushima, Noda et al., 1998).

Atg8p is another ubiquitin-like protein which is modified by serial action of Atg4p, Atg7p and Atg3p and becomes phosphatidylethanolamine conjugated Atg8p. Atg12p-Atg5p-Atg16p and Atg8-PE conjugates are essential for autophagy initiation. Expression level of ATG8 regulates the level of autophagy through regulation of autophagosome size. Recycling of Atg8p is important in case of completion of autophagosome formation. Also Atg8p is the first components of autophagic machinery that serves as a marker protein. Under starvation conditions Atg8p is targeted to membranes of autophagosomes and finally transported to vacuolar lumen (Kirisako, Baba et al., 1999). Atg8p is identified as an essential component for autophagosome formation by Xie *et al*. In some rare cases autophagosomes can be detected in autophagy deficient mutants such as *atg1*Δ *pep4*Δ *vps4*Δ and *atg8*Δ *pep4*Δ (Xie, Nair et al., 2008).

1.4.2. Autophagy Specific Phosphatidylinositol 3-Kinase Complex

Atg6p, Atg14p, Vps34p, Vps15p form complex (Complex I) and function in autophagy while Atg6p, Vps38p, Vps34p and Vps15p complex (Complex II) function in vacuolar sorting. These two complexes share common compenents.

Vps15p is serine/threonine protein kinase that mediates phosphorylation of Vps34p. Function of Vps30p/Atg6p is not well undrestood. Atg14p and Vps38p act as connector molecules that bridge Vps30p/Atg6p and Vps34p to allow a complex formation. Complex I is targeted to where Atg proteins assemble while complex II is localized to the endosome. Localization of complex at PAS depends on Atg14p whereas localization of Atg14p at PAS depends on Atg17p, Atg9p, Atg13p. Atg14p might regulate the function of complex I during autophagy via interaction with other Atg proteins. Actually overexpression of ATG14 increases the autophagic activity. (Stack,

DeWald et al., 1995; Kametaka, Okano et al., 1998; Kihara, Noda et al., 2001; Suzuki, Kirisako et al., 2001; Obara, Sekito et al., 2006; Suzuki, Kubota et al., 2007; Obara and Ohsumi, 2011).

1.4.3. Regulation of Autophagy by TOR Pathway and Atg1 Kinase and Its Regulators

Autophagic machinery should be regulated tightly because degradation of large portion of cytoplasm can be lethal in some conditions. The budding yeast has two proteins TOR1 and TOR2, whose activites are regulated by rapamycin which inhibits growth of cells by mimicking starvation conditions (reviewed by Katewa and Kapahi, 2011). It is well known TOR complex I (TORC1) is rapamycin sensitive and regulate growth by various pathways, while TORC2 is rapamycin insensitive (Katewa and Kapahi, 2011). In *S. cerevisiae*, induction of autophagy is negatively regulated by Tor pathway (via TORC1) through dephosphorylation of Atg13p and complex formation between Atg13p-Atg17p-Atg1p (Suzuki, Noda et al., 2004). However, Atg17p forms complex with Atg29p and Atg31p in a rapamycin-independent manner which reflects that the complex is formed independently of nutrient conditions (Kabeya, Noda et al., 2009).

Besides TOR pathway, in mammalian cells autophagy can be regulated by p53 depending on its localization. p53 can induce or inhibit autophagy which leads to respectively cell survival or cell death (Moreau, Luo et al., 2010). Tasdemir *et al.* proposed that p53 inhibits autophagy by mTOR activation via AMP dependent kinase (Tasdemir, Maiuri et al., 2008).

1.5. Oxidative Stress and Autophagy

Autophagy is required for ROS (reactive oxygen species) tolerance under starvation conditions. In autophagy deficient mutants, mitochondria are considered as the major source of ROS under starvation conditions. It is known that autophagy deficient cells are unable to express ROS scavenging enzymes and electron transport chain components such as Cox4 and Cox2 (Suzuki, Onodera et al., 2011).

Atg32 and Atg33 are known as essential components for mitophagy. Atg32 functions as a mitochondrial receptor during specific degradation of mitochondria, but Atg33 is essential for specific degradation of aged or dysfunctional mitochondria during post-log phase growth rather than starvation (Onodera and Ohsumi, 2005). However mitophagy is not significantly involved in the maintenance of respiratory function. During nitrogen starvation, vacuolar degredation of cytoplasmic components by non selective autophagy rather than selective autophagy is more important. Autophagy is required for maintaining mitochondrial function by providing essential amino acid during protein translation (Suzuki, Onodera et al., 2011).

But in *S. pombe* (fission yeast) it has been shown that selective type of autophagy is required for G_0 maintainance via selective degradation of mitochodria which further inhibits accumulation of ROS (Takeda, Yoshida et al., 2010).

1.6. Ageing

Ageing can be defined as a gradual and progressive deterioration of the health by the time. The mechanisms that underlie ageing and age related diseases such as cancer, cardiovascular and neurodegenerative diseases remain poorly understood. In order to determine the mechanisms that underlie ageing and age related diseases single-celled eukaryote *Saccharomyces cerevisiae* is the most important model organism because of its ease of genetic manipulations, well annotated genome and short generation time (Bitterman, Medvedik et al., 2003).

Two modes of ageing are defined in the budding yeast *S. cerevisiae* which are known as replicative and chronological ageing. Replicative ageing is defined as a number of divisions which an individual yeast cells can undergo before it ceases the division. *S. cerevisiae* cells divide by budding and undergo asymmetrical cell division. Mortimer and Johnston were the first scientists who performed yeast life span analysis by micromanipulation in order to find out number of cell division a mother cell can undergo (Mortimer and Johnston, 1959). The nature of asymmetrical division leads to mother cell specific replicative ageing that cause mother cells to retain an ageing factors (Egilmez and Jazwinski 1989; Kennedy, Austriaco et al., 1994) Extrachromosomal rDNA circles (Sinclair and Guarente, 1997), protein carbonyls and oxidatively damaged proteins (Aguilaniu, Gustafsson et al., 2003; Erjavec, Larsson et al., 2007) are possible

ageing factors that retain within the mother cell during asymmetrical cell division which leads to replicative ageing. Also deletion of RAS1 (Sun, Kale et al., 1994) and FOB1 (Defossez, Prusty et al., 1999) genes individually can increase replicative life span of the budding yeast. FOB1 and RAS1 genes are some of the examples for regulation of replicative ageing at gene level.

Second mode of ageing is chronological ageing. CLS is defined as an amount of the time that cell can remain viable while in a non-dividing state (Fabrizio and Longo, 2003). SOD1 and SOD2 which encode cytoplasmic and mitochondrial superoxide dismutases, respectively, can modulate chronological life span and extend chronological survival by 30% (Longo, Liou et al., 1999).

However in chronological life span experiments %2 glucose is used in liquid medium as the carbon source (Fabrizio and Longo, 2007). Initially, yeast cells use glucose by fermentation. Due to fermentation of glucose, ethanol accumulates within the extracellular enviroment. Several studies revealed that ROS production due to respiration contributes to chronological ageing (Burhans and Weinberger, 2009; Burtner, Murakami et al., 2009). ROS, including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^-) are known as secondary messengers within the cells. CR (caloric restriction) conditions or inactivation of catalases extend chronological life span through formation of H_2O_2 which activates superoxide dismutases within the cells. Fifty five years ago Dr. Denham Harman proposed that ROS (reactive oxygen species) that produced within the mitochondria is the main reason for ageing. This theory is called as the free rediacal theory of ageing (Harman, 2003). Today in contarary to free rediacal theory of ageing, oxidative stress is necessary for extension of chronological age of *S. cerevisiae* cells (Mesquita, Weinberger et al., 2010). Besides ROS production, recent studies proposed that accumulation of acetic acid would be the main ageing factor that limits chronological life span of yeast cell (Burtner, Murakami et al., 2009). Transfer of chronologically aged cells from the expired medium is sufficient to extend CLS is another evidience that medium environment play important role on CLS regulation (Fabrizio, Liou et al., 2003).

Also Cheng and Ivessa have proposed that during CLS the number of the mtDNA (mitochondrial DNA) that migrates into the nucleus increases (Cheng and Ivessa, 2010). At the same time proteasomal pathway collaborates with autophagy in supporting the longevity of G_0 cells (chronologically aged cells) (Takeda, Yoshida et al., 2010).

1.6.1. Role of Caloric Restriction on Ageing

Caloric restriction is one of the most important intervention that extends life span in all common model organisms that used in ageing researches. Steinkraus *et al.* reviewed in their recent article, reduction in glucose level within the media can extend life span of budding yeast also reduction in level of amino acid can extend replicative life span as same as reduction in glucose level. The level of glucose concentration which is used during caloric restriction experiments can be changed between 0.5% to 0.05% (Steinkraus, Kaeberlein et al., 2008).

Three well known kinases, SCH9, HXK2 and TOR are nutrient sensitive and life span extension via their deficiency is linked to activation of other pathways that regulates longevity. TOR can modulate chronological and replicative life span through many different pathways. Under nitrojen starvation condition longevitiy is modulated by TOR pathway through ribosome biogenesis. Deletion of TOR pathway also provides stress resistance to heat and oxidative stress that induces localization of Msn2 transcription factor into the nucleus (Powers, Kaeberlein et al., 2006). Life span extension through MSN2/4 depends on transcription of PNC1 which encodes an enzyme that deaminates nicotinamide which inhibits sirtuins if it is not catabolized (Anderson, Bitterman et al., 2003). Under growth conditions Msn2/4 is localized within the cytoplasm (Beck and Hall, 1999). Upon glucose starvation, TOR inhibition or rapamycin treatment, Msn2/4 is translocated to the nucleus and increase transcription of PCN1 that extends life span (Medvedik, Lamming et al., 2007).

1.6.2. Ageing and Autophagy

It is known that basal autophagic activity decreases with the age that leads to accumulation of toxic materials and dysfunctional organelles within the cells. As it was described above, TOR pathway is one of the most important pathway that has impact on ageing process. Previous studies have showed that TORC1 has roles in a large number of diseases such as diabetes, heart disease and cancer. In these kind of diseases common risk factor is ageing. It has been demonstrated in model organisms (yeast, worms, flies and mice) that cellular ageing requires TORC1 (Katewa and Kapahi, 2011). For instance inhibition of Tor pathway by caloric restriction which leads to life span

extension in various model organisms (Blagosklonny, 2008), is regulated by autophagic pathway. Rapamycin is used for induction of autophagy besides its roles in cancer treatment (Hartford and Ratain, 2007). There are dozens of compounds known as an inducer of autophagy such as rilmenidine, carbamazepine (Hidvegi, Ewing et al., 2010), resveratrol, spermidine (Morselli, Maiuri et al., 2010). Administration of rapamycin or spermidine can prolong the life span of model organisms including *C. elegans*, *Drosophila* and mice. In *C. elegans* life span prolonging effect of rapamycin is lost by deletion of ATG6/Beclin-1 indicates that induction of autophagy by pharmacological agents may prolong life span (Morselli, Maiuri et al., 2010).

Also in another important model organism *C. elegans* it is known that mutation in insulin-like growth factor receptor; DAF-2 leads to life span extension. Recently, Melendez *et al*. discovered that in DAF-2 mutants, inactivation of autophagic machinery through knock down of Beclin-1 which is homolog of ATG6 in *S. cerevisiae,* result in shortened life span (Melendez, Talloczy et al., 2003; Kenyon, 2005). This suggests that autophagy may regulate ageing through conserved Insulin/IGF-1 signaling pathway. Furthermore Hars *et al.* determined that knocking down of both ATG7 and ATG12 leads to significant life span shortening compared to the control in DAF-2 mutant back ground. In DAF-2 mutant back ground, deletion of ATG12 or ATG7 shorten life span more than when genes are deleted on wild type back ground (Hars, Qi et al., 2007).

On the other hand, another nutrient sensitive enzyme, sir2 which is a histone deacetylase and uses NAD^+ as a substrate that can deacetylate acetylated lysine residues in the N-terminal tails of histone H3 and H4 plays roles on modulation of ageing. In mammalian cell culture it is demostrated that Sirt1 (yeast homolog sir2) activity is necessary for the induction of starvation induced autophagy. Autophagy can be induced by resveratrol via SIRT1 (Morselli, Maiuri et al., 2010). Morselli *et al.* proposed that induction of autophagy in the presence of resveratrol or nutrient deficiency depends on Sirt1 in human cells as well as dietary restriction in *C. elegans*. As a result of their research they conclude that autophagy is universally required pathway for the lifespan-prolonging effects of caloric restriction and pharmacological sirtuin-1 activators (Morselli, Maiuri et al., 2010). It is proposed that essential components of autophagic machinery such as atg5p, atg7p and atg8p interact with Sirt1. In the absence of Sirt1, acetylation of multiple autophagy proteins is increased which results in inhibition of autophagy (Lee, Cao et al., 2008). In yeast according to our knowledge there is no

known study about the role of sir2 on autophagy. Maybe the same mechanisms can operate in *S. cerevisiae* as well.

1.7. Transcriptional Regulation of Autophagy

Under starvation conditions, ATG8 is rapidly upregulated in yeast and mammalian cells. But not much is known about transcriptional machinery in yeast *S. cerevisiae* but in *drosophila* FoxO3 is identified as a transcription factor that is necessary for transcription of autophagy related genes (Juhasz, Puskas et al., 2007). Gcn4 is known as a general transcription factor. In yeast several autophagy related genes such as ATG1, ATG13 and ATG14 are downstream targets of gcn4p. In a recent study Natarajann *et al.* have shown that autophagy is triggered under starvation conditions but in *gcn4*Δ cells appearance of autophagic bodies leads to suggestion that gcn4p is not required for autophagosome formation but is required for enhancement of autophagic response (Natarajan, Meyer et al., 2001).

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used media, buffers, solutions and their compositions are presented in Appendix A.

2.2. Methods

2.2.1. Yeast Strains

Wild type (BY4741) strain of *S. cerevisiae* and twenty nine isogenic deletion mutants are all haploids with the mating type 'a' and his3, leu2, met15, ura3 back ground were analysed during replicative and chronological ageing assays. All deletion mutants contain kanamycin resistance gene (MATa, his3,leu2, met15, ura3, deleted geneΔ : KA) instead of the deleted gene. The strains used in this experiment are listed in (Table 2.1).

2.2.2. Identification of Autophagy Related Genes

Autophagy genes are determined through Saccharomyces Genome Database website. As a result of search for autophagy genes, 36 autophagy genes are determined. Only non essential genes (Non–essential means when the gene is knocked out the cell can stay viable and that gene is not essential for the life of the cell.) are studied which is listed in (Table 2.1).

2.2.3. Identification of Autophagy Related Genes That Lead to Ageing

Yeast strains were grown on YPD rich medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) for determination of short-lived and long-lived mutants. Before analysis, yeast strains are removed from frozen stocks (-80ºC) and streak onto YPD agar plates. Cells were kept for 2 days incubation at 30ºC. Cells were inoculated onto agar plates as a single line. For each strain, average 40 individual cells were selected and removed from yeast colonies by using a micromanipulator. 20 of 40 individual daughter cells were collected and selected as starting mother cells. Newly formed daughter cells from these virgin cells were removed and discarded. Every 90 minutes intervals plates were controlled and newly formed daughters were removed until cells stopped dividing. During night periods plates were stored at 4ºC. Replicative life span was determined as the total number of daughter cells that each mother cell generated.

2.2.4. Determination of Oxidative Stress Tolerance of Autophagy Mutants

The oxidative stress tolerances of autophagy gene mutants were examined by oxidative stress generating agent; hydrogen peroxide (H_2O_2).

2.2.4.1. HALO Assay (H_2O_2 Resistance)

HALO assay is used to test the sensitivities of different deletion mutants of autophagy genes against to hydrogen peroxide. The yeast cells were grown overnight in YPD at 30°C. The over night grown cells are dilute in ratio of 1:10 with fresh YPD media and incubated for 3 hours at 30°C at 180 rpm. The OD_{600} values of the cells were adjusted to 0,2 and 400 µl of cells were strewed on YPD plates. After 1 hour incubation at 30°C, 5µl of 8.8M H_2O_2 was dropped into the center of the YPD plate and the plates were incubated at 30°C for 2 days. The diameter of the hole at the center of the plate was measured to determine the H_2O_2 sensitivity of the mutants and the wild type strain.

2.2.5. Chronological and Replicative Ageing Patterns of Wild Type Strain of *S. cerevisiae* (BY4741) Cells upon Boron Treatment

The chronological ageing pattern of yeast cells can be identified by using the colony formation unit (CFU) assay (Parrella and Longo, 2008).

Yeast strains were grown in 5 ml YPD media overnight at 30°C at 180 rpm. 5 ml of overnight grown cell cultures are washed with dH_2O twice and suspended in 5 ml of fresh YPD. 23 ml of fresh YPD media is transferred into 250 ml flasks and 2 ml of overnight grown cell cultures is added into the flasks. The cells were incubated at 30°C at 180 rpm for 18 days. First day is suggested as a day zero and starting from the day zero, 10 µl of cell sample is taken and diluted for the 'Thoma Lam' counting. According to the Thoma Lam counts, approximately 500 cells were strewed onto YPD agar plates and incubated at 30°C for 2 days. The grown colonies are counted and the ability of cells to form a colony is calculated by the formula below;

CFU % = (100 X counted colony number) / (strewed cell number)

This survival is monitored repeatedly in every 72 hrs for each strain until the 18th day of the starting culture is reached.

Table 2.1. Non-essential autophagy genes that were investigated in this study

YHR171W	**ATG7**	Member of the E1 family of ubiquitin-activating enzymes; mediates the conjugation of Atg12p with Atg5p and Atg8p with PE
YDR022C	**ATG31**	Autophagy-specific protein; may form a complex with Atg17p and Atg29p that localizes other proteins to the pre-autophagosomal structure
YPL166W	**ATG29**	Autophagy-specific protein; interacts with Atg17p and localizes to the PAS in a manner interdependent with Atg17p and Atg31p
YBR128C	**ATG14**	Autophagy-specific subunit of PI 3-kinases complex I; Atg14p targets complex I to the PAS
YPL149W	**ATG5**	Conserved protein involved in autophagy and the Cvt pathway; conjugate with Atg12p to form a complex involved in Atg8p lipidation
YNR007C	**ATG3**	E2-like enzyme involved in autophagy and the Cvt pathway; plays a role in formation of Atg8p-PE conjugates
YNL223W	**ATG4**	Conserved cysteine protease required for autophagy; cleaves Atg8p to a form required for autophagosome and Cvt vesicle generation
YBR217W	**ATG12**	Conserved ubiquitin-like modifier involved in autophagy and the Cvt pathway; conjugated to Atg5p to form a complex involved in Atg8p lipidation
YJL178C	**ATG27**	Type I membrane protein involved in autophagy and the Cvt pathway
YIL146C	**ATG32**	Mitochondrial-anchored transmembrane receptor ; interacts with the autophagy adaptor protein, Atg11p, and is essential for mitophagy
YBR131W	**CCZ1**	Protein involved in vacuolar assembly, essential for autophagy and the Cvt pathway
YNL242W	**ATG2**	Peripheral membrane protein required for vesicle formation during autophagy, pexophagy, and the cvt pathway
YFR021W	**ATG18**	Phosphoinositide binding protein required for vesicle formation in autophagy and the cvt pathway
YGL180W	**ATG1**	Protein ser/thr kinase required for vesicle formation in autophagy and the Cvt pathway; structurally required for PAS formation
YKR019C	**IRS4**	EH domain-containing protein involved in regulating PtdIns(4,5) levels and autophagy
YLR423C	**ATG17**	Scaffold protein responsible for PAS organization; regulatory subunit of an autophagy-specific complex that includes Atg1p and Atg13p; stimulates Atg1p kinase activity

(Cont. on next page)

Table 2.1. (Cont.)

YPL120W	**VPS30**	Subunit of PI 3-kinases complexes I and II; Complex I is essential in autophagy and Complex II is required for vacuolar protein sorting
YLR377C	**FBP1**	Fructose-1,6-bisphosphatase, undergoes either proteasome-mediated or autophagy-mediated degradation depending on growth conditions
YCR068W	**ATG15**	Lipase required for intravacuolar lysis of autophagic bodies and Cvt bodies
YPR185W	**ATG13**	Regulatory subunit of the Atg1p signaling complex; required for vesicle formation during autophagy and the Cvt pathway
YLL042C	**ATG10**	Conserved E2-like conjugating enzyme that mediates formation of the Atg12p-Atg5p conjugate
YLR356W	**ATG33**	Mitochondrial mitophagy-specific protein; not required for other types of selective autophagy or macroautophagy; conserved within fungi, but not in higher eukaryotes
YMR159C	**ATG16**	Conserved protein that interacts with Atg12p-Atg5p conjugates to form Atg12p-Atg5p-Atg16p multimers, which localize to the pre-autophagosomal structure and are required for autophagy
YKL103C	**LAP4**	Vacuolar aminopeptidase yscI; zinc metalloproteinase that belongs to the peptidase family M18; often used as a marker protein in studies of autophagy and Cvt pathway
YML018C	**YML018C**	Putative protein of unknown function; physical interaction with Atg27p suggests a possible role in autophagy
YLR189C	**ATG26**	UDP-glucose:sterol glucosyltransferase, conserved enzyme involved in synthesis of sterol glucoside membrane lipids; in contrast to ATG26 from P. pastoris, S. cerevisiae ATG26 is not involved in autophagy
YJR066W	**TOR1**	PIK-related protein kinase and rapamycin target; subunit of TORC1, a complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy
YDR119W	**VBA4**	Protein of unknown function; physical interaction with Atg27p suggests a possible role in autophagy

CHAPTER 3

RESULTS

3.1. Replicative Ageing Patterns of Autophagy Gene Mutants

In order to study the roles of autophagy genes on replicative ageing, we analyzed the life span of cells lacking these genes. Replicative ageing assay (by Micromanupulation Microscope) was performed by means of a micromanuplator (Mortimer and Johnston, 1959).

For the replicative life span analysis, 29 deletion mutants of all non-essential autophagy genes were first screened in fashion of 10 cells for each strain. For the second screening 20 cells for each strain were analyzed at least once. During replicative life span analysis all of the 29 deletion mutants were grown on YPD rich media (2% glucose, 2% peptone, 1% yeast extract and 2% agar). Results are summurized in (Table 3.1), (Table 3.2) and (Figure 3.1).

In our study we determined %30 reduction in life span of *atg17*Δ mutant on BY4741 genetic back ground. It is known that ATG17 encodes a scaffold protein which is responsible for PAS organization. Atg17p plays role as a regulatory subunit of autophagy specific complex which includes Atg13p and Atg1p within the complex that induces kinase activiy of Atg1p. Previous studies showed that on S288C back ground deletion of ATG17 gene shortens life span in rich YPD (YPD media with 2% glucose) media which is consistent with our result on BY4741 back ground (Tang, Watkins et al., 2008) .

ATG15 encodes lipase which is required for intravacuolar lysis of autophagic bodies and cvt bodies. Previous studies showed that under normal conditions (YPD media with 2% glucose) deletion of ATG15 gene has no effect on life span regulation which is consistent with our result (Tang, Watkins et al., 2008).

In contrary to present study (Tang, Watkins et al., 2008) in our study we determined %20 reduction in life span of *atg7*Δ mutant on BY4741 genetic back ground. ATG7 functions as a member of the E1 family of ubiquitin-activating enzymes

and conjugates Atg12p with Atg5p and Atg8p with phosphatidylethanolamine. In drosophila deletion of ATG7 leads to impaired ability to induce autophagy in response to starvation and resulted in reduced replicative lifespan under normal conditions (Juhasz, Erdi et al., 2007).

In our study we determined % 15 reduction in life span of *atg27*Δ mutant on BY4741 genetic back ground. ATG27 is a type I membrane protein involved in autophagy and the Cvt pathway. Previous studies showed that under nitrogen starvation conditions deletion of ATG27 leads to decreased autophagy (Yen, Legakis et al., 2007).

In our study we determined % 13 reduction in life span of *atg14*Δ mutant on BY4741 genetic back ground which is shown for the first time. Atg14 is a autophagy specific subunit of phosphatidylinositol 3-kinase complex I. Atg14p targets complex I to the phagophore assembly site (PAS) which is required for localizing additional ATG proteins to the PAS. Previous studies showed that under starvation conditions deletion of ATG14 leads to inhibition of autophagy (Tsukada and Ohsumi, 1993; Kametaka, Okano et al., 1998; Kihara, Noda et al., 2001).

In our study we determined % 15 reduction in life span of *lap4*Δ mutant on BY4741 genetic back ground which is shown for the first time. LAP4 encodes a vacuolar aminopeptidase and zinc metalloproteinase that often used as a marker protein in studies of autophagy and CVT pathway.

In our study we determined % 22 reduction in life span of *atg12*Δ mutant on BY4741 genetic back ground which is shown for the first time. Atg12p is a conserved ubiquitin-like modifier that involved in autophagy and the Cvt pathway. Previous studies showed that under nitrogen starvation conditions deletion of ATG12 leads to inhibition of autophagy (Tsukada and Ohsumi, 1993; Mizushima, Noda et al., 1998).

3.2. Oxidative Stress Tolerance of Autophagy Related Gene Mutants

It is known that ROS have destructive effects on biological molecules such as lipids, proteins and nucleic acids and cause pathologies such as cardiovascular diseases, cancer and ageing (Minotti and Aust, 1992; Lenaz, Bovina et al., 2002; Rosenfeld and Beauvoit, 2003; Turrens, 2003; Skulachev and Longo, 2005). In order to see the effects of exogenous H_2O_2 treatment, autophagy gene mutants were treated with 8.8M hydrogen peroxide by a Halo assay. We speculate that hydrogen peroxide sensitive mutants are prone to oxidative stress and may age faster than normal cells.

As shown in (Table 3.3) and (Figure 3.2) according to Halo assay results (p-value<0,05) deletion of *atg7*Δ, *atg29*Δ*, atg15*Δ*, yml01c8*Δ and *atg26*Δ genes resulted in high sensitivity against oxidative stress (Table 3.3). Their sensitivity to H_2O_2 was changed respectively from 17% , %25, %13, %56 and 11% compared to the wild type cells (Table 3.3) and (Figure 3.2). In a previous study, ATG7 mutant flies which were treated with hydrogen peroxide and paraquat showed two fold faster rate of death than controls (Juhasz, Erdi et al., 2007). In our study we determined that deletion of ATG7 result in high sensitivity against oxidative stress when compared to wild type strain. YML018C is a protein with unknown function and its function should be further studied to understand the link between autophagy and oxidative stress. Also for the first time we determined that deletion of ATG29, ATG15, YML018C AND ATG26 result in high sensitivity against to oxidative stress when compared to wild type strain.

CHAPTER 4

CONCLUSION

In our study we aimed to find out the relation between ageing and autopahgy. For this purpose we screened replicative ageing patterns of 29 deletion mutants of autophagy genes. According to our results we determined six deletion mutants that were shown reduced replicative life span. Deletion of ATG17 was reduced replicative life span which is consistent with previous study (Tang, Watkins et al., 2008). In our study for the first time we determined that deletion of ATG27, ATG7, ATG12, ATG14 and LAP4 lead to reduced life span compared to wild type strain BY4741. Also It is known that ROS have destructive effects on biological molecules such as lipids, proteins and nucleic acids and cause pathologies such as cardiovascular diseases, cancer and ageing (Minotti and Aust, 1992; Lenaz, Bovina et al., 2002; Rosenfeld and Beauvoit, 2003; Turrens, 2003; Skulachev and Longo, 2005). In order to determine the importance of autophagy against to oxidative stress we performed Halo assay and we found out that five mutants (*atg7*Δ*, yml018c*Δ*, atg15*Δ*, atg29*Δ and *atg26*Δ) were sensitive against to oxidative stress. Their sensitivity to H_2O_2 was changed respectively from 11% and 56% compared to the wild type cells. In previous studies It was shown that ATG7 mutant flies were more sensitive against to oxidative stress creating agents hyrogen peroxide and paraquat (Juhasz, Erdi et al., 2007). Also in yeast a*tg7*Δ mutants showed more sensitive phenotype compared to wild type, against to hydrogen peroxide which was determined for the first time. YML018C, gene with unknown function obviously has some roles against to oxidative stress. Function of YML018C should be further investigated. In our laboratory we determined that expression of GCN4 is upregulated with boron treatment (data not published). We proposed that upregulation of GCN4 maybe can modulate replicative and chronological ageing through activation of autophagic pathway. According to results of chronological and replicative ageing assays we determined that boron shorten replicative and chronological life span in a dose-dependent manner. We hope that our results will contribute to the field of ageing studies.

REFERENCES

Aguilaniu, H., L. Gustafsson, et al. (2003). "Asymmetric inheritance of oxidatively damaged proteins during cytokinesis." Science 299(5613): 1751-1753.

Alexander, D. E. and D. A. Leib (2008). "Xenophagy in herpes simplex virus replication and pathogenesis." Autophagy 4(1): 101-103.

Alvers, A. L., L. K. Fishwick, et al. (2009). "Autophagy and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae." Aging Cell 8(4): 353-369.

Anderson, R. M., K. J. Bitterman, et al. (2003). "Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae." Nature 423(6936): 181-185.

Baba, M., K. Takeshige, et al. (1994). "Ultrastructural analysis of the autophagic process in yeast: detection of autophagosomes and their characterization." J Cell Biol 124(6): 903-913.

Barth, H., K. Meiling-Wesse, et al. (2001). "Autophagy and the cytoplasm to vacuole targeting pathway both require Aut10p." FEBS Lett 508(1): 23-28.

Beau, I., A. Esclatine, et al. (2008). "Lost to translation: when autophagy targets mature ribosomes." Trends Cell Biol 18(7): 311-314.

Beck, T. and M. N. Hall (1999). "The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors." Nature 402(6762): 689-692.

Bernales, S., S. Schuck, et al. (2007). "ER-phagy: selective autophagy of the endoplasmic reticulum." Autophagy 3(3): 285-287.

Bitterman, K. J., O. Medvedik, et al. (2003). "Longevity regulation in Saccharomyces cerevisiae: linking metabolism, genome stability, and heterochromatin." Microbiol Mol Biol Rev 67(3): 376-399, table of contents.

Blagosklonny, M. V. (2008). "Aging: ROS or TOR." Cell Cycle 7(21): 3344-3354.

Burhans, W. C. and M. Weinberger (2009). "Acetic acid effects on aging in budding yeast: are they relevant to aging in higher eukaryotes?" Cell Cycle 8(14): 2300-2302.

Burtner, C. R., C. J. Murakami, et al. (2009). "A molecular mechanism of chronological aging in yeast." Cell Cycle 8(8): 1256-1270.

Cheng, X. and A. S. Ivessa (2010). "The migration of mitochondrial DNA fragments to the nucleus affects the chronological aging process of Saccharomyces cerevisiae." Aging Cell 9(5): 919-923.

Defossez, P. A., R. Prusty, et al. (1999). "Elimination of replication block protein Fob1 extends the life span of yeast mother cells." Mol Cell 3(4): 447-455.

Egilmez, N. K. and S. M. Jazwinski (1989). "Evidence for the involvement of a cytoplasmic factor in the aging of the yeast Saccharomyces cerevisiae." J Bacteriol 171(1): 37-42.

Erjavec, N., L. Larsson, et al. (2007). "Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104p." Genes Dev 21(19): 2410-2421.

Fabrizio, P., L. L. Liou, et al. (2003). "SOD2 functions downstream of Sch9 to extend longevity in yeast." Genetics 163(1): 35-46.

Fabrizio, P. and V. D. Longo (2003). "The chronological life span of Saccharomyces cerevisiae." Aging Cell 2(2): 73-81.

Fabrizio, P. and V. D. Longo (2007). "The chronological life span of Saccharomyces cerevisiae." Methods Mol Biol 371: 89-95.

Harman, D. (2003). "The free radical theory of aging." Antioxid Redox Signal 5(5): 557-561.

Hars, E. S., H. Qi, et al. (2007). "Autophagy regulates ageing in C. elegans." Autophagy 3(2): 93-95.

Hartford, C. M. and M. J. Ratain (2007). "Rapamycin: something old, something new, sometimes borrowed and now renewed." Clin Pharmacol Ther 82(4): 381-388.

Hidvegi, T., M. Ewing, et al. (2010). "An autophagy-enhancing drug promotes degradation of mutant alpha1-antitrypsin Z and reduces hepatic fibrosis." Science 329(5988): 229-232.

Ichimura, Y., T. Kirisako, et al. (2000). "A ubiquitin-like system mediates protein lipidation." Nature 408(6811): 488-492.

Juhasz, G., B. Erdi, et al. (2007). "Atg7-dependent autophagy promotes neuronal health, stress tolerance, and longevity but is dispensable for metamorphosis in Drosophila." Genes & Development 21(23): 3061-3066.

Juhasz, G., L. G. Puskas, et al. (2007). "Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body." Cell Death Differ 14(6): 1181-1190.

Kabeya, Y., T. Kawamata, et al. (2007). "Cis1/Atg31 is required for autophagosome formation in Saccharomyces cerevisiae☆." Biochemical and Biophysical Research Communications 356(2): 405-410.

Kabeya, Y., N. N. Noda, et al. (2009). "Characterization of the Atg17–Atg29–Atg31 complex specifically required for starvation-induced autophagy in Saccharomyces cerevisiae☆." Biochemical and Biophysical Research Communications 389(4): 612-615.

Kamada, Y., T. Funakoshi, et al. (2000). "Tor-mediated induction of autophagy via an Apg1 protein kinase complex." J Cell Biol 150(6): 1507-1513.

Kametaka, S., T. Okano, et al. (1998). "Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, Saccharomyces cerevisiae." Journal of Biological Chemistry 273(35): 22284-22291.

Kametaka, S., T. Okano, et al. (1998). "Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, Saccharomyces cerevisiae." J Biol Chem 273(35): 22284-22291.

Katewa, S. D. and P. Kapahi (2011). "Role of TOR signaling in aging and related biological processes in Drosophila melanogaster." Exp Gerontol 46(5): 382-390.

Kawamata, T., Y. Kamada, et al. (2005). "Characterization of a novel autophagy-specific gene, ATG29." Biochem Biophys Res Commun 338(4): 1884-1889.

Kennedy, B. K., N. R. Austriaco, Jr., et al. (1994). "Daughter cells of Saccharomyces cerevisiae from old mothers display a reduced life span." J Cell Biol 127(6 Pt 2): 1985-1993.

Kenyon, C. (2005). "The plasticity of aging: insights from long-lived mutants." Cell 120(4): 449-460.

Kihara, A., T. Noda, et al. (2001). "Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae." J Cell Biol 152(3): 519-530.

Kihara, A., T. Noda, et al. (2001). "Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae." Journal of Cell Biology 152(3): 519-530.

Kirisako, T., M. Baba, et al. (1999). "Formation process of autophagosome is traced with Apg8/Aut7p in yeast." J Cell Biol 147(2): 435-446.

Klionsky, D. J., J. M. Cregg, et al. (2003). "A unified nomenclature for yeast autophagy-related genes." Dev Cell 5(4): 539-545.

Kraft, C., A. Deplazes, et al. (2008). "Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease." Nat Cell Biol 10(5): 602-610.

Lee, I. H., L. Cao, et al. (2008). "A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy." Proc Natl Acad Sci U S A 105(9): 3374-3379.

Lemasters, J. J. (2005). "Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging." Rejuvenation Res 8(1): 3-5.

Lenaz, G., C. Bovina, et al. (2002). "Role of mitochondria in oxidative stress and aging." Ann N Y Acad Sci 959: 199-213.

Longo, V. D., L. L. Liou, et al. (1999). "Mitochondrial superoxide decreases yeast survival in stationary phase." Arch Biochem Biophys 365(1): 131-142.

Medvedik, O., D. W. Lamming, et al. (2007). "MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae." PLoS Biol 5(10): e261.

Melendez, A., Z. Talloczy, et al. (2003). "Autophagy genes are essential for dauer development and life-span extension in C. elegans." Science 301(5638): 1387-1391.

Mesquita, A., M. Weinberger, et al. (2010). "Caloric restriction or catalase inactivation extends yeast chronological lifespan by inducing H2O2 and superoxide dismutase activity." Proc Natl Acad Sci U S A 107(34): 15123-15128.

Minotti, G. and S. D. Aust (1992). "Redox cycling of iron and lipid peroxidation." Lipids 27(3): 219-226.

Mizushima, N., T. Noda, et al. (1998). "A protein conjugation system essential for autophagy." Nature 395(6700): 395-398.

Moreau, K., S. Q. Luo, et al. (2010). "Cytoprotective roles for autophagy." Current Opinion in Cell Biology 22(2): 206-211.

Morselli, E., M. C. Maiuri, et al. (2010). "Caloric restriction and resveratrol promote longevity through the Sirtuin-1-dependent induction of autophagy." Cell Death Dis 1(1): e10.

Morselli, E., M. C. Maiuri, et al. (2010). "The life span-prolonging effect of sirtuin-1 is mediated by autophagy." Autophagy 6(1): 186-188.

Mortimer, R. K. and J. R. Johnston (1959). "Life span of individual yeast cells." Nature 183(4677): 1751-1752.

Murakami, C. and M. Kaeberlein (2009). "Quantifying yeast chronological life span by outgrowth of aged cells." J Vis Exp(27).

Nakatogawa, H., K. Suzuki, et al. (2009). "Dynamics and diversity in autophagy mechanisms: lessons from yeast." Nat Rev Mol Cell Biol 10(7): 458-467.

Natarajan, K., M. R. Meyer, et al. (2001). "Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast." Molecular and Cellular Biology 21(13): 4347-4368.

Noda, T., J. Kim, et al. (2000). "Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways." J Cell Biol 148(3): 465-480.

Obara, K. and Y. Ohsumi (2011). "PtdIns 3-Kinase Orchestrates Autophagosome Formation in Yeast." J Lipids 2011: 498768.

Obara, K., T. Sekito, et al. (2006). "Assortment of phosphatidylinositol 3-kinase complexes--Atg14p directs association of complex I to the pre-autophagosomal structure in Saccharomyces cerevisiae." Mol Biol Cell 17(4): 1527-1539.

Onodera, J. and Y. Ohsumi (2005). "Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation." J Biol Chem 280(36): 31582-31586.

Parrella, E. and V. D. Longo (2008). "The chronological life span of Saccharomyces cerevisiae to study mitochondrial dysfunction and disease." Methods 46(4): 256-262.

Powers, R. W., 3rd, M. Kaeberlein, et al. (2006). "Extension of chronological life span in yeast by decreased TOR pathway signaling." Genes Dev 20(2): 174-184.

Roberts, P., S. Moshitch-Moshkovitz, et al. (2003). "Piecemeal microautophagy of nucleus in Saccharomyces cerevisiae." Mol Biol Cell 14(1): 129-141.

Rosenfeld, E. and B. Beauvoit (2003). "Role of the non-respiratory pathways in the utilization of molecular oxygen by Saccharomyces cerevisiae." Yeast 20(13): 1115-1144.

Sakai, Y., M. Oku, et al. (2006). "Pexophagy: autophagic degradation of peroxisomes." Biochim Biophys Acta 1763(12): 1767-1775.

Shintani, T., K. Suzuki, et al. (2001). "Apg2p functions in autophagosome formation on the perivacuolar structure." J Biol Chem 276(32): 30452-30460.

Sinclair, D. A. and L. Guarente (1997). "Extrachromosomal rDNA circles--a cause of aging in yeast." Cell 91(7): 1033-1042.

Skulachev, V. P. and V. D. Longo (2005). "Aging as a mitochondria-mediated atavistic program: can aging be switched off?" Ann N Y Acad Sci 1057: 145-164.

Stack, J. H., D. B. DeWald, et al. (1995). "Vesicle-mediated protein transport: regulatory interactions between the Vps15 protein kinase and the Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeast." J Cell Biol 129(2): 321-334.

Steinkraus, K. A., M. Kaeberlein, et al. (2008). "Replicative aging in yeast: the means to the end." Annu Rev Cell Dev Biol 24: 29-54.

Sun, J., S. P. Kale, et al. (1994). "Divergent roles of RAS1 and RAS2 in yeast longevity." J Biol Chem 269(28): 18638-18645.

Suzuki, K., T. Kirisako, et al. (2001). "The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation." EMBO J 20(21): 5971-5981.

Suzuki, K., Y. Kubota, et al. (2007). "Hierarchy of Atg proteins in pre-autophagosomal structure organization." Genes Cells 12(2): 209-218.

Suzuki, K., T. Noda, et al. (2004). "Interrelationships among Atg proteins during autophagy in Saccharomyces cerevisiae." Yeast 21(12): 1057-1065.

Suzuki, S. W., J. Onodera, et al. (2011). "Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction." PLoS One 6(2): e17412.

Takeda, K., T. Yoshida, et al. (2010). "Synergistic roles of the proteasome and autophagy for mitochondrial maintenance and chronological lifespan in fission yeast." Proc Natl Acad Sci U S A 107(8): 3540-3545.

Takeshige, K., M. Baba, et al. (1992). "Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction." J Cell Biol 119(2): 301-311.

Tang, F., J. W. Watkins, et al. (2008). "A life-span extending form of autophagy employs the vacuole-vacuole fusion machinery." Autophagy 4(7): 874-886.

Tasdemir, E., M. C. Maiuri, et al. (2008). "Regulation of autophagy by cytoplasmic p53." Nat Cell Biol 10(6): 676-687.

Thumm, M., R. Egner, et al. (1994). "Isolation of autophagocytosis mutants of Saccharomyces cerevisiae." FEBS Lett 349(2): 275-280.

Tsukada, M. and Y. Ohsumi (1993). "Isolation and Characterization of Autophagy-Defective Mutants of Saccharomyces-Cerevisiae." FEBS Letters 333(1-2): 169-174.

Tsukada, M. and Y. Ohsumi (1993). "Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae." FEBS Lett 333(1-2): 169-174.

Turrens, J. F. (2003). "Mitochondrial formation of reactive oxygen species." J Physiol 552(Pt 2): 335-344.

Wang, C. W., J. Kim, et al. (2001). "Apg2 is a novel protein required for the cytoplasm to vacuole targeting, autophagy, and pexophagy pathways." J Biol Chem 276(32): 30442-30451.

Xie, Z., U. Nair, et al. (2008). "Atg8 controls phagophore expansion during autophagosome formation." Mol Biol Cell 19(8): 3290-3298.

Yen, W. L., J. E. Legakis, et al. (2007). "Atg27 is required for autophagy-dependent cycling of Atg9." Molecular Biology of the Cell 18(2): 581-593.

APPENDIX A

1. MEDIA

a. Glucose (YPD) medium, per liter

1% yeastn extract, 2% peptone, 2% glucose (Media sterilized by autoclaving at 121 ℃ for 15')

b. Glucose (YPD) agar medium, per liter

1% yeast extract, 2% peptone, 2% glucose, 2% agar (Media sterilized by autoclaving at 121 ℃ for 15')

Table 3.1. Replicative life span analysis of deletion mutants of autophagy genes on YPD – First screen

Strain Name	Mean RLS	n (Sample size)
*atg7*Δ	19,9±2,1	10
*atg31*Δ	27,3±2,3	10
*atg29*Δ	24,3±3,3	10
*atg14*Δ	21,1±2,3	10
*atg5*Δ	30,3±2,1	10
*atg3*Δ	22,4±1,7	10
*atg4*Δ	29,5±2	10
*atg12*Δ	21,5±2,1	10
*atg27*Δ	20,6±3	10
*atg32*Δ	25±1,6	10
*ccz1*Δ	23,3±2,3	10
*atg2*Δ	24,1±2,1	10
*atg18*Δ	26,9±3,1	10
*atg1*Δ	25,1±2,5	10
*irs4*Δ	18,9±2,5	10
*tax4*Δ	23,4±1,8	10
*atg17*Δ	19,1±0,9	10
*vps30*Δ	27±2,6	10
*fbp1*Δ	20,6±1,3	10
*atg15*Δ	20,1±2,8	10
*atg13*Δ	22,6±2,3	10
*atg10*Δ	19,6±2,5	10
*atg33*Δ	23,8±2,5	10
*atg16*Δ	25±3	10
*lap4*Δ	22,5±2,6	10
*yml108c*Δ	24,1±2,5	10
*atg26*Δ	23,4±1,8	10
*tor1*Δ	28,5±2,8	10
*vba4*Δ	24,8±1,1	10
BY4741	26,1±2,2	10

Table 3.2. Replicative life span analysis of deletion mutants of autophagy genes on YPD – Second screen

Strain Name	Mean RLS	n (Sample Size)
*atg7*Δ	20,6±1	47
*atg17*Δ	18,1±1	44
*fbp1*Δ	23,1±1	49
*irs4*Δ	24,4±1,2	49
*atg10*Δ	23,8±1,1	50
*atg27*Δ	22,1±0,9	59
*lap4*Δ	22,1±1,2	39
*atg15*Δ	24±1,6	30
*atg14*Δ	22,5±1,6	30
*atg3*Δ	26,6±1,4	26
*atg12*Δ	20,3±1,2	26
BY4741	25,9±0,7	133

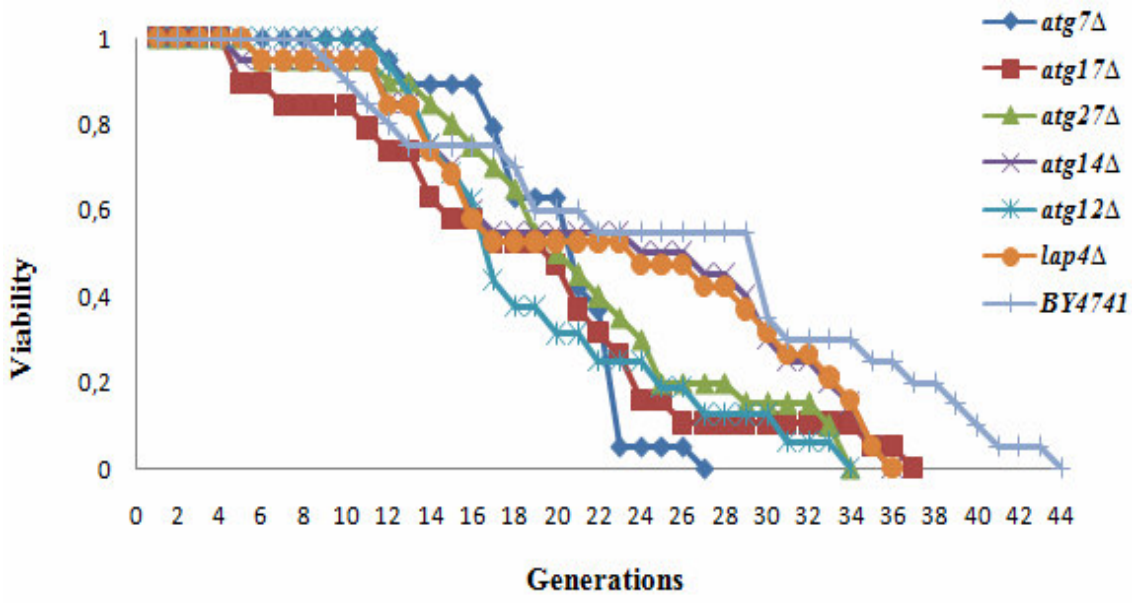

Figure 3.1. Replicative life span analysis of deletion mutants of autophagy genes on YPD

Table 3.3. Statistical analysis of hydrogen peroxide sensitivity by HALO assay

Strain Name	p Value	Mean Diameter	Hydrogen peroxide sensitivity in %
*atg7*Δ	0,024	3,9±0,1	16,7
*atg31*Δ	0,3156	3,1±0,2	
*atg29*Δ	0,0215	4,2±0,2	25,5
*atg14*Δ	0,2299	3,2±0,06	
*atg5*Δ	0,2545	3,5±0,08	4,4
*atg3*Δ	0,5739	3,2±0,1	
*atg4*Δ	0,777	3,4±0,1	1,4
*atg12*Δ	0,9082	3,3±0,1	
*atg34*Δ	0,2614	3,1±0,1	
*atg27*Δ	0,5569	3,5±0,2	4,7
*ccz1*Δ	0,65	3,4±0,07	1,4
*atg2*Δ	0,7467	3,3±0,1	
*atg18*Δ	0,1432	3,1±0,1	
*atg1*Δ	0,6967	3,2±0,2	
*atg17*Δ	0,2362	3,2±0,08	
*vps30*Δ	0,7	3,4±0,08	1,4
*irs4*Δ	0,6996	3,4±0,08	1,4
*tax4*Δ	0,366	3,5±0,1	5,4
*fbp1*Δ	0,0861	3,5±0,06	5,4
*atg15*Δ	0,0108	3,8±0,09	13,4
*atg13*Δ	0,4124	3,4±0,06	2,4
*atg10*Δ	0,2617	3,7±0,3	11,4
*atg16*Δ	0,4317	3,2±0,1	
*lap4*Δ	0,5739	3,2±0,1	
*yml018c*Δ	0,000011	5,2±0,1	55,9
*atg26*Δ	0,038	3,7±0,1	10,7
*tor1*Δ	0,5739	3,2±0,1	
*ymr221c*Δ	0,5626	3,2±0,2	
*vba4*Δ	0,4365	3,5±0,2	4,6
WT		3,3	

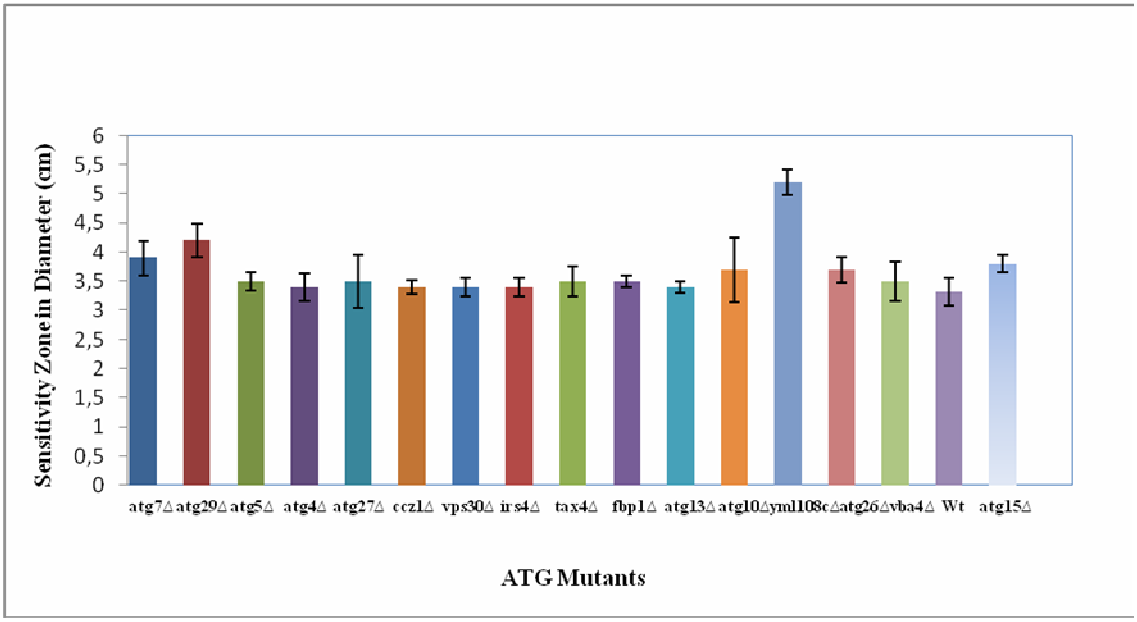

Figure 3.2. Hydrogen peroxide sensitivity of autophagy related gene mutants

3.3. Chronological and Replicative Ageing Patterns of Wild Type Strain of *S. cerevisiae* (BY4741) Cells upon Boron Treatment

Under starvation conditions, ATG8 is rapidly upregulated in yeast and mammalian cells. But not much is known about transcriptional machinery that functions during autophagy induction in yeast *S. cerevisiae* (Juhasz, Puskas et al., 2007).

Gcn4 is known as a general transcription factor. In yeast several autophagy related genes such as ATG1, ATG13 and ATG14 are the downstream targets of GCN4 (Natarajan, Meyer et al., 2001). In our laboratory it is determined that GCN4 is upregulated upon boron treatment. We proposed that maybe boron treatment can modulate replicative and chronological ageing through GCN4 upregulation and autophagy induction.

In order to study the roles of boron on chronological and replicative ageing of the wild type strain of *S.cerevisiae* (BY4741), colony formation assay (CFU) (Parrella and Longo, 2008) and replicative ageing assay (by Micromanupulation Microscope) (Mortimer and Johnston, 1959) was used.

For the replicative ageing analysis, wild type yeast strain (BY4741) were grown on YPD rich medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) in the presence of different amounts of boron (1mM, 5mM, 10mM, 20mM and 30mM). During the life span analysis, in each screening 20 replicas were analyzed for each

group and repeated at least twice. We found out that due to increased boron treatment there were major drop in life spans of wild type yeast strain (BY4741) as shown in (Table 3.4) and (Figure 3.3).

Table 3.4. Statistical analyses of replicative life span analyses upon boron treatment

	N (Sample Size)	Average Life Span
Control	40	24,5±1,6
1mM Boron	40	26,2±1,6
5mM Boron	40	24,6±1,6
10mM Boron	40	23,3±1,4
20mM Boron	40	21,8±1,4
30mM Boron	40	16,3±0,9

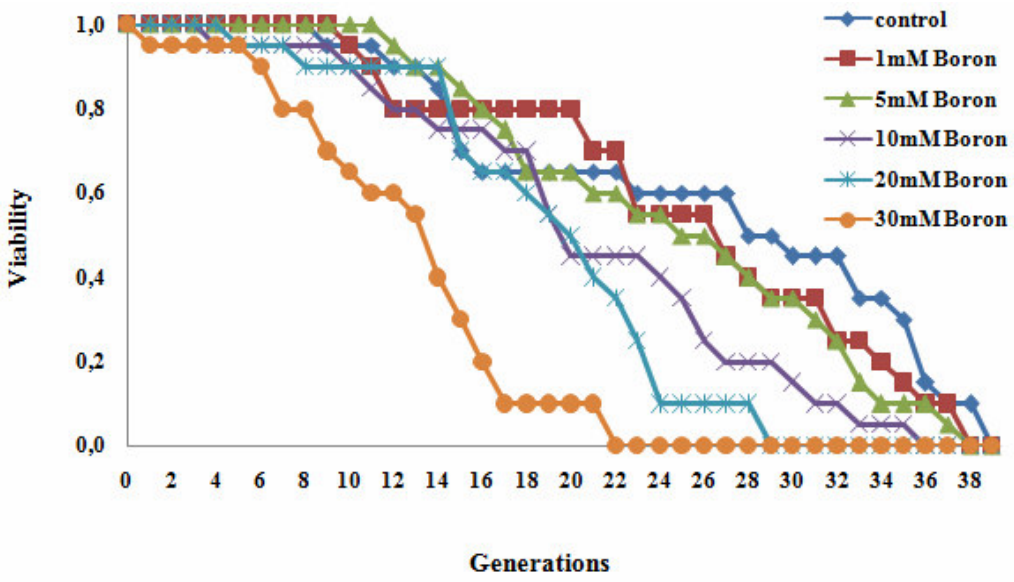

Figure 3.3. Replicative life span analyses of BY4741 yeast cells. Life span analyses for indicated amount of boron treated BY4741 yeast cells

For the chronological ageing analysis, wild type yeast strains (BY4741) were grown in YPD rich medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) in the presence of different amounts of boron (1mM, 5mM, 10mM, 20mM and 50mM). In

YPD media, almost all wild type yeast strains have shown stable ageing patterns, in which they showed a stable decrease in their colony formation abilities as shown in (Table 3.5) and (Figure 3.4).

Table 3.5. Chronological life span analyses of BY4741 (wild type) yeast cells on YPD media. Life span analyses for indicated amount of Boron treated BY4741 yeast cells by Colony Forming Unit (CFU)

	Day 0	**Day 2**	**Day 4**	**Day 6**	**Day 8**	**Day 10**	**Day 12**	**Day 14**	**Day 16**	**Day 18**
Control	100	80±6	77±3	55±8	48±1	52±4	48±7	38±2	34±4	22±1
1mM	100	75±7	74±9	50±9	57±5	49±6	47±2	38±3	38±1	22±1
5mM	100	72±3	69±4	51±7	47±7	55±2	50±4	40±3	29±5	30±7
10mM	100	71±3	78±1	61±8	55±7	53±4	59±6	46±4	43±2	28±3
20mM	100	63±9	64±5	53±6	51±7	54±4	47±3	43±3	34±2	22±10
50mM	100	69±5	65±5	41±4	38±8	37±6	23±2	20±1	12±2	9±0,4

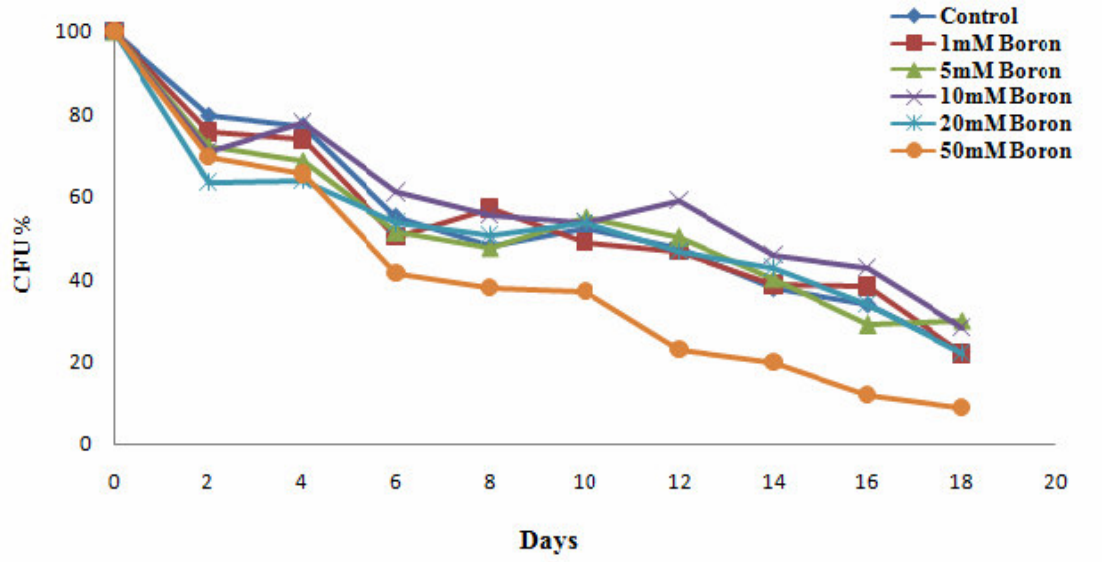

Figure 3.4. Chronological Life Span analyses of BY4741 yeast cells on YPD media. Chronological life span analyses for indicated amount of Boron treated BY4741 yeast cells

According to results of chronological ageing assays we concluded that boron shorten chronological life span in a dose-dependent manner as shown in (Figure 3.4) and (Table 3.5).