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Regulation of Yeast Life Span by Autophagy Related Genes

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Abstract
Aging can be defined as a gradual and progressive deterioration of the health by the time which elevates the risk of death. Autophagy is a conserved pathway from yeast to mammals, which is defined as a catabolic bulk degradation pathway. However, in *Saccharomyces cerevisiae,* autophagy is defined as single cell's adaptation to starvation. There is an emerging link between autophagic pathway and life span determination, however the roles of individual Autophagy Related Genes (ATG) in cellular aging have not been elucidated. In this study, we investigated the replicative and chronological life span of 32 yeast deletion mutants lacking the genes of autophagic machinery. We identified six mutants (*atg7Δ, atg17Δ, atg27Δ, lap4Δ, atg12Δ, atg14Δ*) with shorter replicative life span and four mutants (*ccz1*Δ*, atg10*Δ*,atg15*Δ and *fbp1*Δ) with shorter chronological life span.Our data suggest involvement of several new ATG mutants in life span determination.

Key Words: Autophagy,Aging,Life Span,Yeast, Longevity

INTRODUCTION

Autophagy involves degradation of damaged cellular components by lysosomal/vacuolar enzymes [1,2]. Autophagic pathway is required for maintaining many cellular functions under different circumstances and it is important for viability. Under normal conditions basal level of autophagy regulates turnover of proteins and organelles within the cell. However, it is upregulated in case of cellular damage or limitations in nitrogen and carbon sources. Induction of autophagy plays an essential role in molecular recycling in the cell [3]. Initiation of autophagy begins with the formation of double-membraned vesicles called autophagasomes which is a part of the cytoplasm to be degraded [4]. In subsequent process, autophagasomes fuse with vacuole/lysosome and their ingredients are digested by the resident enzymes present in vacuoles. Digestion products are reused by the cell after they are transported back into the cytoplasm [5]. Genes that play role in autophagy (ATG genes) were mostly identified and characterized in yeast [6]. There are more than 30 autophagy related genes and they have different functions such as induction, vesicle formation, expansion, fusion and transport of the degradation products in different aspects of the pathway. Yeast has been used widely in cellular aging studies due to the conserved mechanisms of aging between yeast and higher organisms. Yeast cells undergo both replicative and chronological aging. Replicative life span (RLS) reflects the number of daughter cells that each mother cell generates before it stops dividing and the chronological life span (CLS) is defined as the duration of the time a cell can remain viable in the stationary phase.

There is an established link between autophagy and aging. In general, inhibition of autophagy causes premature aging whereas activation of it delays aging and extends longevity in many different model organisms [7]. In *C. elegans* and *D. melanogaster* inhibiton of autophagy decreases damaged protein turnover and shortens life span (8,9). Several genome-wide screens have shown that autophagic path is required for chronological life span determination in yeast [10-12]. Calorie restriction extends life span by inducing autophagy and inhibiting autophagy prevents extension in life span in many model organisms [13-19]. Rapamycin, a drug that induces autophagy, extends life span in many organisms, however, deletion of autophagy genes blocks the life span extension provided by rapamycin [20,18]. Additionally, autophagy plays a role in life span modulation by pharmacological agents spermidine and resveratrol [21,22].

Intrugued by the importance of autophagy in life span determination, we analyzed the replicative and the chronological life spans of yeast mutants that are deficient in autophagy related genes to elucidate roles of these genes in aging process.

MATERIALS AND METHODS

Yeast strains and growth conditions

Wild type (BY4741) strain of *S. cerevisiae* and its isogenic deletion mutants were obtained from the yeast deletion collection set (Invitrogen). Cells were grown on YPD agar media (2% glucose, 2% peptone, 1% yeast extractand 2% agar, Applichem, Germany).

Life span analysis

Yeast strains were grown on YPD agar for two days prior to life span analysis. Replicative life span was determined as the total number of daughter cells that each mother cell generated. For each strain, individual daughter cells were collected and selected as starting mother cells. Newly formed daughter cells from these mother cells were removed and discarded. Plates were controlled periodically and newly formed daughter cells were removed until cells stopped dividing. During night periods plates were stored at 4ºC. In the initial screen, life span of ten cells for each strain were analyzed in order to identify candidate mutants with altered life spans. Subsequently more cells for each candidate mutants were analyzed at least by two independent assays. Student t-test was used as a statistical analysis. For the chronological life span analysis, yeast strains were grown in 2 ml YPD media overnight and were suspended in 18 ml fresh YPD in 250ml flasks and incubated at 30°C for 15 days at 180 rpm. The survival rate of cells was measured by counting the colony forming units every 72 h. Each strain was analyzed three times.

Halo assay

A halo assay was used to test the sensitivities of autophagy gene mutants against to hydrogen peroxide. The yeast cells were grown overnight in YPD at 30°C. The cells were diluted in fresh YPD media and incubated for 3 hours. 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 (Sigma, USA) was dropped into the center of the YPD plate and the plates were incubated at 30°C for 2 days. The diameters of the halo at the center of the plates were measured by a ruler. Samples were analyzed 3 times for each strain. Student t-test was used as a statistical analysis.

RESULTS AND DISCUSSION

Replicative life span analyses of autophagy (ATG) mutants

Autophagy is a path by which cells get rid of their internal debri and recycle molecules for other aspects of cellular metabolism. As shown by many others, we reasoned that disruption of autophagy by genetic manupulations may alter the life span of cells, but we aimed to screen to all ATG genes rather than using some of the ATG genes. Yeast mutants for all the non-essential autophagy related genes were analyzed for their relative RLS compared to the wild type cells. Genes whose products play role in autophagy were identified from Saccharomyces Genome Database by GO annotations and listed in Table (1).We first analyzed ten cells for each of thirty two ATG mutants to reduce the number of samples. After the initial analyses, mutants with reduced life span were further analyzed by using higher number of cells. At the end, we confirmed that *atg7Δ*, *atg17Δ*, *atg27Δ*, *lap4Δ*, *atg12Δ*, *atg14Δ* mutants have reduced RLS (Figure 1, Table 2). Deletion of ATG17 gene decreased the RLS by 30%. Atg17 serves as a scaffold protein for formation of the PAS (Pre-Autophagosomal Structure)[23]. It is also required for vesicular expansion and is the regulatory subunit of the Atg1 kinase complex [24]. Cells lacking ATG17 gene are deficient in autophagy and sensitive to nutrient limitations [24]. A previous study has shown that deletion of ATG17 gene shortens life span under normal conditions which is consistent with our results [25].

In contrast to Tang et al., (2008) we observed that deletion of ATG7decreased the RLS by 20%. ATG7 functions as a member of the E1 family of ubiquitin-activating enzymes and conjugates Atg12 with Atg5 and Atg8 with phosphatidylethanolamine (PE). In *Drosophila,* deletion of ATG7 leads to impaired ability to induce autophagy in response to starvation and results in reduced life span. Thus, under normal conditions, it is not surprising that the absence of ATG7 led to reduced RLS in yeast [26]. ATG14 encodes the autophagy specific subunit of the phosphatidylinositol 3 - kinase complex I [27], which targets other players of the complex I to PAS [28,29], thereby leads the complex I to function specifically in autophagy. Atg14 also plays role in directing other proteins such as Atg2, Atg8 and complex of Atg5, Atg12 and Atg16 to the PAS [30]. Atg14 is a conserved protein of autophagy pathway and has homolog in human [31]. ATG14 mutants are defective in autophagy and Cvt paths, and can not survive under nutrient limitations [32]. It has been shown that calorie restrcition can not operate on RLS extention in *atg14Δ* mutants [25]. Our analyses showed that absence of this gene shortened RLS by 13% under normal growth conditions, which supports the previous findings. LAP4 encodes a vacuolar aminopeptidase/zinc metalloproteinase transported to vacuole by the Cvt pathway [33]. There is no previous information about enrollment of *LAP4* in life span determination in yeast. We found that the deletion of this gene decreased the RLS by 15%. Atg12 is a conserved ubiquitin-like modifier and is conjugated with Atg5 under normal conditions [34]. Atg12-Atg5 complex interacts with Atg16 and localizes to pre-autophagosomal structure [35,34]. In *atg12Δ* mutants, Lap4 (pro-aminopeptidase-I) is not processed to mature form [36]. Absence of Atg12 also inhibits autophagic machinery under nitrogen starvation conditions [37,38]. In the present study we observed that the deletion of ATG12 lead to a decrease in RLS by 22%. Atg27 is a phosphotidylinositol 3-phosphate binding protein involved in membrane delivery to PAS [39].

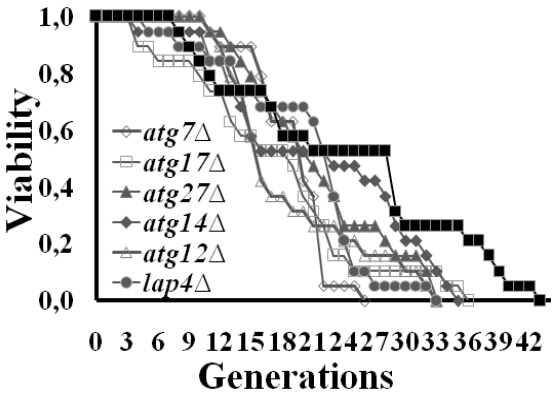

Figure 1. Replicative life span analyses of indicated ATG mutants.

Atg27 plays a vital role in both autophagy and the Cvt pathway. Absence of *ATG27* leads to deficiency in Cvt path and pexophagy. Previous studies have shown that under nitrogen starvation conditions the deletion of ATG27 results in decreased autophagy [39]. Similarly, in our study we found that the deletion of ATG27 decreased the RLS by 15%.

Chronological life span analyses

Chronological life span (CLS) is defined as the time that cells can remain viable in the quiescent state [40]. Several genes that regulate chronological life span through autophagic pathway have been identified recently. Autophagy is needed for cell viabilty because amino acid homeostasis is regulated by autophagy under starvation conditions [16]. To elucidate the roles of autophagy in life span determination, we also analyzed the CLS of ATG related gene mutants under normal growth conditions. Of the 32 mutants (Table 1), only *ccz1*Δ*, atg10*Δ*, atg15*Δ and *fbp1*Δ mutants had a shorter CLS compared to that of wild type cells (Figure 2 and Table 3). CCZ1 encodes a protein which is involved in vacuolar assembly and essential for maintanence of functional autophagic machinery [41,42]. ATG10 encodes a E2 like ubiquitin conjugating enzyme [43] that plays a role in formation of Atg12p-Atg5p conjugate [44]. ATG15 encodes a lipase that is required for degredation of autophagic vesicles in the vacuole [45]. Involvement of *ATG15* in CLS determination has been noticed previously [12]. Absence of *ATG15* shortened the CLS when cells were starved for either phosphate or leucine [12]. FBP1 encodes for the fructose - 1,6 - biphosphatase enzyme which plays roles in gluconeogenesis [46] and which is degraded either by proteasomal or autophagic pathway [47]. It has been previously shown that deletion of two ATG genes, ATG1 or ATG7, reduced CLS under minimal growth conditions [16], but our findings suggested that *atg1Δ* and *atg7Δ* mutants have normal CLS in rich media. We believe the inconsistency betwen the two studies come from the type of the growth conditions used.

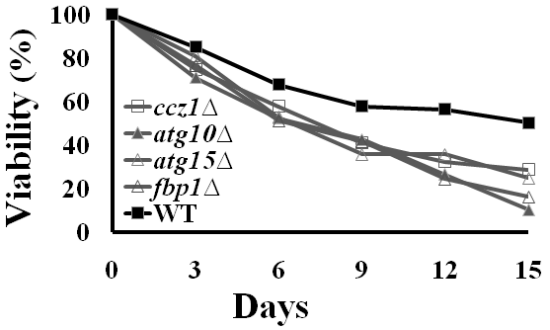

Figure 2. Chronological Life Span analyses of ATG mutants. For the chronological life span analysis, yeast strains were grown in 2 ml YPD media overnight and were suspended in 18 ml fresh YPD in 250ml flasks and incubated at 30 °C for 15 days at 180 rpm. The survival rate of cells was measured by counting the colony forming units every 72 h. Each analysis was performed three times.

Hydrogen peroxide sensitivity of autophagy mutants

Oxidized macromolecules and organelles are recycled by autophagy and thus impairement of autophagy is expected to cause accumulation of oxidative damage and sensitivity to oxidants. We performed a hydrogen peroxide halo assay on autophagy gene mutants to asses their oxidative stress tolerance levels. As shown in Figure (3) our results revealed that *atg7*Δ, *atg29*Δ*, atg15*Δ and*atg26*Δ mutants showed higher sensitivity to hydrogen peroxide compared to that of wild type cells. In a previous study, ATG7 mutant flies which were treated with hydrogen peroxide and paraquat had a shorter life span [26].Our analyses also showed that ATG7 deficient yeast cells were prone to oxidative stress and live shorter. Similary, *atg15*Δ cells also showed hydrogen peroxide sensitivity and had a shorterCLS.

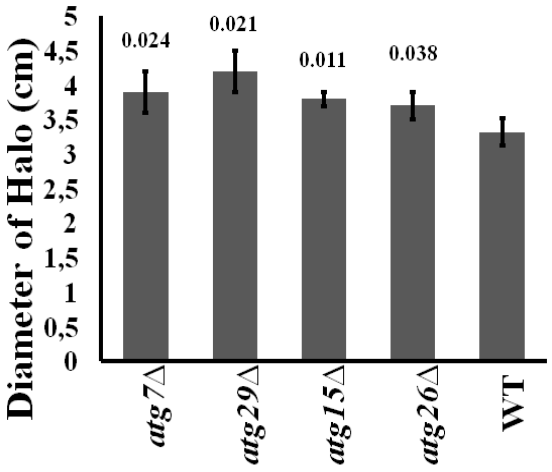

Figure 3. Hydrogen peroxide sensitivities of autophagy mutants. Hydrogen peroxide sensitivity was determined by a Halo assay in which 5 µl of 8.8 M H_2O_2 applied to the center of the YPD plates which were pre-inoculated with cells. The diameter of zones in which cell growth was suppressed was measured. Each strain was tested three times, and the bars show the standart deviation.Numbers above the bars show the p-values derived from the Student's t test.

CONCLUSION

We analyzed 32 autophagy releated gene mutants for their life span and hydrogen peroxide sensitivity. We found that six mutants, *atg7Δ, atg17Δ, atg27Δ, lap4Δ, atg12Δ, atg14Δ,*had shorter RLS and four mutants, *ccz1*Δ*, atg10*Δ*, atg15*Δ and *fbp1*Δ,had shorter CLS. Interestingly, only two mutants with shorter life span (*atg7Δ, atg15*Δ) showed hydrogen peroxide sensitivity. In this study, we aimed to reveal the effect of individual autophagy related genes on yeast longevity and our data did not necessarily exclude the possibility that the shorter RLS/CLS of mutants might be an indirect effect of related mutations.

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Table 1. Autophagy genes/mutants that were analyzed in this study

Systematic Name	Standard Name	Function
YHR171W	***ATG7***	Member of the E1 family of ubiquitin-activating enzymes
YDR022C	***ATG31***	Autophagy-specific protein; may form a complex with Atg17p and Atg29p
YPL166W	***ATG29***	Autophagy-specific protein; interacts with Atg17p
YBR128C	***ATG14***	Autophagy-specific subunit of PI 3-kinases complex I
YPL149W	***ATG5***	Conserved protein involved in autophagy and the cvt pathway
YNR007C	***ATG3***	E2-like enzyme involved in autophagy and the cvt pathway
YNL223W	***ATG4***	Conserved cysteine protease
YBR217W	***ATG12***	Conserved ubiquitin-like modifier
YOL083W	***ATG34***	Receptor protein involved in selective autophagy during starvation
YJL178C	***ATG27***	Type I membrane protein involved in autophagy and the cvt pathway
YIL146C	***ATG32***	Mitochondrial-anchored transmembrane receptor
YBR131W	***CCZ1***	Protein involved in vacuolar assembly, essential for autophagy and the cvt pathway
YNL242W	***ATG2***	Peripheral membrane protein required for vesicle
YFR021W	***ATG18***	Phosphoinositide binding protein required for vesicle formation
YGL180W	***ATG1***	Protein serine/threonine
YKR019C	***IRS4***	EH domain-containing protein involved in regulating PtdIns(4,5) levels and autophagy
YJL083W	***TAX4***	EH domain-containing protein involved in regulating PtdIns(4,5) levels and autophagy
YLR423C	***ATG17***	Scaffold protein responsible for PAS organization
YPL120W	***VPS30***	Subunit of PI 3-kinases complexes I and II
YLR377C	***FBP1***	Fructose-1,6-bisphosphatase
YCR068W	***ATG15***	Lipase required for intravacuolar lysis of autophagic bodies and cvt bodies
YPR185W	***ATG13***	Regulatory subunit of the Atg1p signaling complex
YLL042C	***ATG10***	Conserved E2-like conjugating enzyme
YLR356W	***ATG33***	Mitochondrial mitophagy-specific protein
YMR159C	***ATG16***	Conserved protein that interacts with Atg12p-Atg5p
YKL103C	***LAP4***	Vacuolar aminopeptidase yscI
YML018C	***YML018C***	Putative protein of unknown function
YLR189C	***ATG26***	UDP-glucose:sterol glucosyltransferase
YJR066W	***TOR1***	PIK-related protein kinase and subunit of TORC1
YMR221C	***YMR221C***	Putative protein of unknown function
YDR119W	***VBA4***	Protein of unknown function
YBL078C	***ATG8***	Component of autophagosomes and Cvt vesicles

Table 2 The statistical analyses of the replicative life span assays

Strain Name	*atg7*Δ	*atg17*Δ	*atg27*Δ	*lap4*Δ	*atg12*Δ	*atg14*Δ	*WT*
RLS (Mean ± SEM)	20.6±1	18.1±1.2	22.1±0.9	22.1±1.1	20.3±1.3	22.5±1.6	25.7±0.7
n	47	44	59	39	26	30	151
p-value	7×10^{-5}	7×10^{-7}	3×10^{-3}	7×10^{-3}	8×10^{-2}	5×10^{-4}	
% Decrease in RLS	20	30	15	15	22	13	

Table 3. The statistical analyses of the chronological life span assays

	Day Zero	Day 3	Day 6	Day 9	Day 12	Day 15
***ccz1*Δ**	100	75±3.5	58±0.7	41±3.6	32±2.3	28±2.3
***atg10*Δ**	100	71 ±2.2	52±1.7	42±3.6	26±2.7	10±1.6
***atg15*Δ**	100	81±1.7	53±4.9	36±3	36±1.1	25±1.2
***fbp1*Δ**	100	77±3.6	51±4.2	42±2.5	24±2.1	16±2.4
WT	100	85±3.1	68±4.2	58±2.4	56±2.6	50±1.6

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