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Research Paper

A life-span extending form of autophagy employs the vacuole-vacuole fusion machinery

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Abbreviations: Ape1, aminopetidase I; COP II, coat protein complex II; CR, caloric restriction; Cvt, cytoplasm to vacuole targeting; DHR, dihydrorhodamine; ER, endoplasmic reticulum; POBN, 4-pyridyl-1-oxide *N-tert*-butylnitrone; ROS, reactive oxygen species; SNAREs, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; SREBP, sterol regulatory element binding protein

Key words: autophagy, ergosterol, vacuole, Atg15, life span

While autophagy is believed to be beneficial for life-span extension, it is controversial which forms or aspects of autophagy are responsible for this effect. We addressed this topic by analyzing the life span of yeast autophagy mutants under caloric restriction, a longevity manipulation. Surprisingly, we discovered that the majority of proteins involved in macroautophagy and several forms of microautophagy were dispensable for life-span extension. The only autophagy protein that is critical for life-span extension was Atg15, a lipase that is located in the endoplasmic reticulum (ER) and transported to vacuoles for disintegrating membranes of autophagic bodies. We further found that vacuole-vacuole fusion was required for life-span extension, which was indicated by the shortened life span of mutants missing proteins (*ypt7*Δ, *nyv1*Δ, *vac8*Δ) or lipids (*erg6*Δ) involved in fusion. Since a known function of vacuole-vacuole fusion is the maintenance of the vacuole membrane integrity, we analyzed aged vacuoles and discovered that aged cells had altered vacuolar morphology and accumulated autophagic bodies, suggesting that certain forms of autophagy do contribute to longevity. Like aged cells, *erg6*Δ accumulated autophagic bodies, which is likely caused by a defect in lipase instead of proteases due to the existence of multiple vacuolar proteases. Since macroautophagy is not blocked by *erg6*Δ, we propose that a new form of autophagy transports Atg15 via the fusion of vacuoles with vesicles derived from ER, and we designate this putative form of autophagy as secretophagy. Pending future biochemical studies, the concept of secretophagy may provide a mechanism for autophagy in life-span extension.

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Introduction

Eukaryotic cells have evolved delicate quality control mechanisms. These mechanisms include DNA repair in the nucleus and protein degradation in the cytoplasm. Such mechanisms ensure sustained cell growth. Defects in these mechanisms result in accumulation of DNA mutations or misfolded proteins and lead to cell senescence and aging. The regulation of synthesis and transport of lipids such as cholesterol has also been extensively studied (reviewed in ref. 1). However, the quality control mechanisms for cholesterol, i.e., how cells handle altered sterols, are not as well appreciated as that for other macromolecules.

The lysosome (vacuole in yeast) is likely an organelle that is involved in the quality control of sterol metabolism. Mutations in lysosomal lipase activities are the basis of disordered lysosomal storage of cholesterols in Niemann-Pick type A and B, and other diseases.[1] Instead of serving as a passive storage for sterols, lysosomes monitor the quality of sterols via their multiple functions. Insertion of plant sitosterol, which differs from cholesterol in an extra ethyl group at the C24 position, into the membrane of endoplasmic reticulum (ER) of mammalian cells results in autophagic cell death.[2] Autophagy is the lysosomal degradation of damaged, superfluous or obsolete proteins or organelles (reviewed in refs. 3 and 4). Multiple signals trigger different forms of autophagy. Starvation induces nonselective autophagy as well as autophagic degradation of ER, the organelle that synthesizes sterols.[5]

Autophagy is classified into several forms based on the targets for degradation, including nonselective autophagy and selective autophagy such as mitophagy (degradation of mitochondria) and pexophagy (degradation of peroxisomes). Based on the mechanisms of how targets are transported into vacuoles/lysosomes, autophagy is also divided into three different forms: macroautophagy, microautophagy, and chaperone-mediated autophagy (reviewed in refs. 4, 6 and 7). While macroautophagy involves the formation of an autophagosome and fusion of this autophagosome with the vacuoles, microautophagy refers to a process where vacuoles directly uptake targets by invagination of vacuolar membrane. Chaperone-mediated

autophagy was discovered in mammalian cells but not in yeast cells to date. Another form of autophagy, the cytoplasm-to-vacuole targeting (Cvt) pathway, was only discovered in yeast but has not been detected in mammalian cells so far. Thus, it is likely that more forms of autophagy will be discovered. These multiple forms of autophagy support each other during cell growth. For example, macroautophagy increases the surface area of vacuoles/lysosomes but microautophagy decreases it. Defective macroautophagy leads to constitutive activation of chaperone-mediated autophagy.[8]

Not surprisingly, several papers reported that autophagy is required for life-span extension.[9,10] However, Kenyon's group has recently claimed that autophagy is neither sufficient nor necessary for life-span extension.[11] Clues for resolving these controversies may come from studies on autophagy and lipid metabolism. Studies on fungi revealed a link between vacuoles and the metabolism of ergosterol, the counterpart of cholesterol. Ergosterol is synthesized on ER by a series of enzymes. Although deletion of one of the last few enzymes (Erg6, Erg2, Erg3, etc.,) is not lethal, the mutant accumulates altered sterols.[12] The ER membrane normally does not contain sterols. Insertion of sterols into the ER membrane will change the physical property of the membrane and cause ER stress,[13] which is known to activate autophagy.[14] Altered membranes may be more sensitive to oxidative attack and need to be removed from the cytosol. Accordingly, mutants defective in either ergosterol synthesis (*erg3*Δ) or intra-vacuolar lipid degradation via autophagy (*atg15*Δ) are sensitive to lipid oxidant linoleic acid 13-hydroperoxide but not hydrogen peroxide or other oxidants tested.[15] Atg15, the only lipase in the yeast autophagy pathway identified so far, is an ER integral membrane protein with its enzyme domain in the ER lumen but functions inside vacuoles.[16,17] Moreover, blocking the ergosterol synthesis with ketoconazole upregulates the expression of several ERG (*ERG6, ERG3, ERG4*, etc.,) genes as well as *ATG15* gene in a pathogenic fungus *Trichophyton rubrum*.[18] Thus, we hypothesize that a form of autophagy controls the quality of ergosterols.

Here we tested our hypothesis by analyzing which vacuolar functions are required for life span extension under a longevity context. While the formation of autophagosomes and the degradation of intravacuolar proteins are dispensable for life span extension, the intravacuolar membrane disintegration by Atg15 is critical to life span extension. Consistent with the role of Atg15 in controlling lipid qualities, we observed that *erg6*Δ behaves similar to *atg15*Δ in shortening the life span in CR media and in accumulation of reactive oxygen species. However, *erg6*Δ accumulates oxidized materials inside vacuoles but *atg15*Δ accumulates oxidized materials in the cytoplasm. Thus, we propose that a special form of autophagy, which has been termed secretophagy, relieves the oxidative pressure generated by malfunctions in ergosterol synthesis and thus extends the life span.

Results

Vac8, a vacuolar membrane protein, plays roles in longevity. Recent functional studies of the vacuolar membrane protein Vac8[20,37] suggest a genetic interaction with TOR complex 1. Vac8 interacts with Tco89, a component of TOR1 complex, in a yeast two-hybrid assay (ref. 37; Fig. 1A). Tor complex 1 negatively controls macroautophagy (ref. 38; Fig. 1B). Double deletion of *TOR1* and *VAC8* makes cells arrested after nitrogen starvation.[20] These genetic interactions prompted us to measure the life span of *vac8*Δ mutants as the first step in deciphering the relationship between autophagy and other vacuolar functions and life span. During our analysis of life span, we noticed that virgin cells of *tor1*Δ *vac8*Δ took a very long time to produce their first buds (Fig. 1C). While virgin *vac8*Δ cells finished their first budding in 4 to 5 hours, virgin *vac8*Δ *tor1*Δ cells took about 12 hours to finish their first budding (Fig. 1C). Deletion of *TOR1* alone does not affect this budding phenotype (data not shown). This observation is consistent with Zurita-Martinez's report that Tor1 signaling requires Vac8. Although the double mutant was very slow in producing its first bud, it had a significantly longer life span than *vac8*Δ alone (p-value = 3×10^{-8}; Fig. 1D). The longevity mediated by *tor1*Δ was independent of Vac8, suggesting that other targets of Tor1p such as protein synthesis may also contribute to life-span extension. The life span of *vac8Δ* (17.3), which is shorter than that of wild type (usually 25 to 27), suggested the contribution of some vacuolar functions in longevity. To search for such longevity functions, we analyzed more mutants defective in vacuolar functions including autophagy.

The majority of macroautophagy proteins are dispensable for life-span extension. A short life span of a mutant on YEPD medium may simply suggest a defect in fitness. To analyze how vacuolar functions contribute to life-span extension, we compared the life span of mutants on caloric restricted medium, a known longevity manipulation. If a mutant does not affect the life span on normal medium, but shortens the life span on calorie restricted medium, the affected gene is likely dedicated to longevity. As a first step to analyze the contribution of autophagy, we monitored the extent of macroautophagy of cells incubated in media with different concentrations of glucose (Fig. 2A). In this assay, the maturation of Ape1 is a sign of macroautophagy. To our surprise, the restricted glucose concentration (0.5%) that extends the life span significantly did not upregulate the autophagy activity (lane 2, Fig. 2A); it did not show an obvious effect on Ape1 maturation. Maturation of Ape1 occurred when the concentration of glucose was decreased to 0.2% (lane 3 of Fig. 2A). These results indicated that there was little, if any, effect of caloric restriction on macroautophagy. While it is possible that a mild upregulation of autophagy has a cumulative beneficial effect in each succeeding generation, it is also possible that only selected aspects of autophagy are required for life-span extension.

To understand which steps or which aspects of autophagy contribute to life-span extension, we did a partial screen for autophagy and other vacuole-related functions. We selected mutants defective in each stage of the autophagy process (Fig. 1A) and measured their life span on CR media (Table 1). This measurement revealed several novel relationships between autophagy and life-span extension.

Most mutants defective in the autophagosome formation (step 1 in Fig. 1B) did not affect life span on CR. Deletion of *ATG1, ATG6, ATG7, ATG8* or other genes involved in step 1 did not show a life span that was obviously shorter than that of wild type on CR media (Table 1). There is one scenario that supports the contribution of macroautophagy in life-span extension; these *atg* mutants may have extended life spans on YEPD. Thus, we further compared the life span of selected mutants on YEPD and CR media (Table 2) and also found that the formation of autophagosomes (Atg10) was not required for CR-mediated life-span extension. A striking mutant that shortens the life span on both YEPD and CR media is *atg17*Δ

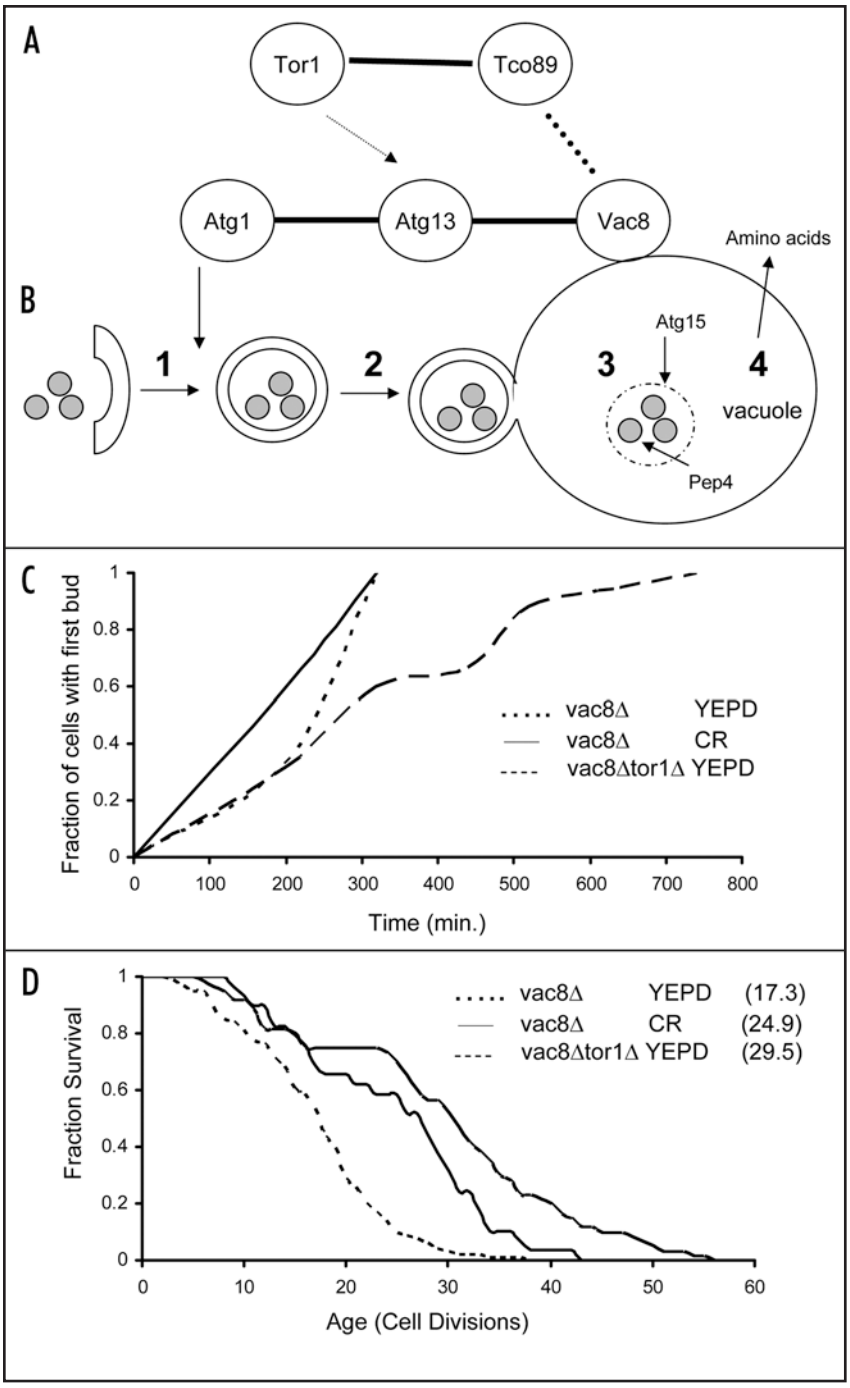

Figure 1. Vac8 is required for life-span extension in wild type but not in *tor1Δ* strain. (A) Summary of Vac8's protein-protein interactions related with Tor1 and Atg1. Solid lines: interactions identified by both affinity-capture assay and yeast two-hybrid assay. Dotted line: interaction identified by yeast two-hybrid assay. Dotted arrow: chemical interactions. Tor1 promotes the phosphorylation of Atg13.[25] Phosphorylated Atg13 inhibits the activity of Atg1.[38] Vac8 is a vacuolar membrane protein that interacts with Atg13 by affinity capture and yeast two-hybrid analysis. Vac8 also interact with Tco89 by yeast two-hybrid. Tco89 is a component of the Tor complex 1. Interaction of Tco89 with Tor1 activates Tor1.[79] (B) Sketch of macroautophagy. Step 1. autophagosome formation. Step 2. Fusion of autophagosome with vacuoles. Step 3. Intravacuolar degradation of autophagic vesicles. Step 4. Recycling of amino acids. (C) *vac8Δ tor1Δ* double mutant is delayed in producing the first bud. Forty five virgin cells of each strain were monitored for the production of the first bud. The fraction of cells with first bud (Y axis) was plotted against the time (X axis). (D) Deletion of *VAC8* shortens the life span. Virgin cells were counted for the number of their daughter cells. The ratio of live cells (fraction survival; Y axis) was plotted against generation (X axis). Numbers in parentheses are the mean life span. The sample sizes for *vac8Δ*, *vac8Δ* CR, and *vac8Δ tor1Δ* are 94, 29 and 60 respectively. The p values are 2 x 10^{-3} between *vac8Δ* and *vac8Δ* CR and 3 x 10^{-8} between *vac8Δ* and *vac8Δ tor1Δ*.

(Table 2). The protein Atg17 controls the magnitude of autophagy[39] but interacts with more than 50 proteins,[40,41] raising the possibility that other functions of Atg17 may also be required for life-span extension. Most autophagy genes were identified in screens under nitrogen starvation stress.[4] Such a nitrogen starvation stress may not happen in most aged cells.

A major purpose of autophagy is to provide amino acids for the cell,[21] which is achieved by intravacuolar protein degradation (Pep4Δ, step 3 in Fig. 1B) and amino acid recycling (*Atg22Δ*, step 4 in Fig. 1B). Blocking these steps (*pep4Δ*, *atg22Δ*) did not shorten the life span on CR media (Table 1). The amino acids resulting from the autophagic degradation of proteins are recycled to the cytoplasm by vacuolar membrane 'effluxers': Atg22, Avt3 and Avt4.[21] Since Atg22 is only one of the three effluxers, we compared the life span of a mutant missing all the amino acids 'effluxers' on YEPD and CR (Fig. 2B). In this triple mutant (*atg22Δ avt3Δ avt4Δ*), caloric restriction still significantly increased the life span. The life span of triple mutant on YEPD is shorter than BY4742, which is likely due to strain background differences. These observations suggest that if macroautophagy contributes to life-span extension, it is not through providing amino acids to the cytosol.

In addition to macroautophagy, we also measured the life span of mutants defective in lipid metabolism (*srt1Δ*), mitochondrial function (*mdm38Δ*), membrane trafficking from ER-to-Golgi (*trs85Δ*), mitophagy (*uth1Δ*), vacuolar protein sorting (*vps1Δ*), peroxisome biogenesis (*pex3Δ*, *pex6Δ*) (Table 1), and the Cvt pathway and pexophagy (*atg11Δ*, Table 2). These mutants either did not affect the life span or did not shorten the life span to the extent of a unique autophagy mutant *atg15Δ*.

Intravacuolar disintegration of autophagic vesicle is required for life-span extension. Although most autophagy genes, especially those involved in protein degradation, are not required for life-span extension, the intravacuolar disintegration of autophagic vesicles that is mediated by Atg15 did contribute to life-span extension in CR media (Table 1). A further comparison of the life span on YEPD and CR media showed that caloric restriction shortens the life span of *atg15Δ* by 28% when compared with YEPD (Fig. 3A). To confirm the effect of Atg15, we put *atg15Δ* mutation in another longevity context. We constructed a *SIR2ox atg15Δ* double mutant and compared its life span with wild-type cells. Overexpression of the histone deacetylase *SIR2* (SIR2ox) extends the life span.[42] In the BY4742 strain background, the SIR2ox-mediated life-span

extension is independent of caloric restriction[43] and thus can be used to further test the contribution of Atg15 to life-span extension. Deletion of *ATG15* blocked the effect of *SIR2ox* in life-span extension; there was no significant difference between the life span of *SIR2ox atg15*Δ and the wild type strain (Fig. 3B). In the presence of *ATG15* (wild type BY4742), overexpression of *SIR2* extends the life span from 26.1 to 34.6 generations.[43] We also obtained similar results in the wild type background (data not shown). Our observations about the life span suggest that intra-vacuolar disintegration of the autophagosome membranes by Atg15 is a key autophagy step for life-span extension especially under longevity conditions.

SNARE proteins are crucial for life-span extension. The intravacuolar disintegration of autophagic vesicles (step 3) occurs after the fusion of the autophagosome with the vacuoles (step 2) (Fig. 1B). We thus tested the contribution of this fusion step to longevity. How vacuoles fuse with autophagosomes is not yet well known. Darsow et al.,[44] reported the contribution of Vam3, a member of the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) for vacuole-vacuole fusion. We thus tested more SNAREs (Vam3, Vam7, Nyv1) for vacuole-vacuole fusion. Both *vam3*Δ (Table 1) and *vam7*Δ (Table 2) greatly shortened the life span. These two proteins are also required for multiple other pathways. Nyv1 is the v-SNARE dedicated to vacuole-vacuole fusion.[45] Ypt7 is a Ras-like GTPase that regulates the assembly of the SNARE pairs during vacuole-vacuole fusion[46] and was thus included in our assay. Deletion of either *YPT7* or *NYV1* (Table 2) blocked the life span extension effect of caloric restriction. They had normal life span on YEPD. Like its effect on *atg15*Δ ($p = 0.0001$), caloric restriction greatly shortened the life span of *ypt7*Δ ($p = 0.0003$) and *nyv1*Δ ($p = 0.0006$) when compared with the life span on YEPD. While the result regarding Vam3 is consistent with the hypothesis that autophagy is required for life-span extension under specialized conditions, the results regarding Nyv1 were surprising. The only reported physical connection between Nyv1 and the autophagosome is a co-immunprecipitation relationship between Nyv1 and Atg8.[47] However, the life span of *atg8*Δ (26.6) on caloric restriction medium (Table 1) is much longer than that of *nyv1*Δ (20.7) (Tables 1 and 2). Thus, we further tested the effect of vacuole-vacuole fusion on life-span extension by analyzing lipid synthesis mutants.

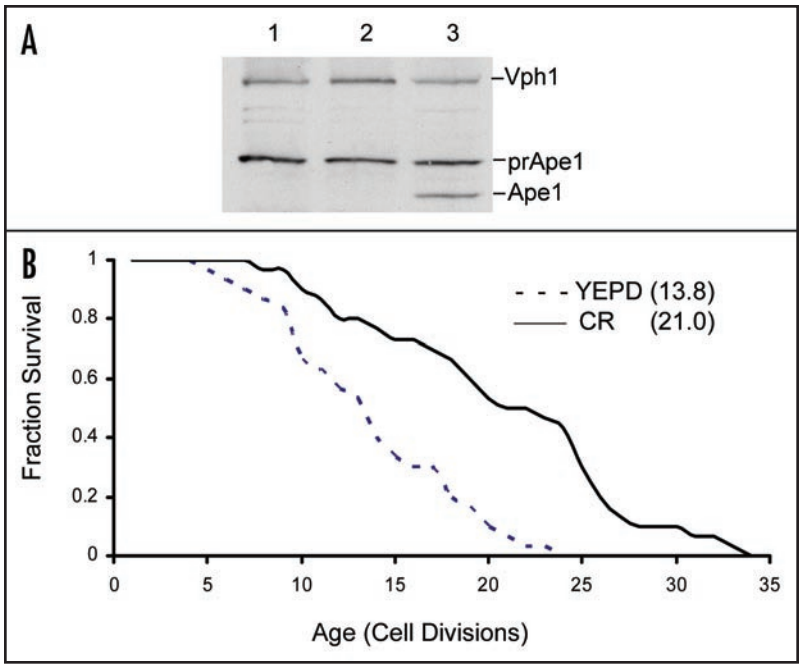

Figure 2. Macroautophagic degradation of proteins is dispensable for life-span extension. (A) Caloric restriction only mildly upregulates the macroautophagic activity. Cell extracts from 0.2 OD log phase cells of LWY2887 were separated by 10% SDS-PAGE and probed for Vph1 and Ape1 after Western blot. Vph1p is visualized as an internal loading control. PrApe1: precursor of aminopeptidase I (Ape1); Ape1: mature Ape1. Cells were LWY 2887 grown in 1. YEP + 2% Dextrose; 2: YEP + 0.5% Dextrose; 3: YEP + 0.2% Dextrose. Note that only lane 3 has matured Ape1 band. (B) Blocking the recycling of amino acids from vacuoles does not block the effect of caloric restriction on life-span extension. The life span of ZFY22 (*atg22*Δ *avt3*Δ *avt4*Δ) was measured on YEP with 2% glucose (YEPD) and YEP with 0.5% glucose (CR). Thirty virgin cells for each sample were analyzed. The p-value is 0.0001.

Vacuole—vacuole fusion is required for life-span extension. In addition to protein components, lipids are also required for vacuole-vacuole fusion. Blocking the synthesis of ergosterol (*erg6*Δ, *erg5*Δ, etc.,) or very long chain fatty acids (*sur4*Δ) generates different extents of fragmented vacuoles, an indication of failure in vacuole-vacuole fusion.[48] We therefore analyzed the life span of these mutants (Table 2).

Selected steps of sterol synthesis are critical for life-span extension. Compared with other *erg* mutants, *erg2*Δ was more compromised in life-span extension on YEPD. In BY4742, the virgin cells from grand colonies of *erg2*Δ mutant did not produce buds when inoculated into a place that is away from a colony on a media plate. We did not observe the beneficial effect of CR on life-span extension in 15 cells from petite colonies of *erg2*Δ mutant (Table 2). While deletion of *ERG5* blocked the effect of caloric restriction, deletion of *ERG6* greatly shortened the life span on CR (Table 2). Unlike other ergosterol synthesis mutants, the mutant *erg28*Δ still supported the life-span extending effect of caloric restriction (Table 2). Deletion of *ERG28* decreases the total 3-hydroxylsterols by 60% but does not accumulate intermediate sterol species such as zymosterol (substrate of Erg6) or fecosterol (substrate of Erg2).[49] Thus, the shortened life span of *erg6*Δ suggested that accumulation of selected intermediate sterols causes a vacuole-fusion defect and shortens the life span.

Blocking the sphingolipid metabolism did not show the same effect as blocking ergosterol synthesis (Table 2). Although deletion of *SUR4* or *LCB4* blocked the effect of caloric restriction (Table 2), the extent was not as severe as deletion of *ERG6*. Intriguingly, *erg6*Δ has more fragmented vacuoles than *sur4*Δ.[48]

Further evidence for the key role of vacuole-vacuole fusion in life-span extension came from the analysis of Vac8's functions. The vacuolar membrane protein Vac8 is required for multiple pathways via its dedicated binding partners.[37] As shown in Table 2, none of the mutants (*atg11*Δ, *vac17*Δ, *tco89*Δ, *nvj1*Δ) missing one of Vac8's known binding partners shortened the life span to the extent of Vac8 on YEPD or to the extent of the vacuole-vacuole fusion mutants (*nyv1*Δ, *ytp7*Δ, *erg6*Δ) on CR, suggesting that some functions of Vac8 with unidentified partners are critical for life-span extension. One of the missing partners is likely involved in vacuole-vacuole

Table 1 Selected autophagy steps are required for caloric restriction-triggered life-span extension

Mutant	MLS on CR (N)*	Autophagy steps affected	Corresponding protein function in autophagy
wild type	29.3 (60)		
*atg1*Δ	30.8 (45)	1	Protein serine/threonine kinase that initiates autophagy[70]
*atg5*Δ	25.2 (31)	1	Conjugated protein of Atg12[70]
*atg6*Δ	30.7 (48)	1	Inovolved in organization of phagphore assembly site[70]
*atg7*Δ	30.4 (30)	1	Mediates conjugation of Atg12–Atg5 and Atg8[70]
*atg8*Δ	26.6 (30)	1	Conjugate to phosphatidylethanolamine and involved in membrane fusion[70]
*atg12*Δ	25.4 (30)	1	Ubiquitin-like modifier[70]
*atg13*Δ	29.2 (45)	1	Binding partner of Atg1 and Vac8[25]
*atg14*Δ	25.6 (30)	1	Autophagy-specific subunit of PI3 kinase complex I[70]
*atg18*Δ	31.5 (30)	1	Formation of pre-autophagosome structure[70]
*atg26*Δ	33.5 (30)		Not involved in autophagy in *S. cerevisiae*[71]
*vam3*Δ	12.8 (60)	2	Vacuolar membrane protein required for fusion with autophagosome[44]
*pep4*Δ	28.7 (45)	3	Proteinase A[72]
*atg15*Δ	17.8 (45)	3	Putative lipase required for intra-vacuolar disintegration of autophagic vesicles[16]
*atg22*Δ	29.9 (45)	4	Vacuolar membrane protein required for recycling of amino acids[21]
*srt1*Δ	28.2 (30)		Synthesis of long chain dolichols in lipid particles[73]
*mdm38*Δ	29.3 (32)		Mitochondrial protein transport[73]
*trs85*Δ	24.1 (27)		Targeting of ER-to-Golgi transport vesicles[75]
*uth1*Δ	22.4 (32)	mitophagy	Mitochondrial outmembrane protein[76]
*vps1*Δ	27.6 (30)		Vacuolar protein sorting[77]
*pex3*Δ	27.2 (31)		Peroxisome biogenesis[78]
*pex6*Δ	28.5 (15)		Peroxisome biogenesis[78]

*MLS, mean life span; N, sample size; CR, calorie restricted media (YEP with 0.5% glucose).

fusion. This conclusion echoes a previous report about Pfa3, the palmitoyltransferase of Vac8. Palmitoylation of Vac8 attaches Vac8 to the vacuole membrane and is required for vacuole-vacuole fusion.[22,50] Cells of *pfa3*Δ have normal vacuoles in normal media but highly fragmented vacuoles in calorie-restricted media.[51]

In *nyv1*Δ, *ypt7*Δ or *erg6*Δ, highly fragmented vacuoles coincide with shortened life span on CR. For Vac8-binding partners, none of the known partners have fragmented vacuoles in *vac8*Δ when mutated and none of them is involved in life-span extension as Vac8 is. Thus, we conclude that vacuole-vacuole fusion is a prerequisite for life-span extension.

Caloric restriction challenges vacuolar functions related with vacuole fusion. To further explore the relationship between vacuolar functions and CR-mediated life-span extension, we chose to analyze *erg6*Δ among the several fusion mutants (*ypt7*Δ, *nyv1*Δ, *erg6*Δ in Table 2) in which caloric restriction greatly shortens the life span. This choice was based on a previous report that overexpressing *ERG6* promotes vacuole-vacuole fusion.[52]

One major function of vacuoles is to provide resistance to alkaline pH via vacuolar H^+-ATPase.[53] The proper assembly and function of the vacuolar ATPase depends on sphingolipids but not ergosterol; deletion of *SUR4* makes cells sensitive to alkaline media (pH 7.4) (ref. 54; top right, Fig. 4A). The sensitivity of *sur4*Δ or *erg5*Δ was not affected by the concentration of glucose (right, Fig. 4A). However, the sensitivity of *erg6*Δ to pH 7.4 increased as the concentration of glucose in the media decreases (right, Fig. 4A). On YEP with 2% glucose, *erg6*Δ was less sensitive to pH 7.4 than *sur4*Δ. On YEP with 0.05% glucose, *erg6*Δ was more sensitive to pH 7.4 than *sur4*Δ. Since ergosterol is not directly involved in the assembly of vacuolar H^+-ATPase, the alkaline sensitivity (Fig. 4A) suggests a possible relationship between vacuole-vacuole fusion and life-span extension; proper vacuole-vacuole fusion maintains vacuolar membrane integrity that is essential for rapid assembly of vacuolar H^+-ATPase and for other vacuolar trafficking.

If the shortened life span of *erg6*Δ on CR is caused by a defect in vacuole-vacuole fusion, restoring the fusion defect may restore the life span. The above result that caloric restriction made *erg6*Δ cells sensitive to alkaline pH led us to test the effect of acidic pH. Moreover, it has been reported that vacuole-vacuole fusion relies on the vacuolar H^+ gradient.[55] Thus, we lowered the pH of CR media to 3.5 and analyzed the vacuole morphology of *erg6*Δ cells (Fig. 4B and C). Intriguingly, low pH promoted vacuole-vacuole fusion in *erg6*Δ cells incubated in CR media. The proportion of cells with normal fusion (less than six vacuolar vesicles per cell) in CR pH 3.5 (about 70%) was close to that of cells in YEPD (about 80%) and both were higher than that of cells in CR (40%). To evaluate the effect of the promotion of vacuole-vacuole fusion on life span, we compared the life span of *erg6*Δ cells in CR media and CR media with low initial pH (pH 3.5). As predicted, low extracellular pH restored the life span of *erg6*Δ strain (Fig. 4D). These results suggest that vacuole fusion as well as vacuole membrane integrity is critical for life-span extension.

Aged cells have altered vacuolar membranes. The contribution of vacuolar membrane integrity to life-span extension was further supported by the analysis of the vacuole membrane dynamics in aged cells. We analyzed the fluid phase endocytosis and vacuolar

morphology of aged cells (Fig. 5A). We isolated cells at generation 6–7, a stage where most cells are capable of producing buds (Fig. 1D). Thus, we can compare the difference between the aged mother cell and its daughter (young cell). In daughters (white arrows in FM 4–64 panels of Fig. 5A), we observed that FM 4–64 entered cells at 15 min and began to appear on the vacuole membrane between 15 to 30 min (Fig. 5A), which is consistent with a previous report.[29] In contrast, mother cells did not show clear vacuolar structure at 15 min. The dye began to appear on the vacuole membrane at or after 30 min in the aged mother cells. In some aged mother cells, although the shapes of vacuoles were visible (the black arrow in the left DIC image of 45 min, Fig. 5A), there was no FM 4–64 defined boundaries, suggesting an alteration in the vacuole membrane.

The differences between aged and young cells were more evident in an assay with Vac8-GFP, which visualizes the vacuolar membrane structure (Fig. 5B). We isolated aged cells (generations 6–7) from LWY7235 carrying VAC8-GFP. The mean life span of LWY7235 was 15 to 17 (data not shown). At generations 6 to 7, some cells already showed end phenotypes. Shown in 30 m (Fig. 5B) is an example of multibuds from one aged mother cell, one end phenotype. FM 4–64 was transported to the sites of Vac8 in the bud at 15 min (white arrow heads in the 15 min row) but not in its mother cell. After 30 min, FM 4–64 reached to Vac8 sites in both the mother cell and the bud. It was noticeable that most mother cells had one large round vacuole (block arrows), while the buds had vacuoles with several vacuolar vesicles. In addition to the large round vacuole, some aged cells also accumulated intravacuolar vesicles with both FM 4–64 and Vac8-GFP signals (Arrows in 3 hr row of Fig. 5B), an indication of invagination of vacuolar membranes. Mother cells at generations 6 to 7 are at an early stage of aging, but showed vacuolar morphologies distinct from their daughters (young cells). The deterioration of vacuolar membranes early in a cell's life suggests that maintaining the vacuolar membrane integrity is likely a rate-limiting factor for life-span extension.

Ergosterol synthesizing mutant accumulates oxidized vesicles in vacuoles. To explore the relationship between ergosterol metabolism and Atg15, we analyzed the generation and accumulation of reactive oxygen species (ROS) in cells, since aged cells and short-lived mutants all accumulate ROS.[35,36] Yeast cells incubated with 4-POBN produced a stable nitroxide ESR spectrum with hyperfine splitting constants (A_N = 14.15 G, A_H = 2.65 G) consistent with those reported previously for the α-hydroxyethyl adduct of 4-POBN (Fig. 6A).[31] This signal most likely originates from the hydroxyl (·OH) radical, which can react with endogenously produced ethanol to generate the α-hydroxyethyl radical, which in turn can react with 4-POBN to produce the stable α-hydroxyethyl-POBN adduct.[31,32] 4-POBN has been shown to effectively compete for free radicals inside cells.[30] Thus, the intensity of the α-hydroxyethyl-POBN adduct provides a measure of the rate of ·OH radical formation in cells. Although *erg6*Δ or *atg15*Δ did not show any obvious difference than wild type in YEPD media, they showed different degrees of ROS accumulation in calorie-restricted media. Caloric restriction did not change the intensity of ROS signal in wild type, but increased the signal in the two mutants especially in *atg15*Δ.

To determine whether there is a sequential relationship between the ROS accumulation in *erg6*Δ and *atg15*Δ, we labeled intracellular ROS using dihydrorhodamine 123.[36] In YEPD media, the fluorescence signals in *erg6*Δ (Fig. 6B, part g) and *atg15*Δ (Fig. 6B, part k) were higher than that in wild type (Fig. 6B, part c). This was apparently different from results shown in Figure 6A. The spin trap technique measures all ROS in cells but dihydrorhodamine 123 labeling likely measures the oxidized materials on or in membranes. Thus, the apparent discrepancy between these two assays suggests that *erg6*Δ and *atg15*Δ have more membrane damage than wild type strain. The location of the oxidized materials can be used to arrange the order of ROS generation and removal.

Although neither *erg6*Δ nor *atg15*Δ were defective in uptaking autophagosomes, which is evident by the accumulation of autophagic vesicles inside vacuoles (Fig. 6B, part e, f, i, j), they accumulated ROS in different locations especially in calorie-restricted media. In *erg6*Δ cells, we frequently observed strong fluorescent signals inside vacuoles with a giant autophagic vesicle (arrows in Fig. 6B, part f, h). However, in *atg15*Δ cells, fluorescent signals never appeared in vacuoles (arrows in Fig. 6B, part j, l), although these vacuoles had multiple small autophagic vesicles. These autophagic vesicles are not obvious in still images as shown in Figure 6B, part j but were obvious under microscope. The fluorescent signals in *atg15*Δ only appeared in the cytoplasm (Fig. 6B, part l) and are similar to what happened in wild type cells (Fig. 6B, part c). While high levels of ROS in *atg15*Δ (Fig. 6A) suggested that Atg15 is required for the removal of ROS-damaged materials from the cytosol, the accumulation of ROS inside the vacuoles of *erg6*Δ cells (Fig. 6B, part h) suggested that macroautophagy alone was not enough to remove the ROS-damaged materials on time.

Table 2 Vacuole membrane integrity is critical for life-span extension*

Mutant	MLS on YEPD (N)	MLS on CR (N)	Effect of CR (%)	p-value
wild type	26.9 (51)	31 (44)	+15.2	0.03
*atg17*Δ	12.2 (22)	10.4 (36)	-15.2	0.50
*atg10*Δ	26.5 (48)	24.8 (48)	-6.1	0.31
*vam7*Δ	10.8 (60)	10.4 (60)	-3.7	0.90
*ypt7*Δ	24.2 (60)	17.8 (60)	-26.4	0.0003
*nyv1*Δ	26.7 (60)	20.7 (60)	-22.5	0.0006
*erg2*Δ-petite#	15.7 (14)	17.1 (15)	+8.6	0.76
*erg3*Δ	22.3 (60)	20.4 (60)	-8.5	0.34
*erg5*Δ	24. 1(60)	25.8 (60)	+7.3	0.29
*erg6*Δ	22.1(60)	16.9 (60)	-23.5	0.0002
*erg28*Δ	25.2 (60)	28.2 (60)	+14.3	0.035
*sur4*Δ	24.4 (49)	24.4 (60)	0	0.65
*lcb4*Δ	32.5 (48)	28.6 (55)	-11.9	0.12
*lcb5*Δ	22.2 (53)	25.2 (54)	+13.7	0.04
*atg11*Δ	31.5 (53)	27.5 (56)	-12.6	0.07
*vac17*Δ	27.0 (51)	31.6 (48)	+17.1	0.05
*tco89*Δ	24.2 (52)	26.8 (54)	+10.7	0.07
*nvj1*Δ	21.6 (50)	24.6 (55)	+13.7	0.046

*MLS, mean life span. The sample size (N) is in parenthesis. Effect of CR was the difference between MLS of CR and YEPD divided by the MLS on YEPD. A p-value less than 0.05 reveals significant differences between the life span on YEPD and CR media. #Virgin cells from grand colonies of *erg2*Δ did not bud on either YEPD or CR plates, we only listed the data from cells originated from petitie colonies of *erg2*Δ strain.

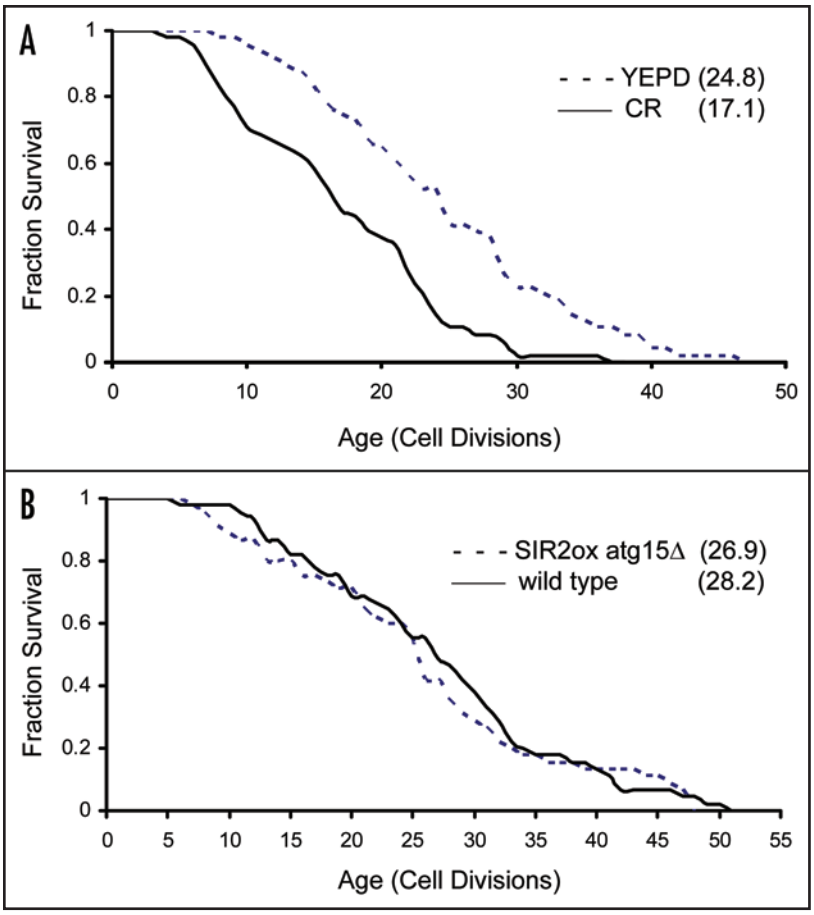

Figure 3. Intravacuolar lipid degradation is required for longevity. (A) Deletion of *ATG15* blocks the effect of caloric restriction on life-span extension. The sample sizes were 48 for both YEPD and CR. The p-value is 0.0001. (B) Deletion of *ATG15* blocks the effect of *SIR2* overexpression on life-span extension. Forty-five virgin cells of each sample were analyzed. The p-value is 0.49.

Discussion

Our data presented here indicate that the formation of autophagosomes (Atg1, Atg5, Atg8, etc.,), the recycling of amino acids (Atg22), and selected autophagy (Atg11 for the Cvt pathway and pexophagy, Uth1 for mitophagy) are all dispensable for life-span extension (Tables 1 and 2). However, the accumulation of autophagic vesicles in aged cells (Fig. 5B) strongly suggests that some forms of autophagy contribute to life-span extension. The indispensability of intravacuolar disintegration of autophagy body membranes (Atg15) and vacuole-vacuole fusion machineries (ergosterol, Nyv1, Ypt7, etc.,) for life-span extension led us to propose that a new form of autophagy extends the life span (Fig. 7; see below for details).

Aged cells are defective in autophagy. The accumulation of abnormal proteins in cells from aged animals led to the proposal that autophagy declines with age (reviewed in ref. 56). From their analyses of the distribution of the lysosomal membrane protein LAMP-2A, Cuervo and colleagues proposed that the age-dependent decline of chaperone-mediated autophagy is caused by lysosomal membrane alteration.[57] In this work, we observed the age-dependent alteration of vacuolar membrane (Fig. 5). The large round vacuole in aged cells is similar to vacuoles of *fab1*Δ or *vac14*Δ,[58] which has shortened life span.[19] One feature of these mutants is the accumulation of phosphatidylinositol 3-phosphate (PI3P).[58,59] Intriguingly, Ohsumi's group recently reported that macroautophagy transports PI3Ps to the vacuole.[60] Thus, the large round vacuole suggests an imbalance of lipids. Our result that Erg6 is critical for life-span extension implies the significance of proper sterols in life-span extension. In support of this, caloric restriction upregulates the expression of *ERG4*.[80] Erg4 catalyzes the last step of ergosterol synthesis and its steady state protein level is the lowest among the last few Erg proteins.[61] Upregulation of Erg4 thus presumably decreases the levels of intermediate sterols and decreases the chance of ROS attack. The contribution of ergosterol metabolism and Atg15 to caloric restriction-mediate life-span extension suggests a link between ergosterol metabolism and certain forms of autophagy.

Vesicles originated from ER have different fates. One quality control point for sterol metabolism in yeast is likely the formation of vesicles from ER. In mammalian cells, the activity of the transcription factor sterol regulatory element binding protein (SREBP) is controlled by the ER to Golgi transport. In the presence of cholesterol, SREBP is immobilized in the ER membrane. In the absence of cholesterol, coat protein complex II (COPII) subunits (Sec23/24) capture SREBP and transport SREBP into Golgi, where SREBP is liberated and becomes activated (reviewed in ref. 62). Although ER is the site of sterol synthesis, ER membrane contains very low levels of sterols (reviewed in refs. 1 and 63). The newly synthesized sterols on ER are transported to target sites (plasma membrane, lipid particles, etc.,) by multiple sterol-transporting mechanisms (reviewed in ref. 64). Nutrition perturbation or mutations in sterol synthesizing enzymes such as *erg6*Δ will cause accumulation of altered sterols such as zymosterol, the substrate of Erg6.[12] These altered sterols tend to elude the normal sterol-transporting systems and insert into the ER membrane, which may in turn cause ER membrane deformation and disturb normal ER functions such as secretion.

Most proteins synthesized on ER are transported to the Golgi complex by COPII vesicles (reviewed in ref. 65). Atg15 is a transmembrane protein with its enzymatic domain in the lumen of ER.[16] Atg15 is proposed to be transported via the path ER → Golgi → multi-vesicle bodies (MVB) → vacuole (ref. 17; pathway b1 in Fig. 7). Because of the topology, the enzymatic domain will be encapsulated in vacuoles as depicted in Figure 7 (top right). Thus, a seed activity of Atg15 is required. Ohsumi and coworkers identified that autophagosome formation relies on some COPII subunits (Sec16, Sec23 and Sec24) but not other subunits (Sec13, Sec31).[66,67] During COPII vesicle formation, Sec23/Sec24 interacts with the initializing protein Sar1 and recruits cargoes, while Sec13/Sec31 works in later steps to accelerate the packing process.[65] Insertion of altered sterols into the membrane may change the property of lipid-protein interactions and thus delay the assembly of Sec13/Sec31 to the vesicle. Vesicles lacking Sec13/Sec31 cannot fuse with the Golgi complex but have a high tendency to be attacked by ROS due to the double bonds in sterol molecules. ROS attacked vesicles will be treated as a target of autophagosome (pathway b2 in Fig. 7). With regard to Atg15

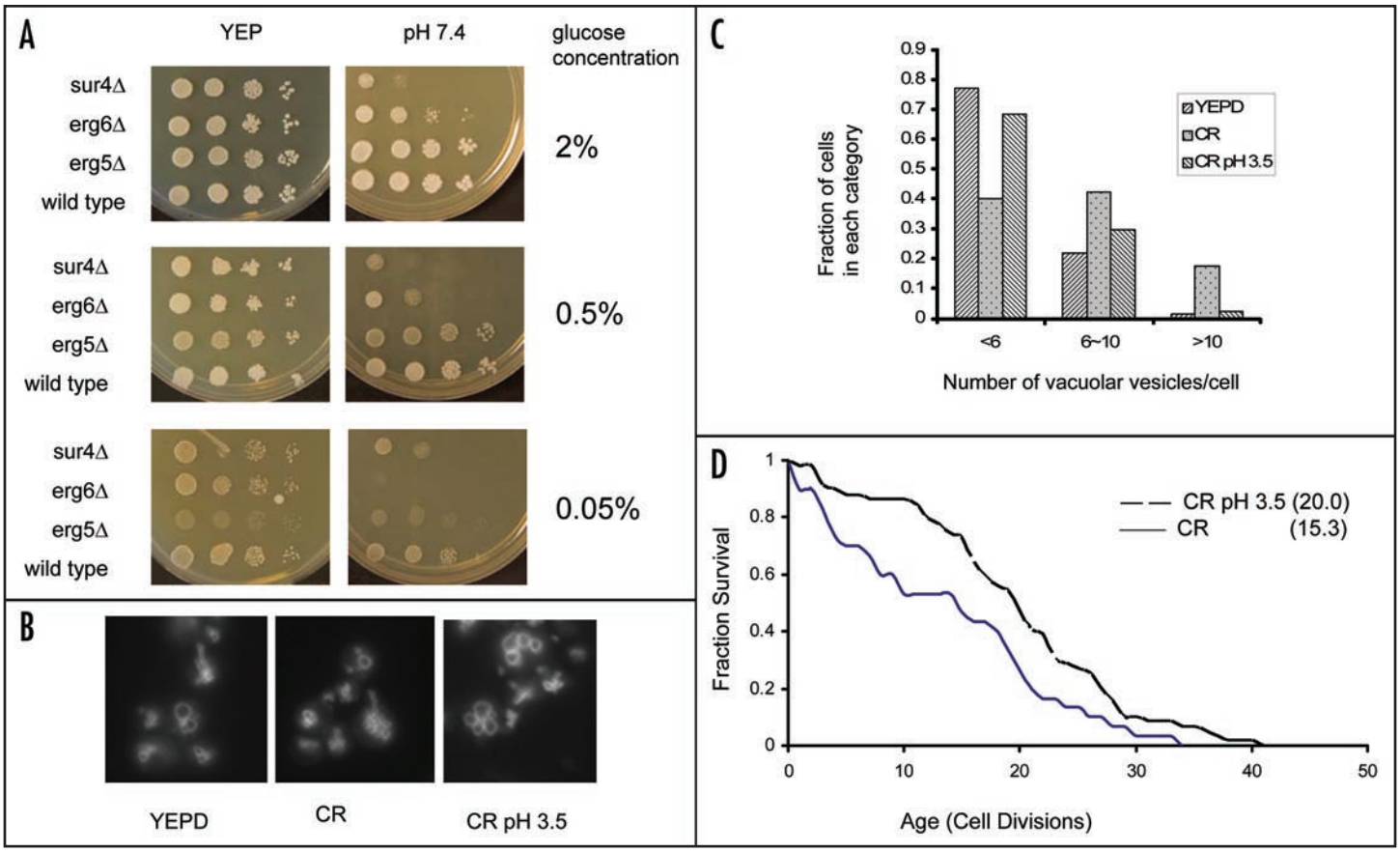

Figure 4. Caloric restriction exacerbates the vacuolar defects in erg6Δ cells. (A) Comparison of growth of selected lipid-synthesizing mutants on alkaline media. Left: YEP media plus the indicated concentration of glucose without buffering. Right: YEP plus the indicated glucose and buffered to pH 7.4 with 50 mM Tris.Cl. Stationary phase cells from YEPD plates were re-suspended in sterile water and diluted. Five µl of diluted cells were spotted onto the indicated plates. The dilutions from left to right are: 0.1, 0.01, 0.001 and 0.0001 OD_{600}. (B) Vacuole morphology of *erg6*Δ cells in different media. Cells were incubated overnight to mid log phase, labeled with FM 4–64 for 1 hr, washed off the excess dye, chased for 4 hours in the indicated media and photographed with fluorescent microscope. (C) Distribution of *erg6*Δ cells with different numbers of vacuolar vesicles. Pictures obtained from (B) were analyzed in the following way. First, cells were divided into three categories based on the number of visible vacuolar vesicles in each cell. Then, the fraction of each category was plotted (C). Cells with less than six vesicles indicate a wild-type vacuolar morphology. Cells with more than six vesicles were categorized as fragmented vacuole, which indicates a defect in vacuole-vacuole fusion. The sample size (total cells) is 315 for YEPD, 222 for CR, and 101 for CR pH 3.5. (D) Low extracellular pH restores the life span of *erg6*Δ on CR. The life span of *erg6*Δ cells on CR and CR pH 3.5 media were measured and plotted. The sample size was 27 for CR and 58 for CR pH 3.5. CR media was adjusted to pH 3.5 with 1 N HCl. The p-value is 0.001.

enzyme activity, the autophagosome pathway also results in an Atg15 molecule separated from its targets (top right in Fig. 7).

Based on previous studies[52,63,65,66,68] and the results presented in this paper, we propose a novel autophagy pathway (pathway b3 in Fig. 7) for the defective COPII vesicles. Sterol-profiling data indicates that vacuole membranes have a higher zymosterol to ergosterol ratio (1:8) than that of secretory vesicles (1:38) and plasma membrane (1:20).[63] Blocking the function of Erg6 (*erg6*Δ) may cause accumulation of its substrate zymosterol on ER membranes and change the proportion of sterols in COPII vesicles. Insertion of zymosterols into ER membranes would make the defective COPII vesicle membrane more similar to the membrane of vacuoles than that of normal secretory vesicles. Thus, these defective COPII vesicles could adopt Nyv1 (v-SNARE) into their membranes and thus fuse with vacuoles. Consistent with this hypothesis, multiple studies have reported the requirement of specific sterols in vacuole-vacuole fusion.[52,68,69] This form of autophagy exposes Atg15 to the acidic vacuole lumen and allows Atg15 to exert its function (bottom vacuole on the right of Fig. 7). This form of fusion alone is not enough to fully exert Atg15's activity. An active vacuole-vacuole fusion (pathway c) employing the normal vacuole fusion machinery (Vac8, Nyv1, Ypt7, etc.,) allows this seed Atg15 to liberate other Atg15 molecules. Blocking vacuole-vacuole fusion in strains with high tendency to attract ROS attack such as *erg6*Δ thus results in the accumulation of autophagic vesicles inside vacuoles (Fig. 6B, part f, h). On the other hand, remedying vacuole fusion defect of *erg6*Δ restores the life-span extension (Fig. 4). For brevity, we refer to this proposed novel autophagy pathway (b3 in Fig. 7) as secretophagy in the following text.

Secretophagy is beneficial to life-span extension. We envision multiple potential beneficial effects of the secretophagy pathway to life-span extension. First, it could provide seed Atg15 activity and allow the completion of other forms of autophagy. Atg15 is a short-lived protein in the vacuole lumen[16] and a constitutive purveyor of seed Atg15 activity is required for sustained cell growth. Second, it could monitor the quality of sterols on ER and remove defective COPII vesicles from the cytoplasm and thus decrease the chance of such vesicles being attacked by ROS. Third, it could transport sterols to the vacuole membrane, which may enhance the vacuole

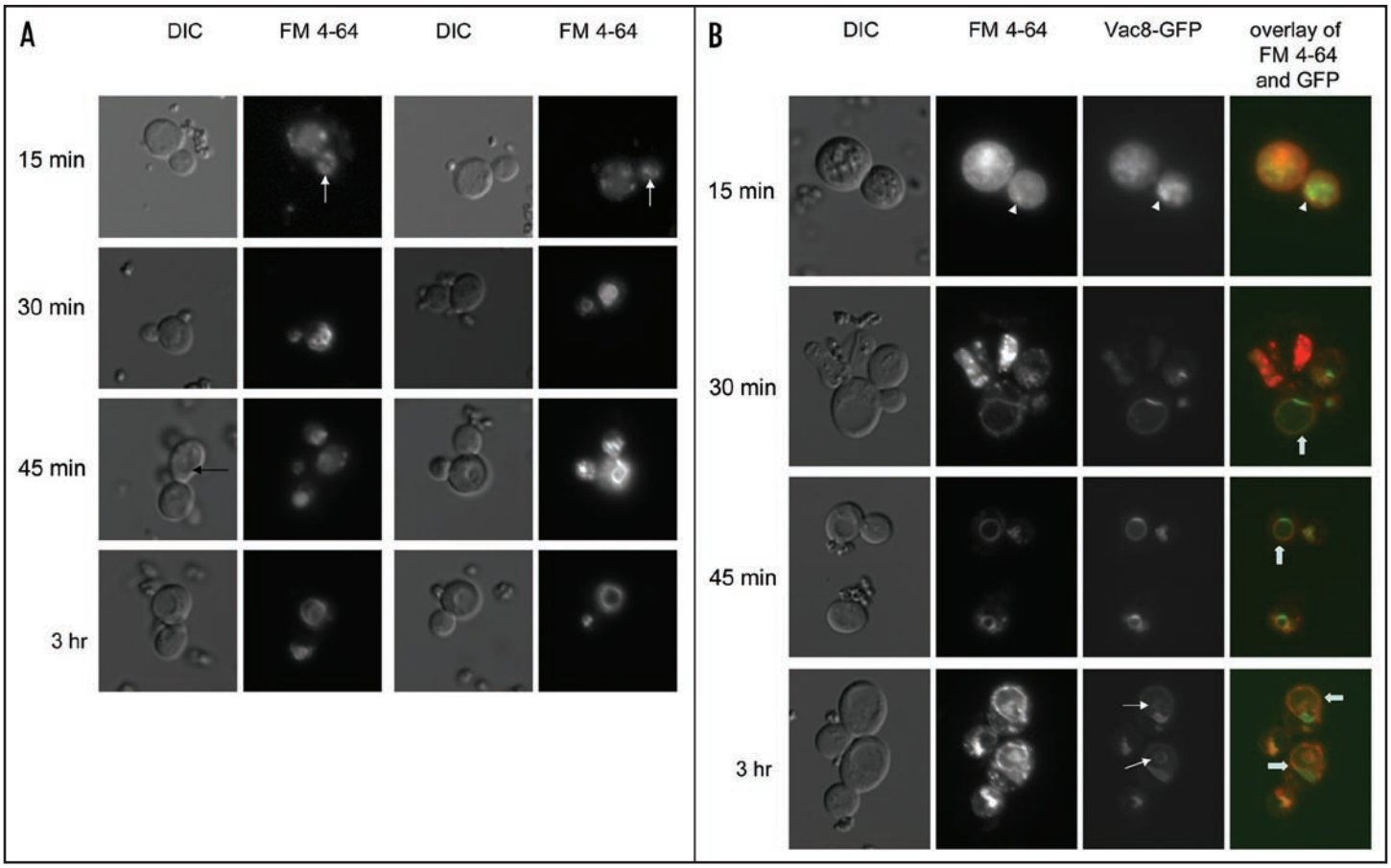

Figure 5. Aged cells have abnormal vacuolar morphology and function. Early log phase BY4742 cells (A) or LWY7235 (VAC8-GFP) (B) were labeled with biotin, inoculated to fresh YEPD, and incubated for 6 doubling times. Cells were sorted with streptavidin-maganetic beads. After sorting, cells were labeled with FM 4–64 on ice for 30 min. and then chased for 3 hours. At the indicated time points, an aliquot of cells were collected, washed with YEPD plus 15 mM each of sodium azide and sodium fluoride, and stored on ice. Cells were then observed under fluorescent microscope and photographed. (A) Mother cells of BY4742 uptake FM 4–64 more slowly than their buds do. The non-yeast look beads in DIC images are streptavidin magnetic beads. The black arrow indicates the shape of the vacuole in a mother cell. White arrows in the FM 4–64 images of 15 min row indicate the vacuolar structures in buds. (B) LWY7235 mother cells have altered vacuole membrane. Arrow heads in the 15 min row indicate that FM 4–64 reached the Vac8 sites in the bud. Block arrows indicate the single round vacuole in aged mother cells. Arrows in the 3 hr row indicate the intravacuolar vesicle containing Vac8.

membrane integrity. Thus, we postulate that secretophagy is a life-span extending form of autophagy. Biochemical analysis of the composition of oxidized materials inside *erg6*Δ vacuoles (Fig. 6B, part h) as well as membranes of aged vacuoles will elucidate detailed mechanisms of how secretophagy extends the life span.

Materials and Methods

Strains, plasmids, media and yeast manipulation. BY4742 (wild type), described before,[19] and knock-out strains in BY4742 background (purchased from Invitrogen) were used for life-span analysis unless otherwise specified. The *vac8*Δ*tor1*Δ double mutant in BY4742 was kindly provided by Dr. Maria Cardenas.[20] *SIR2* overexpression in BY4742 (*SIR2ox-LEU2*) was kindly provided by Dr. Brian Kennedy.[19] *SIR2ox* strain (Mat α) was crossed with BY4741 (Mat a). The resulting diploid was sporulated to obtain *SIR2ox* (Mat a) strain, which in turn was crossed with *atg15*Δ*::Kan*^{R} strains in BY4742. The diploid was sporulated to obtain the *SIR2ox-LEU2 atg15*Δ*::Kan*^{R} strain. ZFY22 (*atg22*Δ *avt3*Δ *avt4*Δ in SEY6210) was kindly provided by Dr. Daniel Klionsky.[21] Yeast strain LWY2887 was described before.[22] YEPD: yeast extract (1%)-peptone (2%)-dextrose (2%). CR: yeast extract (1%)-peptone (2%)-dextrose (0.5%) or as indicated in the text. Solid media contains 2% agar. Vacuole morphology was analyzed with FM 4–64 as described before.[22]

Autophagy assay. Most assays for yeast autophagy rely on nitrogen starvation,[3] which may perturb glucose metabolism. To evaluate the effect of caloric restriction on autophagy, we employed the measurement of the maturation of a vacuolar protease aminopeptidase I (Ape1) in *vac8*Δ cells.[23] The precursor Ape1 is transported to the vacuole either by the cytoplasm-to-vacuole (Cvt) pathway under nutrient condition or by the macroautophagy pathway under nitrogen starvation condition and is matured in the vacuole.[24] Deletion of *VAC8* blocks the Cvt pathway[22,25] but not the macroautophagy pathway.[23] Thus, the maturation of Ape1 in *vac8*Δ cells can be used as a criterion for macroautophagy. Cell extract from LWY2887 was prepared and analyzed for aminopeptidase I (Ape1) and Vph1 by Western blotting as described before.[26]

Life-span analysis. We used the replicative life span, a measure of how many buds a mother cell produces in her entire life, to judge the effect of experimental treatment on yeast longevity. Cells were revived from -80°C and grown at 30°C for 2–3 days. Cells from the fresh

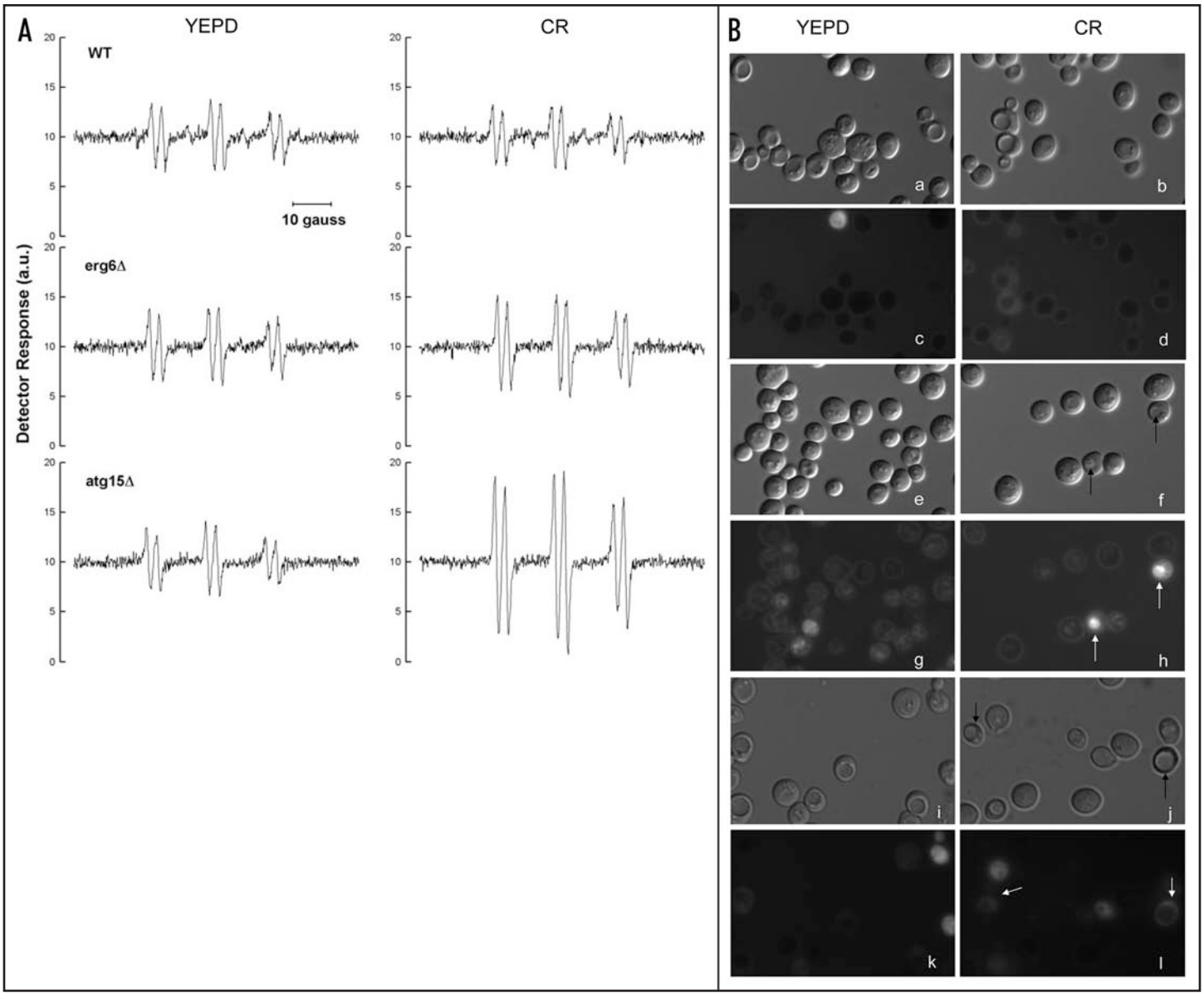

Figure 6. Caloric restriction induces accumulation of oxidative materials in *erg6*Δ and *atg15*Δ cells. (A) ESR spectra of POBN-radical adducts produced by whole yeast cells incubated in YEPD (left) or caloric restricted media (YEP + 0.5% glucose, right). One set of spectra from ESR was re-plotted with SigmaPlot. The heights of peaks correspond to levels of reactive oxygen species. Note the heights of peaks in *atg15*Δ CR are higher than other samples indicating that *atg15*Δ produced higher amount of POBN-radical adduct than other samples in CR medium. (B) The accumulation of reactive oxygen species inside cells is different in different mutants. Stationary phase cells (60 hrs) were labeled with dihydrorhodamine 123 at concentration of 5 μg/ml and incubated for 2 hrs. DIC images and fluorescence images under FITC channel were taken. Left: cells incubated in YEPD. Right: cells incubated in CR medium. Wild type: a, b (DIC), c, d (fluorescent). *erg6*Δ; e, f (DIC), g, h (fluorescent). *atg15*Δ: i, j (DIC), l, k (fluorescent).

colonies were used for life-span analysis. To decrease the perturbation of heterogeneity among colonies on YEPD plates, we compared the life-span of granddaughters. It was previously reported that stationary phase cells have a shorter life span whereas the granddaughters from a stationary phase cell exhibits the normal life span.[27] Thus, we prepared the cells for life-span assay in the following manner. The revived cells were spotted onto fresh plates using a micromanipulator and allowed to undergo 1–2 rounds of cell division. Thirty to sixty virgin cells were selected and incubated for one doubling time. Buds were picked and subjected to life-span analysis. Cells were grown at 30°C during the day and stored at 4°C at night. Daughter cells were removed by gentle agitation with a dissecting needle and recorded every 1–2 cell divisions. Cell death was diagnosed when it lysed or stopped budding in 24 hours. The survival rate (Y axis; ratio of bud-producing cells) was plotted against the generation (X axis) to obtain the survival curve. The mean life span (average of all cells) was used to compare the life span between different strains or different incubation conditions. A two-tailed Wilcoxon Rank-Sum test was employed to analyze whether the difference between YEPD and CR was significantly different. A p value less than 0.05 was considered significant.

Isolation and analysis of aged cells. Aged cells were isolated with a method targeting the surface proteins on the mother cell as previously described.[28] Early log phase cells (OD_{600} around 0.5) were labeled

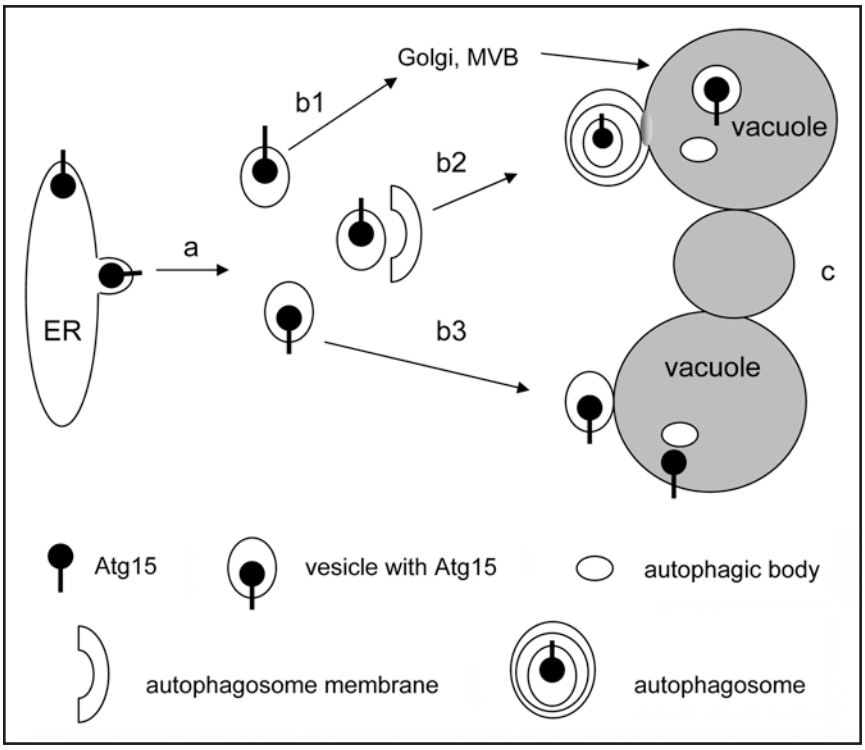

Figure 7. A proposed new form of autophagy employs the vacuole fusion machinery. a: ER membrane with Atg15 is budded as COPII vesicles. The content and quality of sterols in these vesicles determine their fates. b1: If the membrane contains normal sterols, full COPII vesicle will form and be transported to the Golgi. Then Atg15 will be transported to the vacuole by the multiple vesicular body (MVB) pathway. b2: If the membrane contains abnormal sterols, COPII may not be able to fully assemble on time. Such vesicles may be encapsulated by autophagosome and then fuse with vacuoles. In both b1 and b2 pathways, the lipase domain of Atg15 (black circle in the diagram) is still encapsulated by the autophagic bodies inside vacuoles and cannot access its substrate. b3: If the content and species of sterols in the Atg15 vesicle membrane are similar to those in vacuole membranes, the vesicle may fuse with vacuoles directly by using some proteins (Nyv1, Ypt7, etc.,) in the vacuole-vacuole fusion machinery. This proposed new form of autophagy, secretophagy, exposes the lipase domain to the lumen of the vacuole and allows Atg15 to work in an acidic environment. c: Vacuole-vacuole fusion (Vac8, Nyv1, Ypt7, etc.,) allows the Atg15 on vacuole membrane to work on more autophagic bodies. Caloric restriction may upregulate the secretophagy pathway to extend the life span (see text for detail).

with E-Z link biotin (Sulfosuccinimidyl-6-(biotinamido)Hexanoate) (Prod# 21335, Pierce Co., Rockford, Illinois) in pH 8.0 phosphate buffer on ice for 30 min. The excess biotin was washed with 100 mM glycine in phosphate buffer. Labeled cells were re-suspended in YEPD to OD_{600} around 0.01 and incubated for 6 doubling times (about 11 hrs in a 30°C shaker). The biotin-labeled old cells were purified with streptavidin magnetic beads (Cat# MMI-105, BioClone Inc., San Diego, California). The aged cells were analyzed for fluid phase endocytosis as described by Heese-Peck et al.[29]

Spin trapping. The spin trap 4-pyridyl-1-oxide N-tert-butylnitrone (POBN) has been shown to enter cells and react with oxygen and carbon-centered radicals to produce stable electron spin resonance spectra in mammalian cell systems.[30-32] We labeled yeast cells with POBN with the following procedure. Overnight yeast cells were inoculated into fresh media to an initial OD_{600} of 0.02. Cells were incubated in a 30°C shaker for 3 hrs. The spin trap POBN (Sigma, P9271) was dissolved in water, filter sterilized, and added to the culture to a final concentration of 20 mM. Cells were incubated for a further 5 hrs. At that point, the OD_{600} reached 0.3 to 0.5. Cells were collected by centrifugation and re-suspended into their own supernatant to a concentration of 5 OD/ml. Aliquots of 0.6 ml of cells were subjected to electron spin resonance measurements.

Electron spin resonance (ESR) measurement. ESR spectra were measured on a Bruker EMXPlus X-band ESR spectrometer (Billerica, Massachusetts) at room temperature according to a previous report.[33] Instrument conditions were as follows: frequency, 9.774 GHz; microwave power, 20.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; time constant, 20 ms; receiver gain, 1 x 10^5; sweep width, 100 G with 1024 point resolution. Twelve individual scans were averaged to produce a single spectrum.

Dihydrorhodamine 123 staining. Stationary phase cells (60 hrs in a 30°C shaker) were labeled with dihydrorhodamine 123 (Sigma, D1054). The dye was dissolved in ethanol at a concentration of 2.5 mg/ml and added to the culture at a final concentration of 2.5 μg/ml. Cells were further incubated for 2 hrs, collected by centrifugation and analyzed by fluorescence microscopy. Dihydrorhodamine 123 can be oxidized to rhodamine 123 intracellularly by peroxidases and electron donors from oxidized materials.[34] Rhodamine 123 is fluorescent and cannot cross the membranes. Thus, dihydrorhodamine 123 can be used as a tracer for oxidative materials inside cells.[35,36] To monitor the fluorescence, we used an FITC filter. Photographs were taken within a few seconds due to the rapid bleaching of the fluorescence signal.

Acknowledgements

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