V-ATPase Disassembly at the Yeast Lysosome-Like Vacuole Is a Phenotypic Driver of Lysosome Dysfunction in Replicative Aging

Fiza Hashmi and Patricia M. Kane

Department of Biochemistry and Molecular Biology
SUNY Upstate Medical University, Syracuse, NY USA

Address correspondence to: Patricia M. Kane
kanepm@upstate.edu

Running title: V-ATPase disassembly in replicative aging

1   Declines in lysosomal acidification and function with aging are observed in organisms

2   ranging from yeast to humans. V-ATPases play a central role in organelle acidification and V-

3   ATPase activity is regulated by reversible disassembly in many different settings. Using the yeast

4   *Saccharomyces cerevisiae* as a replicative aging model, we demonstrate that V-ATPases

5   disassemble into their V_1 and V_0 subcomplexes in aging cells, with release of V_1 subunit C

6   (Vma5) from the lysosome-like vacuole into the cytosol. Disassembly is observed after ≥ 5 cell

7   divisions and results in overall vacuole alkalinization. Caloric restriction, an established

8   mechanism for reversing many age-related outcomes, prevents V-ATPase disassembly in older

9   cells and preserves vacuolar pH homeostasis. Reversible disassembly is controlled in part by the

10   activity of two opposing and conserved factors, the RAVE complex and Oxr1. The RAVE

11   complex promotes V-ATPase assembly and a *rav1Δ* mutant shortens replicative lifespan; Oxr1

12   promotes disassembly and an *oxr1Δ* mutation extends lifespan. Importantly, the level of Rav2, a

13   key subunit of the RAVE complex, declines in aged cells. These data indicate that reduced V-

14   ATPase assembly contributes to the loss of lysosome acidification with age, which affects

15   replicative lifespan.

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INTRODUCTION

It is well-documented that lysosomal acidification is compromised with age across many organisms (Nixon, 2020). Reduced lysosomal acidification has multiple downstream functional consequences. Lysosomal hydrolases represent a major arm of the cellular proteostatic machinery and operate optimally at the acidic pH, so cargo degradation is compromised at higher pH (Vilchez, Saez, & Dillin, 2014). Lysosomes are the terminal compartment for multiple autophagy pathways, so clearance of autophagic cargoes and recycling of nutrients, both critical in aging cells, is slowed (Hansen, Rubinsztein, & Walker, 2018; Kaushik et al., 2021). Iron and other heavy metals are sequestered and buffered in the acidic lysosomes; loss of sequestration can induce both oxidative stress (Diab & Kane, 2013; Kurz, Terman, Gustafsson, & Brunk, 2008) and deficiency in mitochondrial iron-sulfur proteins (Chen et al., 2020). Reduced lysosomal storage can also create toxic imbalances in amino acids such as cysteine that contribute to loss of mitochondrial function (C. E. Hughes et al., 2020). Recent work has highlighted the central role of the lysosome in nutritional signaling and many aspects of this signaling are linked to acidification (Perera & Zoncu, 2016). It is clear that loss of lysosomal acidification can impact many processes associated with age-related functional decline, but the mechanisms behind increases in lysosomal pH are not fully understood.

The highly conserved vacuolar H^+- ATPase (V-ATPase) acidifies the lumen of lysosomes and lysosome-like vacuoles, as well as endosomes and the late Golgi apparatus, in all eukaryotes (Collins & Forgac, 2020). V-ATPases are multi-subunit protein complexes that couple ATP hydrolysis to proton pumping into organelle lumens. The V-ATPase consists of two subcomplexes: a peripheral V_1 subcomplex oriented toward the cytosol that is responsible for ATP hydrolysis connected to a membrane-embedded V_0 subcomplex containing the proton pore.

45   V-ATPase subunit sequences are conserved across eukaryotes and recent V-ATPase structures

46   indicate very strong structural similarity between yeast and mammalian V-ATPases (Oot &

47   Wilkens, 2020).

48   V-ATPase activity is highly regulated and responsive to multiple environmental

49   conditions. Reversible disassembly is a versatile mechanism of V-ATPase regulation that fine-

50   tunes the activity of the proton pump to meet cellular demands (Collins & Forgac, 2020;

51   Jaskolka, Winkley, & Kane, 2021; Wilkens, Khan, Knight, & Oot, 2023). In reversible

52   disassembly, the V_1 subcomplex is released from the V_0 subcomplex inhibiting both ATP

53   hydrolysis and proton pumping (Kane, 1995; Sumner et al., 1995). V_1 subunit C is dissociated

54   from both subcomplexes and also becomes cytosolic during disassembly (Kane, 1995).

55   Disassembly is post-translational and rapidly reversible (Kane, 1995). It was first observed in the

56   yeast *S. cerevisiae* and the tobacco hornworm *M. sexta* upon acute glucose deprivation and was

57   reversed by glucose replenishment (Kane, 1995; Sumner et al., 1995). Since that time, it has

58   become clear that reversible disassembly occurs in many different settings and in response to

59   diverse signals. For example, unlike yeast cells, most mammalian cells appear to promote V-

60   ATPase reassembly under conditions of nutrient deprivation and mTOR inhibition, possibly as a

61   means of promoting lysosomal proteolysis and nutrient recycling (Ratto et al., 2022; Stransky &

62   Forgac, 2015). In neurons, V-ATPases are reversibly disassembled as part of each synaptic

63   vesicle cycle (Bodzeta, Kahms, & Klingauf, 2017). In cardiomyocytes, lipid overload can

64   promote V-ATPase disassembly in endosomes, ultimately contributing to the long-term insulin

65   resistance (Liu et al., 2017). Reversible disassembly of V-ATPases can also be manipulated by

66   both host cells and pathogens to prevent or facilitate infection (Kohio & Adamson, 2013).

67   Several mechanisms for loss of lysosomal acidification with age have been proposed. In

68   yeast, the long-lived plasma membrane proton pump, Pma1, accumulates in mother cells, and it

69   has been proposed that increased proton export through Pma1 disrupts the balance of Pma1 and

70   V-ATPase activities and compromises organelle acidification (Henderson, Hughes, &

71   Gottschling, 2014). Early loss of vacuolar acidification in yeast has been correlated with

72   defective mitochondrial morphology and function. Both the deacidification and mitochondrial

73   morphology phenotypes are suppressed by overexpression of the V-ATPase catalytic subunit,

74   *VMA1*, or an ER-localized assembly factor, *VPH2*, suggesting a possible deficiency in these

75   factors with age (A. L. Hughes & Gottschling, 2012). In *C. elegans*, stability of the *VMA1*

76   transcript is controlled by a microRNA, miR-1, which can globally control lysosomal

77   acidification (Schiffer et al., 2021). In several systems, mRNA expression for one or more V-

78   ATPase subunit genes has been reported to decrease with age (Ghavidel et al., 2018), but in most

79   cases protein levels have not been assessed. All of these mechanisms could contribute to loss of

80   vacuolar/lysosomal acidification. Wilms et al. (Wilms et al., 2017) demonstrated that the mTOR

81   effector Sch9 promotes V-ATPase assembly and extends yeast chronological lifespan.

82   However, despite the importance of reversible disassembly in regulation of V-ATPase

83   activity, V-ATPase assembly state during aging has not been explored extensively. Here we show

84   that V-ATPase assembly does change with age in a yeast replicative aging model. Increased V-

85   ATPase disassembly in older cells is accompanied by decreased vacuolar acidification that does

86   not appear to stem from reduced V-ATPase subunit levels. Instead, we provide evidence that

87   reduced activity of the RAVE (Regulator of H^+-ATPase of Vacuolar and Endosomal membranes)

88   assembly complex may give rise to net V-ATPase disassembly and increased lysosomal pH with

89   age.

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RESULTS

V-ATPases are more disassembled and vacuoles more alkaline after ~5 cell divisions.

Given the evidence that vacuoles and lysosomes are less acidic in older cells, we hypothesized that V-ATPase assembly and activity might also be changing with age. Replicative aging in the yeast *S. cerevisiae* is a widely accepted model for aging (He, Zhou, & Kennedy, 2018). Briefly, yeast cells divide asymmetrically with each cell division giving rise to a new, "rejuvenated" daughter cell from an established mother cell. Each cell division leaves a bud scar on the mother, allowing visual assessment of age. The yeast V_1C subunit (Vma5) is released from both V_1 and V_0 during V-ATPase disassembly, so we first visualized Vma5-GFP localization in a mixed age population of cells (Figure 1). (Note that vacuoles are visible as indentations under DIC (differential interference contrast) optics on the left of the images.) In parallel, we monitored replicative age of each cell by staining with calcofluor white, which labels the bud scars on mother cells. As shown in Figure 1a, Vma5-GFP is tightly localized to the vacuolar membrane in cells with few or no bud scars. In contrast, in older cells with more bud scars, Vma5-GFP exhibited a marked decrease in fluorescence at the vacuolar membrane and a notable increase in cytosolic fluorescence. In order to compare Vma5-GFP localization between cells, we quantitated maximum fluorescence from a line scan across each cell. As shown in Figure 1b, the line scan from a young cell has prominent peaks corresponding to the edges of the vacuole with a high maximum fluorescence signal, while the older cells have less prominent peaks. We conducted the same analysis across populations of cells of mixed age, normalized to the maximum fluorescence signal of young cells and binned the results by the number of bud scars. As shown at the left of Figure 1c, young cells, defined as having less than 5 bud scars, displayed Vma5-GFP localization at the vacuolar membrane. However, the normalized

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114   maximum fluorescence signal, representative of vacuole localization, decreases significantly in

115   cells with five bud scars or more. This early onset of V-ATPase disassembly aligns with previous

116   reports indicating changes in lysosomal pH early in replicative aging (A. L. Hughes &

117   Gottschling, 2012).

118   Vma5 is a V_1 subunit that bridges the V_1 and V_0 subcomplexes of the V-ATPase. The V_1

119   subcomplex also contains three copies of Vma2, and the V_0 subcomplex contains the largest

120   subunit, Vph1, which comprises part of the proton pore (Figure 2a). Although Vma5 appears to

121   be the subunit that is released most completely from the membrane by reversible disassembly

122   (Tabke et al., 2014), the rest of the V_1 sector also dissociates from the vacuolar membrane. We

123   assessed the cellular distribution of Vma2-GFP, a V_1 subunit, and Vph1-GFP, a V_0 subunit

124   (Figure 2b). As shown in Figure 2b, there was less membrane-bound Vma2-GFP in older cells

125   relative to younger cells as assessed by line scans as in Figure 1. However, vacuolar Vph1-GFP

126   levels were the same between old and young cells (Figure 2c). These results are consistent with

127   disassembly of the V-ATPase as cells age.

128   Although comparable levels of Vph1 at the vacuole suggests that expression of V_0

129   subunits and V_0 assembly are intact, V_1 subunits could become cytosolic because of reduced V_1

130   subunit levels in older cells. To address this question, we isolated populations old and young

131   yeast cells by biotinylating the cell walls in a mixed age population, allowing growth to continue

132   for several generations, and then obtaining "old" cells by biotin-streptavidin affinity

133   chromatography (Jin, Cao, & Liu, 2021). Daughter cells that emerged after biotinylation cannot

134   bind to magnetic streptavidin beads and represent the "young" population. The age distribution

135   was determined by counting bud scars in each population and binning by the number of buds per

136   cell (Figure 2d). When prepared by this method, the population of old cells peaks at 20-24 bud

137   scars, while there was a median value of 0-4 bud scars in the young population. Cell lysates were

138   prepared from each population and analyzed by SDS-PAGE and immunoblotting. As shown in

139   Figures 2e and f, there is no significant difference in the cellular levels of V_1 subunits Vma1,

140   Vma2, and Vma5 between young and old populations. These results indicate that the cytosolic

141   populations of V_1 subunits arise from disassembly of the V-ATPase, rather than inability to

142   assemble because of lack of V-ATPase subunits.

143   Reversible disassembly of V-ATPases is employed in a number of contexts to provide

144   dynamic regulation of the complex in response to changing cellular conditions. Disassembled V_1

145   and V_0 subcomplexes lack ATPase and proton transport activity and ATP-driven proton pumping

146   is restored upon reassembly. In order to test whether the lower levels of assembled V-ATPases in

147   old cells result in reduced capacity for acidification, we measured the response of young and old

148   cells to an acute glucose deprivation (an abrupt shift to 0% glucose), which promotes

149   disassembly of the yeast V-ATPase, followed by readdition of glucose to a 2% final

150   concentration, which promotes reassembly and reactivation of the complex (Kane, 1995).

151   Young and old yeast cells obtained as described above were loaded with the ratiometric

152   pH sensor BCECF-AM (2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl

153   ester) which localizes to the vacuole in yeast cells (Diakov, Tarsio, & Kane, 2013). Both young

154   and old cells were shifted to medium with no glucose for ~30 minutes. Fluorescence of cell

155   suspensions was then monitored continuously (Figure 3a), and glucose was added at the

156   indicated time. This assay revealed clear age-dependent differences in the vacuolar pH response

157   to glucose stimulation. Young cells exhibit a rapid drop in vacuolar pH upon glucose addition.

158   This drop was previously shown to be V-ATPase-dependent and to correlate with V-ATPase

159   assembly (Martinez-Munoz & Kane, 2008). In old cells, however, there was a smaller pH

160   decrease after glucose addition, indicating a more alkaline vacuole and consistent with the lower

161   levels of V-ATPase assembly observed by microscopy during growth in glucose-replete

162   conditions in Figures 1 and 2. Quantitative analysis of pH at defined time points across

163   biological replicates (Figure 3b) indicates that vacuoles in old cells are significantly more

164   alkaline than their younger counterparts at each of the indicated time points. These results

165   suggest an age-related alteration in vacuolar pH regulation through the inability of V-ATPase to

166   reassemble. As a result, vacuoles in old cells display a more alkaline pH than vacuoles in young

167   cells.

168   *Caloric restriction restores V-ATPase assembly and vacuolar acidification in older cells.*

169   Caloric restriction (CR) is defined as a reduction in caloric intake in the presence of

170   adequate nutrition (Longo & Anderson, 2022). CR promotes both cellular health and longevity.

171   Extensive research, conducted in diverse model organisms ranging from yeast to worms, flies,

172   and rodents, suggests that CR can have significant anti-aging effects and promote overall health

173   (Longo & Anderson, 2022). In the context of the yeast *S. cerevisiae*, adjusting the concentration

174   of glucose in the growth medium from the 2% used above to 0.5% is a common way to induce

175   CR. This treatment does not significantly reduce growth rate over several cell divisions

176   (Supporting information, Figure 1).

177   As shown in Figure 4, CR reverses the V-ATPase disassembly in older cells. Notably, the

178   V_1 subunits Vma5-GFP (Figure 4a) and Vma2-GFP (Figure 4b) are recruited to the vacuolar

179   membrane in both young and old cells, and there is no significant difference in fluorescence at

180   the vacuolar membrane with age. (Fluorescence intensities of Vph1-GFP continue to be similar

181   between old and young cells (Figure 4c).) This reversal suggests that CR extends V-ATPase

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182   assembly beyond the replicative age of ≥ 5 bud scars when cells grown in higher glucose begin to

183   show disassembly.

184   We hypothesized that given the improvement in V-ATPase assembly, vacuolar pH in old

185   cells might also be restored. We grew cells under CR conditions, loaded the vacuoles with

186   BCECF-AM and monitored vacuolar pH before and after addition of glucose as described above.

187   Figure 4d demonstrates that the glucose-stimulated decrease in vacuolar pH, which was

188   compromised in older cells grown in 2% glucose (Figure 3), was restored to the level of young

189   cells in cells after growth under CR conditions. This observation indicates that CR has a direct

190   impact on both V-ATPase assembly and vacuolar acidification in aging cells. In addition, it

191   further highlights the potential connection between V-ATPase assembly, vacuolar acidification

192   and aging.

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194   *Regulators of V-ATPase assembly state affect replicative lifespan.*

195   If reversible disassembly of the V-ATPase plays a central role in aging of yeast cells, we

196   hypothesized that the cellular factors that regulate V-ATPase assembly might also affect

197   replicative lifespan. The RAVE complex plays a crucial role in regulation of the V-ATPase by

198   reversible disassembly (Jaskolka, Winkley, et al., 2021; Seol, Shevchenko, & Deshaies, 2001). It

199   consists of three subunits: Rav1, Rav2, and Skp1. The RAVE complex is required for reassembly

200   of V-ATPase complexes disassembled by glucose deprivation. In mutants lacking Rav1 or Rav2,

201   V-ATPases are predominantly disassembled into V_1 and V_0 subcomplexes and vacuolar

202   acidification is lost (Seol et al., 2001; Smardon, Tarsio, & Kane, 2002). The RAVE complex

203   associates with V_1 subcomplexes in the cytosol. Although V_1 subunit C is also released from the

204   vacuolar membrane, very little is associated with cytosolic RAVE-V_1 (Jaskolka, Tarsio,

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205 Smardon, Khan, & Kane, 2021). In contrast, recent studies suggest that Oxr1, a protein originally

206 associated with resistance to oxidative stress, promotes disassembly of V-ATPases (Khan et al.,

207 2022; Khan & Wilkens, 2024; Klossel et al., 2024) suggesting that RAVE and Oxr1 have

208 opposing effects on the assembly state of V-ATPases as diagrammed in Figure 5a.

209 To further explore the functional significance of these assembly regulators in aging cells,

210 we examined the replicative lifespans of deletion mutants lacking Rav1 or Oxr1 (Figure 5b).

211 Replicative lifespan was measured on YEPD, pH 5 plates, conditions that are optimal for growth

212 of *rav1Δ* strains. Deletion of Rav1 shortened replicative lifespan (median 17 cell divisions, n=

213 30) by 26% relative to wild-type cells (median 23 cell divisions, n= 30). In contrast, the deletion

214 of Oxr1 extended replicative lifespan by 47.8% over wild-type (median 34 cell divisions, n=30).

215 These results reinforce the significance of V-ATPase assembly in replicative aging and suggest

216 that RAVE pro-assembly activity (disrupted in the *rav1Δ* mutant) and Oxr1 anti-assembly

217 activity (lost in *oxr1Δ*) may be central determinants of lifespan.

218 Given these results, we asked whether there were differences in levels of RAVE subunits

219 or Oxr1 between young and old cells. We isolated young and old populations of cells containing

220 myc13-tagged Rav1 or Rav2 by biotinylation and streptavidin magnetic separation as described

221 above (Figure 2), then assessed the levels of the tagged proteins in cell lysates. Although there is

222 no significant difference in Rav1-myc13 levels between young and old cells (Figure 6a), we

223 consistently observed a significant decrease in protein levels of Rav2-myc13 in older cells

224 (Figure 6b). We also assessed expression of *RAV2* in young and old cells by quantitative PCR but

225 observed no significant difference in mRNA levels (Figure 6c). Because Rav2 is required for

226 RAVE complex function in promoting V-ATPase assembly (Seol et al., 2001; Smardon et al.,

227   2002), these results suggest that partial loss of RAVE function in old cells could contribute to

228   reduced V-ATPase assembly and replicative aging.

229   To further explore the basis of the extended replicative lifespan in *oxr1Δ* cells, we

230   observed Vma5-GFP localization in a population of *oxr1Δ* cells of mixed age. As shown in

231   Figure 6d, *oxr1Δ* mutants localize Vma5-GFP to the vacuole even in older cells with ≥5 bud

232   scars. In order to quantitate this effect, we again binned cells by the number of bud scars as

233   described above, and compared Vma5-GFP localization to localization in young daughter cells

234   (Figure 6e). In contrast to wild-type cells, there is no significant difference in Vma5-GFP

235   localization in the *oxr1Δ* mutant until cells have divided 25 or more times. We also isolated

236   young and old populations from an HA-tagged Oxr1 strain. As shown in Figure 6f, the levels of

237   Oxr1 do not change with age. However, loss of RAVE function could ultimately favor Oxr1-

238   induced disassembly.

239   Intriguingly, these results suggest that the *oxr1Δ* mutation mirrors the effects of CR on V-

240   ATPase assembly and longevity. The results also indicate that direct manipulation of V-ATPase

241   assembly by mutation of critical assembly factors can affect replicative lifespan. Specifically,

242   promoting V-ATPase assembly through the RAVE complex appears to be important for

243   preserving replicative lifespan, while the opposing effects of Oxr1 on the V-ATPase tend to

244   shorten lifespan. We hypothesized that restoration of V-ATPase assembly in older cells grown

245   under CR conditions (Figure 4) might be supported by restoration of Rav2 levels. To test this, we

246   isolated young and old populations from Rav2-myc13 tagged cells grown under CR conditions.

247   Under these conditions, V-ATPase assembly and function restored in older cells (Figure 4) and as

248   shown in Figure 6g, Rav2 levels are also restored.

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DISCUSSION

The results described here establish V-ATPase disassembly as an important factor in the reduced vacuolar acidification observed in aging yeast cells. We demonstrate an increase in V-ATPase disassembly at relatively early replicative ages (5-9 cell divisions) similar to the age at which Hughes and Gottschling first observed compromised vacuolar acidification (A. L. Hughes & Gottschling, 2012). We show that promoting assembly of the V-ATPase through CR can restore V-ATPase assembly and vacuolar pH in older cells and that deletion of Oxr1, a negative regulator of V-ATPase assembly, can extend replicative lifespan. Taken together, these data support V-ATPase disassembly as a significant age-related factor behind reduced vacuolar and lysosomal acidification and the associated declines in function. This mechanism does not necessarily conflict with those proposed previously. If V-ATPases are more disassembled in older cells, cells may be even less able to tolerate an imbalance between Pma1 activity at the plasma membrane and V-ATPase activity at the vacuole (Henderson et al., 2014). We see no difference in protein levels of the core V_1 subunits Vma1 and Vma2, or Vma5, between young and old cells. The presence of similar levels of Vph1 in vacuoles of young and old cells suggests that V_0 subunits are also expressed at similar levels, since V_0 assembly occurs in the ER and reduced levels of any V_0 subunit reduces V_0 subcomplex levels at the vacuole (Kane, Kuehn, Howald-Stevenson, & Stevens, 1992). The data presented here suggest a post-transcriptional regulatory mechanism, but increased levels of a V_0 assembly factor like Vph2 (A. L. Hughes & Gottschling, 2012) might still promote assembly and improve acidification.

Many questions remain to be investigated in the future. The reduction in Rav2 protein levels with aging appears to be post-transcriptional but we do not yet know whether this arises from reduced translation or increased degradation of the protein. Perhaps more importantly, even

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273 though reduced levels of Rav2 levels could help explain increased V-ATPase disassembly, we do

274 not know whether the Rav2 reduction occurs as a result of some general vulnerability in aging

275 cells or is deliberately programmed (Gladyshev et al., 2021). The significant increase in lifespan

276 and prolonged V-ATPase assembly in *oxr1Δ* cells seems to argue against any growth advantage

277 from increased V-ATPase disassembly in older cells, at least at the single cell level.

278 Many aging pathways are tightly linked to nutritional signaling, and reversible

279 disassembly is often driven by nutritional signaling pathways. Given the rapid disassembly of the

280 V-ATPase upon acute glucose deprivation (Kane, 1995), it was initially surprising that growth

281 under CR conditions suppresses V-ATPase disassembly with age. However, we previously

282 showed that the acute disassembly response required glucose concentrations well below 0.5%

283 (Parra & Kane, 1998). In mammalian cells, V-ATPase assembly generally increases in response

284 to nutrient deprivation (Ratto et al., 2022; Stransky & Forgac, 2015). CR could mimic this effect

285 in aging cells, preserving lysosomal acidification and function. Signals involved in reversible

286 disassembly of the V-ATPase are incompletely understood. However, it is intriguing that in yeast,

287 RAVE appears to play a central role in glucose signaling during acute glucose deprivation.

288 RAVE is released from the vacuolar membrane upon acute glucose deprivation and recruited

289 back to the membrane upon glucose restoration, even in the absence of V_1 subunit C and the V_1

290 subcomplex (Jaskolka, Winkley, et al., 2021). The RAVE complex appears to be a major

291 determinant of V-ATPase assembly state in multiple situations, including aging.

292 Reduced V-ATPase assembly could certainly be a factor in the age-related decline in

293 lysosomal acidification and function in higher eukaryotes. Reversible disassembly actively

294 occurs in higher eukaryotes including mammalian cells, so the apparatus for age-induced

295 assembly regulation is available. Rabconnectin-3 complexes of higher eukaryotes are the

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296 functional homologues of the yeast RAVE complex (Ratto et al., 2022; Yan, Denef, &

297 Schupbach, 2009). Oxr1 belongs to a family of TLDc proteins that are also found in mammals,

298 and several of these proteins have been shown to bind to mammalian V-ATPases (Eaton, Brown,

299 & Merkulova, 2021; Wilkens et al., 2023). These data suggest that the core elements for

300 controlled V-ATPase disassembly during aging are present in other cells. Here, we observed V-

301 ATPase disassembly in a yeast replicative aging model, which is most comparable to mammalian

302 cell types that continue to divide, such as adult stem cells (He et al., 2018). However, V-ATPase

303 activity is also critical in the yeast chronological aging model, which is more analogous to long-

304 lived, non-dividing mammalian cells. In this model increased V-ATPase assembly has been

305 associated with longevity (Wilms et al., 2017). Taken together, these data suggest that V-ATPase

306 assembly state is linked to multiple aging models and could easily play a role in aging in higher

307 eukaryotes.

308

309 **METHODS**

310 *Yeast strains and plasmids*

311 All strains analyzed were in the BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or BY4742

312 (*MATα his3Δ1 leu2Δ0 lys1Δ ura3Δ0*) background. BY4742 strains containing Vma5-GFP::*HIS3*

313 and Vph1-GFP::*HIS3* were constructed as part of the genome-wide GFP-tagging project (Huh et

314 al., 2003) and purchased from Thermo Fisher. *VMA2* was C-terminally tagged with GFP by PCR

315 amplification from pFA6a-GFP-KanMX6 and genomic integration (Longtine et al., 1998). The

316 strain containing *oxr1Δ::kanMX* and Vma5-GFP::*HIS3* was obtained by crossing BY4741

317 *oxr1Δ::kanMX* from the haploid deletion collection and BY4742 Vma5-GFP::*HIS3*, sporulating

318 the diploid, and obtaining spores with the desired genotype by tetrad dissection. The pRS316

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319   Oxr1-HA plasmid (Khan & Wilkens, 2024) was transformed into *oxr1Δ* and transformants were

320   selected for on SC medium (fully supplemented minimal medium) lacking uracil. Rav1-

321   myc13::*kanMX* and Rav2-myc13::*kanMX* strains were described previously (Smardon et al.,

322   2002). The tagged alleles were PCR amplified from the original strains and integrated into

323   BY4741.

324   *Fluorescence microscopy*

325   Strains expressing GFP-tagged Vma5, Vma2, or Vph1 were grown to log phase in SC

326   medium containing either 2% or 0.5% glucose overnight, pelleted by centrifugation, then

327   suspended in fresh medium containing 2% or 0.5% glucose and grown for an additional 2 hours.

328   Cells were stained with calcofluor white (CW) by diluting a 1 mg/ml CW stock to a final

329   concentration of 10 μg/ml with cells, 5 min prior to imaging. Cells were visualized with a 100x

330   oil (NA 1.4) objective on a Zeiss Imager.Z1 fluorescence microscope with a Hamamastu CCD

331   camera and AxioVision software. Cells were viewed through differential interference contrast

332   (DIC) optics or fluorescence was visualized using a DAPI filter set for CW and a GFP filter set

333   for GFP-tagged subunits. Bud scars were counted from CW staining; in mixed age populations,

334   cells with 5 or more bud scars were designated as old and those with less than 5 as young. To

335   obtain a more precise count of bud scars for binning, cells were visualized on multiple focal

336   planes. GFP fluorescence was then determined for each of the binned ranges. Images of GFP-

337   tagged proteins were captured then processed in FIJI. To assess vacuolar localization, a line was

338   drawn across a cell and through the vacuolar membrane. From this line scan, a plot illustrating

339   fluorescence intensity along the line was generated, with peaks indicating areas of elevated

340   fluorescence at the vacuolar membrane. By quantifying the peak intensity (maximum

341   fluorescence), we quantitated the vacuolar localization for each GFP-tagged subunit. Each

342   biological replicate corresponds to a distinct culture of yeast cells. Maximum fluorescence was

343   quantitated for at least 20 young and 20 old cells per biological replicate. When cells were

344   binned by age, at least 20 cells per biological replicate were counted for each bin. To normalize

345   maximum fluorescence across replicates, the maximum fluorescence from line scans of young

346   and old cells in each biological replicate was averaged and then divided by the average of the

347   young cells in that replicate. The normalized fluorescence for each biological replicate was

348   plotted, along with average intensities across replicates +/- s.e.m. (standard error of the mean).

349   Statistical significance was determined by t-test for the young and old cell comparisons and by

350   ANOVA for the binned samples. In order to show the range of values for the young cells across

351   experiments, the values for the young cells in each biological replicate were averaged and the

352   values for the individual replicates were divided by the average and shown as points on each

353   graph.

354   *Age Enrichment*

355   Age enrichment was performed as described by Jin et al. (Jin et al., 2021). Cells were collected

356   from a 50 mL fresh overnight culture in YEP (yeast extract-peptone medium) supplemented with

357   2% or 0.5% glucose to an OD_{600} of 1.0 and washed twice with cold phosphate-buffered saline

358   (PBS), pH 7.4. Cells were pelleted by centrifugation and washed three times in cold sterile PBS,

359   then labeled with 1.6 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) at room temperature for 30

360   min with gentle agitation. After labeling, the cells were washed three times with cold PBS, pH

361   8.0, to remove free biotin, and resuspended in YEP supplemented with 2% or 0.5% glucose for

362   growth overnight. After 16 hours, cells were pelleted by centrifugation and resuspended in 35 ml

363   of cold PBS, pH 7.4, mixed with 250 μl of a magnetic streptavidin bead suspension (Pierce), and

364   incubated for 60 min at 4°C. The mixture, in a 50 mL conical tube, was loaded onto a magnetic

17

365   separation column (Permagen) at 4°C to separate biotinylated cells and allow unbound cells to

366   settle, and supernatant was removed gently by pipetting. Magnetically separated cells were

367   subsequently washed three times by resuspending in 35 mL PBS, pH 7.4 supplemented with 2%

368   or 0.5% glucose, repeating magnetic separation, and discarding the supernatant. After washing,

369   cells were resuspended in 200 mL of YEP or SC supplemented with 2% or 0.5% glucose and

370   allowed to grow for an additional 4 hours before obtaining the final "old" mother cells and

371   "young" daughter cells. Cells were loaded on the magnetic separation column as described above

372   and daughter cells obtained from the supernatant were pelleted by centrifugation to obtain a

373   concentrated population of young cells. After the final wash, the magnetic beads were

374   resuspended in 1 ml PBS 7.4 and centrifuged at 4000g to concentrate the old population. The two

375   populations were then stored at -80°C for further biochemical analysis or used immediately for

376   pH measurements (see below).

377   *Whole Cell Lysates and Immunoblots*

378   Cell pellets from age-enriched young and old cell populations were resuspended in hot

379   cracking buffer (8 M urea, 5% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 6.8) and glass beads.

380   The mixture was vortexed for 10 sec and incubated at 95 °C for 30 sec repeatedly for a total of 5

381   min. Cellular debris was pelleted by centrifugation at $16,000 \times g$ for 2 min, and supernatants

382   containing whole-cell extracts were used immediately or stored at −80 °C until use.

383   After determination of protein concentrations by Bradford assay, equal concentrations of

384   protein for each sample were separated by SDS-PAGE and transferred to a nitrocellulose

385   membrane. Blots were blocked for 1 hr in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl,

386   0.05% Tween-20) plus 5% nonfat milk before incubating overnight with primary antibodies at 4

387   °C with agitation. Primary antibodies (all used at a 1:500 dilution) included mouse monoclonal

18

388   antibodies: 7A2 against Vma5, 10D7 against Vph1, 8B1 against Vma1, and 13D11 against Vma2

389   (Kane et al., 1992). In addition, anti-myc monoclonal 9E10 (Santa Cruz Biotechnology), anti-HA

390   monoclonal (BioLegend), and anti-GAPDH (Proteintech) antibodies were purchased and used at

391   1:500, 1:500, and 1:10000, respectively. After washing three times with TBST buffer, HRP-

392   conjugated anti-mouse secondary antibody (Bio-Rad) was added at a final dilution of 1:2000 and

393   incubated for 60 min at room temperature. The blot was washed again, incubated with Bio-Rad

394   Clarity Western ECL substrate and imaged in an Azure Sapphire FL Biomolecular Imager.

395   Images were quantified using FIJI. Molecular mass markers were included on every blot. In

396   images of blots, the mass of the marker nearest in size to the protein is indicated.

397   *Vacuolar pH Measurements*

398   Vacuolar pH was measured using the ratiometric fluorescent dye BCECF-AM (Invitrogen) as

399   described previously(Diakov et al., 2013). Age-enriched populations of cells were loaded with

400   BCECF-AM in YEP supplemented with 2% or 0.5% glucose. After washing with YEP media to

401   remove dye, cells were resuspended in YEP, deprived of glucose, and incubated on rotator for 30

402   min. For fluorescence measurement, 20 μl of cell suspension was diluted into 3 ml 1 mm MES

403   pH 5.0 buffer, and fluorescence intensity at excitation wavelengths 450 and 495 nm and emission

404   wavelength 535 nm and was monitored continuously in a Horiba Jovin Yvon Spectrafluor Max

405   fluorometer with temperature maintained at 30°C. The fluorescence ratio for each sample was

406   calibrated for each strain in every experiment by clamping the pH to a range of values from 5.0

407   to 7.0 as described, and the resulting calibration curve was used to convert the experimental

408   fluorescence ratios to vacuolar pH.

409   *Replicative Life Span*

410   Replicative life span assays were performed on YEP, 2% glucose plates buffered to pH 5.

411   Daughter cells were sequentially removed by micromanipulation (Steffen, Kennedy, &

412   Kaeberlein, 2009). Survival curves are pooled data from experiment-matched controls. The

413   number of divisions for 30 mother cells were scored for each curve. p values for replicative life

414   span survival curve comparisons were calculated with a Cox regression model. Kaplan-Meier

415   survival curves were plotted with GraphPad Prism.

416   *RT-PCR of RAV2 from young and old cells*

417   RNA was extracted from young and old cells, obtained by age enrichment as described above,

418   using the NEB Monarch Total RNA Miniprep Kit . RT-PCR was conducted using the NEB Luna

419   Universal One-Step RT-qPCR Kit and performed on a Bio-Rad CFX384 Touch System. Data

420   analysis was conducted using CFX Maestro Software to determine expression levels.

421

425   **Author contributions**: F.H. performed experiments, analyzed data, prepared figures, and wrote

426   the first draft of the manuscript; P.M.K. obtained funding for the project, analyzed data, prepared

427   figures, and contributed to writing of the manuscript.

428   **Conflict of interest**: The authors declare that they have no conflicts of interest with the contents

429   of this article.

430   **Data availability**: The data that support the findings of this study are openly available in

431   Upstate.figshare.com at https://upstate.figshare.com. DOI: 10.58120/upstate.26023660

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661

FIGURE LEGENDS

Figure 1: V-ATPases are more disassembled in yeast cells of older replicative age. (a)
BY4742 cells expressing Vma5-GFP grown in SC containing 2% glucose. Differential
interference contrast microscopy (DIC) was used to visualize vacuoles. Bud scars are stained
with calcofluor white (CW) to determine replicative age. **(b)** Left panel provides representative
example of quantitative measurements using line scans. The young cell is from the dashed white
box in the Vma5-GFP image of **Figure 1a** and the old cell is from the dashed red box. Plot
profiles are superimposed for the young cell (black) and old cell (red). **(c)** Quantitation of
maximum fluorescent intensity across five biological replicates, after normalization to the
average intensity in the youngest bin for each replicate. Each biological replicate (dot) represents
at least 20 cells and bars represent the mean +/- s.e.m. Significance was calculated by ordinary
one-way ANOVA. "Old" for subsequent experiments (without age enrichment) is categorized at
$\geq$5 bud scars. **** represents a p-value <0.0001.

Figure 2: Age-enriched populations of cells have comparable levels of V-ATPase subunits.
(a) Diagram of V-ATPase showing the relative positions of Vma5, Vma2, and Vph1 (Image was
prepared with Biorender.com). **(b)** BY4742 cells expressing Vma2-GFP (V_1B) grow in SC with
2% glucose. CW was used to visualize bud scars. The CW images for young cells were
overexposed relative to those for the old cells in order to visualize the low level staining in cells
with few bud scars. Normalized maximum fluorescence was obtained through line scan
quantitation using FIJI as in **Figure 1**. Means +/- s.e.m. of three biological replicates are shown;
each replicate is represented by a dot. Significance was calculated by unpaired Student's *t* test,
*** p value=0.0009 . **(c)** Cells expressing Vph1-GFP were visualized and analyzed as described

21

456   in **2b**. **(d)** After biotin-streptavidin age enrichment, bud scars were counted for 100 cells in

457   young and old populations, binned by bud count, and the age distribution plotted. **(e)** Lysates

458   were prepared from age-enriched populations defined as in **1c** and equal protein concentrations

459   were separated by SDS-PAGE and examined by immunoblot for V-ATPase protein levels in

460   young (Y) vs. old (O) cell populations defined as in **1c**. **(f)** Band intensities were quantified using

461   FIJI, ratios of V-ATPase subunit levels to the GAPDH internal control were calculated, and ratios

462   were normalized to the young population for each biological replicate. Significance calculated by

463   unpaired Student's *t* test. Data are presented as mean (horizontal bars) ± s.e.m. (whiskers) of

464   three biological replicates. n.s.= not significant.

465

466   **Figure 3: Vacuolar pH is more alkaline in old cells. (a)** Vacuolar pH responses were measured

467   for wild-type BY4742 age-enriched young and old populations as described in Methods.

468   Glucose-deprived cultures were loaded with BCECF-AM. Fluorescence intensity values were

469   collected every 10 sec at excitation wavelengths 450 and 490 nm and emission wavelength 535

470   nm, and glucose was added to a final concentration of 2% after 3 min. The ratio of fluorescence

471   signals from the two excitation wavelengths was calculated and converted to pH via a calibration

472   curve. **(b)** Fluorescence measurements at 1 min. (before glucose addition), 5 min (2 min. after

473   glucose addition), and 8 min. (5 min after glucose addition). Calculated pH measurements are

474   presented as mean (horizontal bars) ± s.e.m. (whiskers) of three biological replicates. * indicates

475   a p value of 0.02, ** represents a p value of 0.001.

476

477   **Figure 4: Caloric restriction (0.5% glucose) restores V-ATPase assembly and vacuolar pH**

478   **in old cells. (a), (b), (c)** Strains used in **Figures 1** and **2** show recruitment of Vma5-GFP **4a** and

479   Vma2-GFP **4b** to the vacuolar membrane after growth in SC with 0.5% glucose while Vph1-GFP

480   4**c** remains unchanged. Maximum fluorescence was measured and normalized as described in

481   Methods and **Figure 1**. CW was visualized in young and old cells as described in **Figure 2b**. No

482   significant differences between young and old cells was observed as calculated by unpaired

483   Student's *t* test. **(d)**Young and old cell populations were obtained by biotin-streptavidin age-

484   enrichment from cells grown in YEP supplemented with 0.5% glucose, and vacuolar pH

485   responses were measured. Data were collected and analyzed as in **Figure 3b**. Quantitated data

486   are presented as mean (horizontal bars) ± s.e.m. (whiskers) of three biological replicates.

487

488   **Figure 5: Effects of *rav1Δ* and *oxr1Δ* on replicative lifespan. (a)** Schematic of reversible

489   disassembly highlighting the roles of the RAVE complex and Oxr1 (Image was prepared with

490   Biorender.com). **(b)** Kaplan-Meier curves comparing the replicative lifespan (RLS) of *rav1Δ*

491   (green), *oxr1Δ* (red), and wild-type cells (black). Median number of replicative generations is

492   shown in parentheses for each strain, and the difference between *rav1Δ* and *oxr1Δ* median values

493   and wild-type are expressed as %. Deletion of *OXR1* significantly increases yeast RLS

494   (p<0.001), and deletion of RAVE component *RAV1* shortens RLS (p < 0.01).

495

496   **Figure 6: Rav2 level is reduced during replicative aging, but restored by CR. (a)** Analysis of

497   Rav1 levels in young and old cells. Age-enriched populations were obtained from BY4741 cells

498   containing Rav1-myc$_{13}$ (young (Y) versus old (O) cells), and cell lysates were prepared,

499   separated, and quantitated as described in Methods and **Figure 2**. Normalized data are presented

500   as mean (horizontal bars) ± s.e.m. (whiskers) of three biological replicates. ns=not significant **(b)**

501   Immunoblot analysis of BY4741 Rav2-myc$_{13}$ from young and old cells prepared as in **6a**.

502　　Normalized data are presented as mean (horizontal bars) ± s.e.m. (whiskers) of three biological

503　　replicates. P-value 0.0094. **(c)** Quantitative RT-PCR comparing expression of *RAV2* mRNA

504　　between young and old cells. **(d)** Wild-type and *oxr1Δ* cells containing Vma5-GFP were grown

505　　in SC containing 2% glucose. DIC used to visualize vacuoles and CW used to visualize bud scars

506　　as described in **Figure 2b**. **(e)** BY4741 *oxr1Δ* cells were binned by the number of bud scars and

507　　normalized maximal fluorescence quantitated as described in **Figure 1**. *** indicates a P value

508　　of 0.001 in comparison to the youngest bin; other bins are not significantly different from the

509　　youngest bin. **(f)** Immunoblot analysis of a BY4741 *oxr1Δ* strain expressing Oxr1-HA from a

510　　low copy plasmid. Young and old cell populations were isolated as in **6a**. Data are presented as

511　　mean (horizontal bars) ± s.e.m. (whiskers) of three biological replicates. **(g)** Immunoblot analysis

512　　comparing Rav2-myc$_{13}$ levels in cells grown in YEP supplemented with 2% glucose and 0.5%

513　　glucose (CR conditions). Samples were prepared and analyzed as in **6b**. Quantification of data

514　　are presented as mean (horizontal bars) ± s.e.m. (whiskers) of three biological replicates. ***

515　　indicates a P value of 0.0001, n.s.= not significant.

516

517　　**Supporting information, Figure 1: Cells show similar growth rates over 12 hours in 2%**

518　　**and 0.5% glucose.** BY4742 cells at log phase were diluted into YEP containing either 2% or

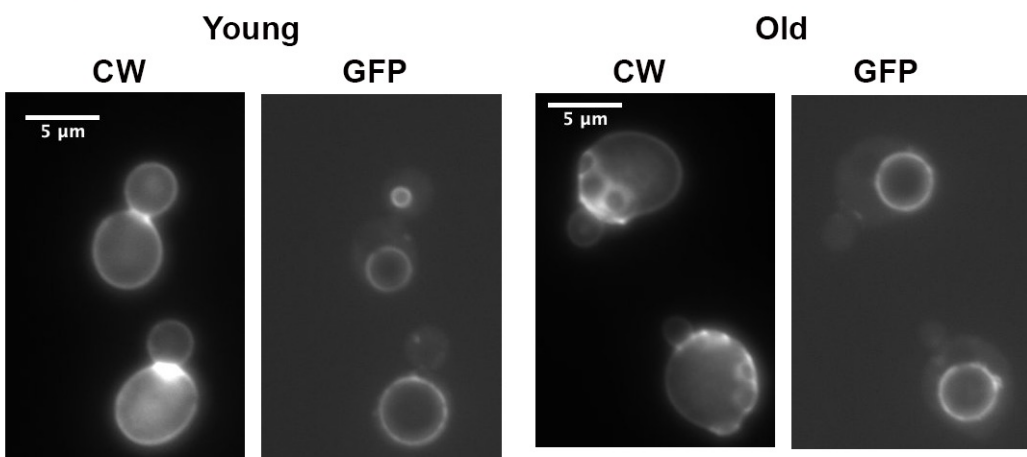519　　0.5% glucose as indicated. OD$_{600}$ was measured every hour for 12 hours.

520

521

522
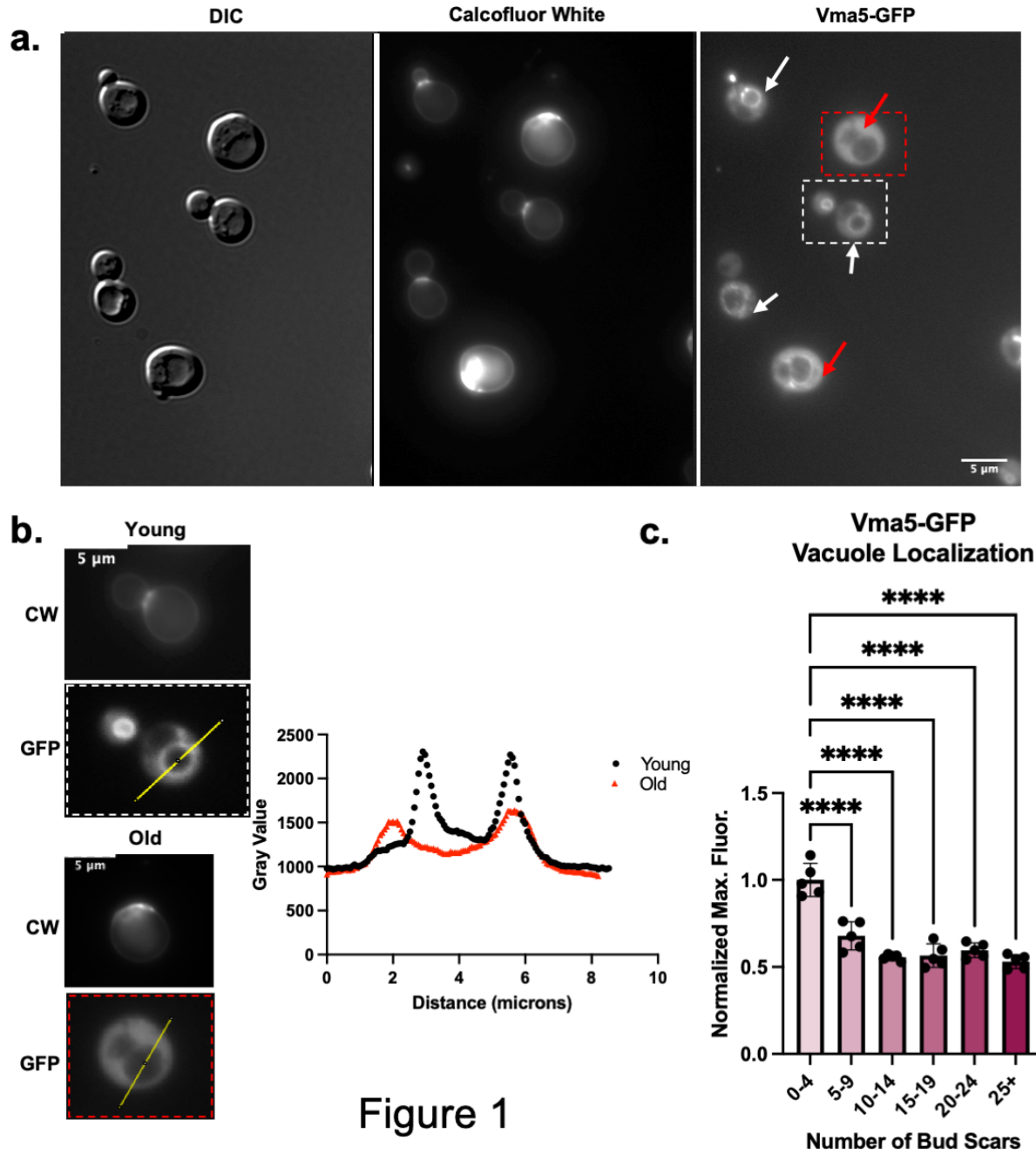
Figure 1
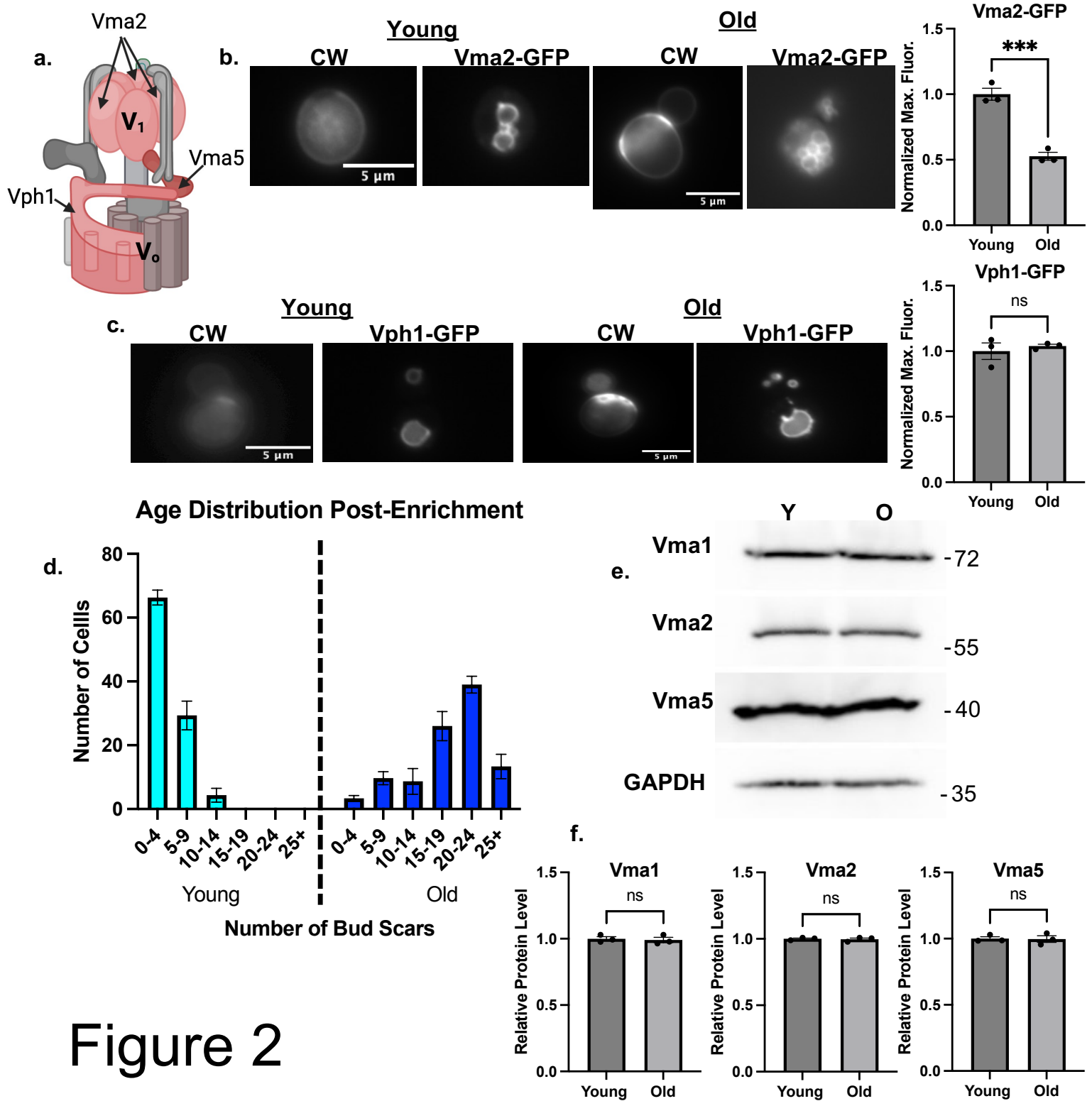
Figure 2

Figure 3

a. Vma5-GFP

Young
CW   GFP

Old
CW   GFP

5 µm

CR Vma5-GFP
ns

b. Vma2-GFP

Young
CW   GFP

Old
CW   GFP

5 µm

CR Vma2-GFP
ns

c. Vph1-GFP

Young
CW   GFP

Old
CW   GFP

5 µm

CR Vph1-GFP
ns

d.

Glucose

Young
Old

Figure 4

a.

V1

Oxr1 →
← RAVE

V1

Vma5

ACTIVE

INACTIVE

b.

Figure 5

Figure 6