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Atg39 selectively captures inner nuclear membrane into lumenal vesicles for

delivery to the autophagosome

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19

Abstract

21   Mechanisms that turnover components of the nucleus and inner nuclear membrane

22   (INM) remain to be fully defined. We explore how components of the INM are selected

23   by a cytosolic autophagy apparatus through a transmembrane nuclear envelope-

24   localized cargo adaptor, Atg39. A split-GFP reporter shows that Atg39 localizes to the

25   outer nuclear membrane (ONM) and thus targets the INM across the nuclear envelope

26   lumen. Consistent with this, sequence elements that confer both nuclear envelope

27   localization and a membrane remodeling activity are mapped to the Atg39 lumenal

28   domain; these lumenal motifs are required for the autophagy-mediated degradation of

29   an integral INM protein. Interestingly, correlative light and electron tomography shows

30   that the overexpression of Atg39 leads to the expansion of the ONM and the enclosure

31   of a network of INM-derived vesicles in the nuclear envelope lumen. Thus, we propose

32   an outside-in model of nucleophagy where INM is delivered into vesicles in the nuclear

33   envelope lumen, which can be targeted by the autophagosome.

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Introduction

The function of the nuclear envelope (NE) is conferred by its biochemical constituents that populate an inner nuclear membrane (INM) with peripherally associated nuclear lamina, an outer nuclear membrane (ONM), and a nuclear pore membrane[1]. The latter defines connections between the INM and ONM where embedded nuclear pore complexes (NPCs) control molecular traffic between the nucleus and cytoplasm[2,3]. Although we have a considerable understanding of the mechanisms that underly molecular exchange through NPCs, it is less well understood how the NE proteome is turned over under either physiological or pathological conditions.

The need to clear damaged or defective proteins from the nucleus and NE is underscored by the accumulation of nuclear protein aggregates in several human diseases[4]. Further, both NPCs[5] and the nuclear lamins accumulate damage with age[6] and defects in nuclear transport[7–9] and NPC injury may be a cause of certain forms of neurodegeneration including amyotrophic lateral sclerosis[10]. Interestingly, the protein constituents of NPCs are also characterized by long half lives in neurons[11–13], which may indicate that they are challenging to productively turnover. Indeed, it is hard to conceptualize how cells might remove these massive macromolecular assemblies without compromising NE integrity. Nonetheless, there is evidence that NPCs may be excised from the NE in both metazoan[14] and in yeast model systems[15–17]. While the endosomal sorting complexes required for transport (ESCRT)[14,16] and the macroautophagy[16,17] machinery have been implicated in these events, the molecular and morphological steps in these pathways are just beginning to come to light.

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60   Like NPCs, there is evidence that the lamins can be turned over with several molecular

61   links implicating macroautophagy in this process[18–21]. Macroautophagy (hereafter called

62   "autophagy") is a catabolic mechanism that delivers protein aggregates, lipids and parts

63   of (and in some cases, whole) organelles to lysosomes for degradation[22]. It begins with

64   the formation of a phagophore membrane that is defined by the covalent coupling of the

65   ubiquitin-like protein LC3 (Atg8, in yeast) directly to phosphatidylethanolamine[23]. The

66   phagophore expands around the cargo, ultimately sealing the cargo inside a closed

67   double membrane organelle called the autophagosome[24]. The autophagosome

68   ultimately fuses with lysosomes (or vacuoles, in yeast) where cargo is degraded[25].

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70   Interestingly, there is evidence that LC3 can direct a form of nuclear autophagy

71   (nucleophagy) by binding to Lamin B1 in the context of oncogene activation: this

72   interaction plays a part in the selective clearance of Lamin B1 from the INM[18]. However,

73   how a cytosolic phagophore selectively targets the INM or nucleoplasm across the

74   double membraned NE remains unknown. Indeed, in most cases of selective organelle

75   targeting, autophagy cargo adaptors bind to specific proteins and recruit the autophagy

76   machinery to initiate phagophore expansion around themselves[23,26,27]. While such a

77   nuclear-specific cargo adaptor has not been identified in metazoans, budding yeast

78   have Atg39 (a.k.a. Esm1[28]), a putative type II transmembrane protein[28] that localizes at

79   the NE and is required for the autophagic degradation of both INM and nucleoplasmic

80   proteins[17,29–33], but not NPCs[16]. Thus, Atg39 is the essential cog in the macroautophagic

81   clearance of INM, but how INM is recognized and delivered to the cytoplasm remains

82   unknown.

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84   Here, we explore the mechanism of Atg39-mediated nucleophagy in budding yeast. The

85   data support that Atg39 acts from the ONM and connects to the INM through its lumenal

86   domain. The lumenal domain has functional elements that are required for NE

87   remodeling and the capture of INM cargo into NE-blebs that can be targeted by

88   autophagy. By using correlative light and electron microscopy (CLEM) and tomography

89   and focused ion beam-scanning EM (FIB-SEM) to visualize NE bleb ultrastructure, we

90   observe the capture of INM into vesicles in the NE lumen. We propose a model where

91   nucleophagy proceeds through an outside-in mechanism where putative translumenal

92   interactions coordinate INM and ONM remodeling to ultimately deliver INM cargo to the

93   autophagosome.

94

95   **Results**

96   **Atg39 accumulates at the ONM**

97   Key unknowns to unraveling the nucleophagy mechanism are determining whether

98   Atg39 acts from the ONM or the INM (or both), and whether, like other cargo

99   adaptors[34–36] it has any inherent membrane remodeling activity. To address the former,

100   we took advantage of a recently developed split-GFP reporter system used to catalogue

101   the INM proteome[37]. The system exploits a series of mCherry-tagged reporter proteins

102   that are expressed as fusions to GFP[11], a 4 kD fragment of GFP, and are localized in

103   the nucleus (GFP[11]-mCherry-Pus1; Fig. 1a) and ER. The two ER reporters differ in that

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104   GFP[11] either faces the lumen (mCherry-Scs2$_{TM}$-GFP[11]) or the cytosol (GFP[11]-mCherry-

105   Scs2$_{TM}$; Fig. 1a). In these backgrounds, the rest of GFP (GFP[1-10]) was expressed on

106   either the N- or C-terminus of Atg39 (Fig. 1a) from a galactose-inducible (*GAL1*)

107   promoter; the reconstitution of a fluorescent GFP provides evidence of physical

108   proximity with which to infer localization.

109

110   Taking advantage of the mCherry-Scs2$_{TM}$-GFP[11] ER lumenal reporter (Fig. 1a, i), we

111   first confirmed the proposed type II topology[28,29] of Atg39 as only Atg39-GFP[1-10], but not

112   GFP[1-10]-Atg39 resulted in visible GFP fluorescence localized at the NE (Fig. 1b). We

113   also noted that there were NE extensions or "blebs" at sites of reconstituted GFP

114   fluorescence (Fig. 1b, arrows) suggesting that Atg39 expression impacted NE

115   morphology, consistent with prior data[28]. These blebs were also observed when GFP[1-

116   10]-Atg39 was expressed alongside the cytosolic-facing ER reporter (Fig. 1c, top panels,

117   arrow). Consistent with the conclusion that GFP[1-10]-Atg39 is localized specifically to the

118   NE, the ER reporter itself was distributed throughout the NE and ER but the GFP

119   fluorescence was only observed at the nuclear periphery and within the blebs extending

120   from the NE. This was particularly evident when line profiles were drawn that bisected

121   the entire cell and nucleus: only the NE, and not the cortical (c) ER peaks of the

122   mCherry fluorescence (magenta) overlapped with the reconstituted GFP-Atg39

123   fluorescence (green)(Fig. 1d).

124

125   As the ER reporters can access both the ONM and INM (Fig. 1a), we tested whether

126   Atg39 could reach the INM using the nucleoplasmic reporter (Fig. 1a, iii). The

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127   extralumenal domain of Atg39 is predicted to be unstructured with a MW of 16 kD.

128   Thus, even with the addition of the GFP$^{1-10}$, it should, in principle, be able to pass

129   through the peripheral channels along the pore membrane, which are thought to restrict

130   passage of extralumenal domains larger than ~60 kD[37,38] . Interestingly, although we

131   observed low levels of GFP fluorescence when GFP$^{1-10}$-Atg39 was expressed with the

132   nucleoplasmic reporter (Fig. 1c, bottom panels), this fluorescence was intranuclear and

133   did not accumulate along the nuclear periphery even at low levels of expression (see

134   timecourse of cells treated with galactose, Extended Data Fig. 1a). We interpret these

135   data in a model where Atg39 can cross the pore membrane, but is then liberated from

136   the INM likely through an INM Associated Degradation (INMAD)-type mechanism[39–42].

137   Such a model predicts the existence of a degron sequence in Atg39, likely in its N-

138   terminus. Consistent with this idea, deletion of the N-terminus of Atg39 resulted in the

139   accumulation of a reconstituted nuclear rim fluorescence when GFP$^{1-10}$ -atg39-(139-

140   398)(Fig. 1a) was expressed with the nucleoplasmic reporter, even after several hours

141   of growth in galactose and thus high levels of expression (Fig. 1e,f, Extended Data Fig.

142   1b), whereas deletion of the C-terminal lumenal (L) domain comprising amino acids

143   169-398 (GFP$^{1-10}$ -atg39-ΔL)(Fig. 1a) mirrored the full-length protein, albeit with a more

144   visible pool at the nuclear periphery at lower levels (Fig. 1g,h, Extended Data Fig. 1c).

145   Thus, there are sequence elements in the N-terminus of Atg39 that might trigger its

146   removal from the INM. Taken together, the data are most consistent with a model in

147   which Atg39 localizes at the ONM and may in fact be restricted from accumulating at

148   the INM.

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150 **The Atg39 lumenal domain is required for ONM targeting and bleb formation**

151 A model in which Atg39 acts from the outside of the nucleus – in predicts physical

152 interactions that connect Atg39 to the INM through the NE lumen/perinuclear space. To

153 explore this, we generated a deletion series of Atg39 to systematically evaluate the

154 sequence elements that conferred ONM localization; we also evaluated the ability of

155 these constructs to induce NE blebs. As a translumenal interaction is predicted to confer

156 ONM accumulation, we first generated C-terminal deletions that sequentially removed

157 lumenal regions that secondary structure prediction suggested were alpha (α) helical in

158 nature (Fig. 2a). Deletion of the terminal two alpha helical segments (GFP-atg39-(1-

159 333)) did not impact NE targeting nor the number of NE blebs observed in each cell

160 (Fig. 2b,e). In contrast, the removal of α2 (GFP-atg39-(1-312)) led to a marked

161 decrease in the number of NE blebs/cell with no obvious impact on NE targeting (Fig.

162 2b,e). These data suggest that the formation of NE blebs requires a discrete sequence

163 motif in the Atg39 lumenal domain. They further imply that the NE remodeling observed

164 is not simply an artifact of overexpression but may in fact represent a membrane

165 deforming activity present in the Atg39 lumenal domain.

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167 Removal of α1 abrogated NE accumulation as both GFP-atg39-(1-275) and GFP-atg39-

168 ΔL no longer exclusively accumulated at the NE and were found throughout the cortical

169 ER as well (Fig. 2b). Thus, these data support a model in which the lumenal domain of

170 Atg39 has sequence elements required for both NE accumulation (α1) and NE

171 remodeling (α2). In analogy to KASH-proteins that accumulate at the ONM by binding to

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172   INM SUN proteins[43], we propose that Atg39 accumulates at the ONM by forming a

173   translumenal bridge through direct or indirect interactions with the INM.

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175   NE targeting and remodeling activities appear to be largely restricted to the lumenal

176   domain as N-terminal deletions of Atg39 did not appreciably impact the accumulation of

177   GFP-atg39-(70-398), GFP-atg39-(120-398) or GFP-atg39-(139-398) at the NE, although

178   we did observe more elaborate NE-blebs (Fig. 2c, arrows and Fig. 2e) in these cells that

179   could be correlated to higher levels of these constructs (Extended Data Fig. 2a). This

180   was intriguing as it suggested that interactions with the autophagy machinery, which are

181   mediated through the Atg8 and Atg11-binding motifs[29] (Fig. 2a) did not contribute to the

182   formation of the NE-blebs. Consistent with this conclusion, similar numbers of GFP-

183   Atg39 containing NE-blebs were observed in *atg8Δ* and *atg11Δ* strains compared to

184   wild type cells (Fig. 2d,e) with GFP-Atg39 expressed at similar levels in these strains

185   (Extended data Fig. 2b). Thus, the Atg39 lumenal domain is necessary for NE targeting

186   and remodeling, which can occur independently of engagement with the core autophagy

187   machinery.

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189   **The Atg39 lumenal domain is required for nucleophagy**

190   The data support a model in which Atg39 can accumulate and mediate NE remodeling

191   from the ONM by virtue of sequence motifs in its lumenal domain. To evaluate the

192   importance of these motifs in the nucleophagic clearance of INM proteins, we tested

193   whether they were required for the autophagic degradation of the established integral

194   INM Atg39-cargo, Heh1 (also called Src1)[29,32] under conditions of nitrogen starvation.

195   We tested the degradation of Heh1-GFP using a standard autophagy assay that relies

196   on the visualization of a stable fragment of GFP (GFP') by Western blot, which is

197   liberated from Heh1-GFP by vacuolar proteases. Consistent with published data[29], we

198   observed a ~65% degradation of the total pool of Heh1-GFP in cells grown in medium

199   lacking nitrogen, which was mitigated in the *atg39Δ* strain (Fig. 3a,b). This effect was

200   specific for Heh1-GFP as the relative degradation of Heh1's paralogue, Heh2-GFP, and

201   the nucleoporin Nup82-GFP, were not significantly impacted by *ATG39* deletion (Fig.

202   3c,d).

203

204   To test the role of the Atg39 lumenal domain motifs in nucleophagy, we next examined

205   Heh1-GFP degradation in strains expressing lumenal truncations from the endogenous

206   *ATG39* chromosomal locus (i.e. under the control of the *ATG39* promoter). Consistent

207   with its importance for executing nucleophagy, the sequential trimming of the C-terminal

208   lumenal domain resulted in a reduction of Heh1-GFP degradation that reflected the NE

209   targeting and remodeling analysis (Fig. 2). For example, consistent with the

210   dispensability of the terminal two α-helical segments for NE targeting and remodeling

211   (Fig. 2b,e), the *atg39-(1-333)* allele fully complemented the degradation of Heh1-GFP

212   as the wild type *ATG39* gene (Fig. 3a,b). In contrast, the sequential removal of the α2

213   (*atg39-1-312*) and α1 (*atg39-1-275*) coding segments, required for NE remodeling and

214   NE targeting, respectively, resulted in a progressive loss of the ability of these alleles to

215   contribute to Heh1-GFP degradation (Fig. 3a,b). Thus, the lumenal sequence motifs that

216   are required for Atg39 NE targeting and blebbing are also needed to effectively execute

217   nucleophagy under conditions of nitrogen starvation.

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218 **Atg39-containing NE-blebs are delivered to the vacuole by autophagy**

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220 Because of the obvious overlap between the requirement of the lumenal motifs for NE

221 targeting/remodeling and nucleophagy, we considered the possibility that the NE-blebs

222 formed upon Atg39 overexpression may in fact represent a morphologically relevant

223 intermediate in nucleophagy, albeit exaggerated due to its high levels. Were this to be

224 the case, several criteria needed to be met. First, if the NE-blebs were an intermediate

225 in nucleophagy, they would need to be delivered to the vacuole in a mechanism

226 requiring core autophagy genes. Second, the expectation would be that the NE-blebs

227 would contain cargo specific for Atg39-mediated nucleophagy. Third, the NE

228 ultrastructure driven by Atg39 would need to reflect characteristics of protein-mediated

229 membrane remodeling as opposed to simply membrane expansion or the formation of

230 membrane stacks or lamellae that are common artifacts of the overexpression of NE

231 and ER membrane proteins[44–52].

232

233 To address these criteria, we first tested whether the NE-blebs were delivered to

234 vacuoles. To induce autophagy, we treated Atg39-GFP expressing cells with rapamycin

235 while arresting Atg39-GFP production by inhibiting the *GAL1* promoter with the addition

236 of glucose to the medium; delivery of Atg39-GFP to vacuoles (labeled with the FM 4-64

237 dye) was monitored by fluorescence microscopy at 30 min intervals (Fig. 3e). To ensure

238 that Atg39-GFP could be visualized in vacuoles, these experiments were performed in a

239 strain lacking Pep4, a vacuolar protease required for activation of vacuolar hydrolases[53].

240 As shown in Fig. 3e, we observed internal vacuolar GFP fluorescence in ~25 % of

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241   rapamycin-treated cells beginning at the 60 min time point, which progressively

242   increased to ~95 % by the end of the experiment (150 min; Fig. 3f). Consistent with the

243   idea that Atg39-GFP is progressively being cleared by autophagy, we observed a

244   coincident reduction in Atg39-GFP fluorescence at the nuclear periphery (Fig. 3g).

245   Further, we did not observe GFP fluorescence in the vacuole in carrier-alone (DMSO)

246   treated samples despite some reduction to its levels at the nuclear periphery (Fig.

247   3e,f,g). We interpret the latter to reflect that Atg39-GFP production is inhibited upon

248   addition of glucose to the medium and there is a dilution of Atg39-GFP signal through

249   the ~1.5 cell divisions that occur during the 150 min time course. Therefore,

250   overexpressed Atg39-GFP can be delivered to the vacuole under conditions of

251   autophagy induction by rapamycin treatment.

252

253   To further confirm that overexpressed Atg39-GFP was degraded by autophagy, we also

254   observed the production of GFP' after treating Atg39-GFP expressing cells with

255   rapamycin (Extended Data Fig. 2c). Consistent with the conclusion that this GFP

256   fragment was the product of a vacuolar protease, it was not observed in a *pep4Δ* strain

257   (Extended Data Fig. 2c). Importantly, this autophagy-dependent degradation of Atg39-

258   GFP also required the Atg39 lumenal domain as GFP' was not visible upon treatment of

259   atg39-ΔL-GFP expressing cells with rapamycin (Extended Data Fig. 2d). Lastly, we

260   confirmed that Atg39-GFP is targeted by autophagy by testing the production of GFP' in

261   both *atg8Δ* and *atg11Δ* strains. Deletion of *ATG8,* and to a lesser extent *ATG11,*

262   abolished its production (Extended Data Fig. 2e). We conclude that Atg39-GFP

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263   containing blebs can be delivered and degraded in the vacuole through an autophagy-

264   dependent mechanism.

265

266   **Atg39-derived NE-blebs specifically capture the INM**

267   The second criteria that would provide confidence that the NE-blebs are a potentially

268   physiological intermediate in nucleophagy would be the selective incorporation of Atg39

269   cargo into the blebs. To test this, we monitored the distribution of several integral

270   components of the NE (Fig. 4a) expressed at endogenous levels including NPCs

271   (Nup85), spindle pole bodies (Spc42) and integral INM proteins Heh1 and Heh2, in the

272   context of mCherry-Atg39 expression. Of these, only Heh1 has been shown to be

273   targeted by Atg39-dependent nucleophagy[29,32], whereas Nup85 can be degraded by

274   NPC-phagy[17]. And indeed, we did not observe any appreciable accumulation of NPCs

275   within the NE-blebs, nor components of the SPB (Fig. 4b, right "GFP" panels, 4c). In

276   striking contrast, Heh1-GFP co-localized with mCherry-Atg39 within at least 50% of the

277   blebs (Fig. 4b, arrowheads, Fig. 4c). As Heh1-GFP is expressed at low levels[54] often

278   below our threshold for detection even at the NE, we suspected that these numbers

279   were an underestimate. In fact, Heh1-GFP is produced from one of two alternatively

280   spliced forms of the *HEH1-GFP* transcript[55]. By examining the localization of a

281   truncation made before the splice site, a more abundant INM-localized heh1-ΔL-GFP

282   (Fig. 4a) can be visualized in virtually all Atg39-containing NE-blebs (Fig. 4b,c). Further,

283   the brighter heh1-ΔL-GFP allowed the continual monitoring of heh1-ΔL-GFP

284   sequestration into the blebs by timelapse microscopy, which occurs concomitantly with

285   bleb formation (Extended Data Fig. 3, arrowheads).

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286

287   Surprisingly, the paralogue of Heh1, Heh2, was not captured with Atg39 in the blebs

288   (Fig. 4b,c) suggesting that there may be some selectivity for subdomains of the INM. To

289   explain this observation, we recently uncovered that a substantial fraction of Heh2 is

290   bound to NPCs[56], which might prevent Heh2's capture into the NE blebs. To test this

291   idea, we examined the localization of a truncation of Heh2 (heh2-ΔWH; Fig. 4a) that

292   retains INM-targeting information but lacks the ability to bind NPCs, which is conferred

293   by a C-terminal winged-helix (WH) domain[56]. Consistent with the conclusion that binding

294   to NPCs prevents capture of Heh2, heh2-ΔWH-GFP was found in 50% of the visible

295   Atg39-containing NE-blebs (Fig. 4b,c). Thus, taken together, Atg39-dependent NE-

296   blebs can selectively capture the INM over other elements of the NE suggesting that the

297   overexpression of Atg39 might be recapitulating key early steps in an Atg39-dependent

298   nucleophagy pathway.

299

300   **NE-blebs contain a network of INM-derived vesicles in the NE lumen**

301   Lastly, to evaluate whether Atg39 overexpression leads to changes in NE morphology

302   that might illuminate early steps in nucleophagy, we turned to CLEM and tomography.

303   We first examined cells prepared from cultures expressing high levels (Extended Data

304   Fig. 4a) of Atg39-GFP (Fig. 5a, Extended Data Fig. 4b, Supplementary Video 1).

305   Correlation of regions of Atg39-GFP fluorescence (Fig. 5a, Extended Data Fig. 4b,

306   insets) with their position in electron micrographs revealed an impressive proliferation of

307   membranes. These membranes were derived from the NE as direct continuity could be

308   observed with the ONM in single tomographic slices and in 3D reconstructions; ONM

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309 ultimately enclosed the entire structure (Fig. 5a, ii, iv-x, arrowheads point to ONM

310 continuity). Most strikingly, captured within the expanded ONM were additional bilayers

311 that are traced in teal (Fig 5a, iv, viii, x), which were most logically derived from the INM.

312 As these membranes were circular within single tomographic slices, we speculated that

313 they were vesicles. Consistent with this, a 3D model of these segmented membranes

314 revealed that they were spherical and similarly sized with a median diameter of ~115

315 nm (Fig. 5a, iii, 5b). Interestingly, a subset of vesicles were connected by membrane

316 constrictions as if undergoing fission (Figure 5a, iii, ix and x, arrows).

317

318 The vesicle diameter measurements were likely an underestimate as it was not possible

319 to capture a large number of entire vesicle volumes within the relatively thin ~200 nm

320 thick tomograms. We therefore also performed FIB-SEM on Atg39-GFP expressing

321 cells. This approach allowed the visualization of 52 whole cell volumes (Extended Data

322 Fig. 5a) revealing cells with expansive networks of NE blebs that emanated from

323 multiple sites on single nuclei (Extended Data Fig. 5b,c, Supplementary Video 2). In

324 these 3D images, we measured the diameter of 982 INM derived vesicles, which had a

325 median diameter of ~164 nm (Fig. 5b). Interestingly, we also observed lipid droplets

326 associated with virtually all of the NE blebs (Extended Data Fig. 5b,c). Thus, the

327 overexpression of Atg39 leads to the generation of a network of likely INM-derived

328 vesicles within the NE lumen alongside an expansion of the ONM with associated lipid

329 droplets. While there are certainly many examples of overexpressed membrane

330 proteins driving changes to membrane morphology, the observed Atg39-dependent

331 morphology is most analogous to those observed in NE egress pathways[57].

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333   To gain more insight into the biogenesis of the Atg39-induced compartments, we next

334   performed CLEM on cells expressing lower levels of a GFP-Atg39 fusion (Extended

335   data Fig. 4a). We examined the ultrastructure at NE sites of local GFP-Atg39

336   accumulation and where emerging blebs were visible by fluorescence microscopy (Fig.

337   5c, Extended data Fig. 4c-f). Ultimately, we examined 26 individual Atg39 focal NE

338   accumulations within 23 cells. Of these, 3 could not be attributed to any obvious

339   morphology with 2 being localized at sites where mitochondria were adjacent to the NE

340   (Fig. 5d, Extended data Fig. 4c). In 9 cells, we could unambiguously correlate the

341   fluorescence to either NVJs (3 out of 9) or regions of PMN (6 out of 9)(Fig. 5d, Extended

342   Data Fig. 4d), which may be consistent with recent work supporting a role for Atg39 in

343   PMN[33]. And, in a single cell we observed likely extensions to the NE that contained

344   NPCs (Fig. 5d, Extended data Fig. 4e).

345

346   The most prevalent morphology (10 out of 26; Fig. 5d) correlated with GFP-Atg39 is

347   presented in Fig. 5c and Extended Data Fig. 4f. As shown in Fig. 5c, we observed direct

348   continuity between the INM and vesicles within the NE lumen with a ~25 nm constriction

349   or bud neck at the INM. As in the high level expression scenario, these vesicles were

350   again similarly sized (median diameter of 139 nm, Fig. 5b) and were sometimes found

351   in a series where membrane connections could be visualized, segmented and 3D

352   modeled (Fig. 5c panel, iii, iv, Supplementary Video 3). Simultaneously, we observed a

353   clear expansion of the ONM, presumably necessary to accommodate the presence of

354   the extra volume occupied by the vesicles in the NE lumen. Thus, we interpret these

355   structures as precursors to the more elaborate compartments observed upon high level

356   expression of Atg39. These data suggest that Atg39 may have the ability to coordinate

357   membrane remodeling between the INM and ONM and capture components of the INM

358   into vesicles in the NE lumen.

359

360   **Discussion**

361   Atg39 has recently emerged as a key player in the autophagic degradation of nuclear

362   and INM components[17,29–32,58], however, how nuclear cargo is selectively packaged and

363   delivered to the cytosolic autophagosome, through the double membraned NE,

364   remained open questions. Here, we provide a framework for answering these questions

365   by proposing an outside-in model of nucleophagy that depends on Atg39 acting from its

366   position at the ONM (see model, Extended Data Fig. 5d). There, Atg39 can engage with

367   the cytosolic autophagy machinery through its cytosolically-exposed N-terminal domain

368   while connecting to the INM through its lumenal domain. Evidence in support of this

369   model includes the demonstrated importance of the Atg39 lumenal domain not only for

370   NE targeting and NE remodeling (Fig. 2) but also for the nucleophagic degradation of a

371   model Atg39 INM cargo, Heh1-GFP (Fig. 3). Such a mechanism may prove relevant to

372   other forms of nuclear autophagy as well. For example, PMN requires the generation of

373   an ONM-vacuole contact site by the pairing of Nvj1 and Vac8[59]: the close apposition of

374   the INM and ONM at these sites is thought to be mediated by the Nvj1 lumenal

375   domain[60]. Interestingly, however, Nvj1 overexpression does not lead to any obvious

376   membrane deformation[59]. Therefore, recent evidence supporting a role for Atg39 in

377   PMN[33] might suggest a model in which Atg39 contributes a membrane remodeling

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378   activity capable of co-evaginating the INM and ONM, which is a pre-requisite for both

379   nucleophagy mechanisms.

380

381   Perhaps the most exciting element of this model is the proposal that the INM and

382   associated nuclear content is captured within NE lumenal vesicles derived from the INM

383   (Fig. 5; Extended Data Fig. 4,5). Although we acknowledge that these structures are

384   observed upon Atg39 overexpression, we argue that they are likely bona fide early

385   intermediates in a physiological nucleophagy mechanism. Our confidence with this

386   conclusion is based on several data. First, there is the remarkable ability of the Atg39-

387   driven NE-blebs to selectively capture established INM cargo over other components of

388   the NE (Fig. 4). Second, the NE-blebs can be targeted by autophagy provided the

389   signal, yet to be defined, is supplied (Fig. 3e,f). These data also suggest that the

390   interaction between Atg39 and the autophagy apparatus may be regulated in some way,

391   a common theme with selective autophagy cargo adaptors[61,62]. Third, the

392   overexpression of Atg39 lacking lumenal elements, in particular the predicted α helix 2,

393   does not drive analogous morphologies (Fig. 2), further fortifying that they are a specific

394   consequence of Atg39 and not simply an accumulation of an overexpressed integral NE

395   protein.

396   The enclosure of INM within lumenal vesicles is also attractive because it provides a

397   harmonious mechanism for how the INM could be selectively removed without

398   impacting NE integrity. The observed ~25 nm membrane necks where the vesicle

399   membranes connect with the INM are strongly suggestive of a protein-scaffold that

400   would maintain their stability and ultimately drive membrane scission. This observation,

18

401   in addition to the chain-like concatenation of the INM-derived vesicles, strongly

402   resembles morphologies associated with ESCRT function[63,64]. The hypothesis that

403   ESCRTs may ultimately be involved in a scission event that would liberate the INM-

404   derived vesicle into the lumen is attractive not only because of the many links between

405   autophagy and ESCRTs[44,65–69], but also because Heh1, the only well-established INM

406   protein cargo of Atg39[29], directly engages with ESCRTs in pathways that ensure NE

407   integrity[70,71].

408   Lastly, the removal of INM contents through the proposed mechanism evokes

409   comparisons to the nuclear to cytosolic translocation of so-called "Mega" RNPs in some

410   *Drosophila* neurons that proceeds through a vesicular intermediate in the NE lumen[72].

411   Thus, we anticipate that the removal of intranuclear contents through the NE lumen will

412   prove to be a generalizable principle of protein, and perhaps RNA, quality control, that

413   will be relevant beyond the yeast system and has in fact already been hypothesized[73].

414   Ultimately understanding whether this is the case will require the identification of a

415   mammalian functional homologue to Atg39, which so far remains elusive but is the

416   focus of active investigation.

417

418

425

Author Contributions

427   Conceptualization: S.C., P.J.M., D.J.T., T.J.M., C.P.L.; Methodology: S.C., P.J.M.,

428   D.J.T., N.R.A.; Investigation: S.C., P.J.M., D.J.T., N.R.A.; Validation: S.C., P.J.M.,

429   D.J.T., N.R.A.; Formal analysis; S.C., P.J.M., D.J.T., N.R.A.; Writing – original draft:

430   S.C., P.J.M., D.J.T., T.J.M., C.P.L.; Writing – review & editing: All authors; Supervision:

431   M.C.K., T.J.M., C.P.L.; Funding acquisition: T.J.M., C.P.L.

432

Competing interests

434   The authors declare no competing interests.

435

436   **Figure legends**

437   **Fig. 1** - **Atg39 accumulates at the ONM.**

438   **a**, Schematic of localization and topology of split-GFP constructs. The GFP[11]-mCherry

439   reporter fusion proteins (i, ii, iii) are constructed as shown in key with localization

440   diagrammed. INM and ONM are inner and outer nuclear membrane, respectively. At

441   bottom are schematics of the Atg39 GFP[1-10] fusions and truncations lacking N or C-

442   termini. The Atg39 transmembrane (TM) domain is depicted as a grey oval. Numbers

443   indicate amino acids.

444   **b**, Deconvolved fluorescence micrographs of cells expressing indicated Atg39 fusion

445   proteins and mCherry-reporters (see inset). GFP, mCherry and merged images shown.

446   Arrowhead points to NE bleb. Scale bar is 5 µm.

447   **c**, Deconvolved fluorescence micrographs of cells expressing GFP[1-10]-Atg39 and

448   indicated mCherry-reporter (inset). GFP, mCherry and merged images shown. Scale

449   bar is 5 µm. Asterisks indicate vacuolar autofluorescence.

450   **d**, Normalized line profiles of GFP (green) and mCherry (magenta) fluorescence (in

451   arbitrary units, a.u.) bisecting cells as indicated by dotted lines in corresponding top and

452   bottom merge panels from **c**. Position of NE (nuclear envelope) and cER (cortical ER) is

453   indicated by dotted lines.

454   **e**, As in **c,** but with cells expressing GFP[1-10]-atg39-(139-398).

455   **f**, As in **d**, but with cells from **e**.

456   **g**, As in **c**, but with cells expressing GFP[1-10]-atg39-ΔL. Asterisks indicate vacuolar

457   autofluorescence.

458   **h**, As in **d**, but with cells from **g**.

21

459

460 **Fig. 2** - **The Atg39 lumenal domain is required for NE targeting and bleb**

461 **formation.**

462 **a**, Schematic of Atg39 with AIM (Atg8 interacting motif, light orange), Atg11-binding

463 region (blue), predicted transmembrane (TM) helix (grey) and predicted alpha (α)

464 helices 1-4 (colored ovals). Numbers are amino acids.

465 **b**, Deconvolved fluorescence micrographs of the indicated GFP fusion constructs.

466 Numbers indicate amino acid position from **a**. Scale bar is 5 μm.

467 **c**, As in **b**.

468 **d**, Deconvolved fluorescence micrographs of GFP-Atg39 in the indicated strains. Scale

469 bar is 5 μm.

470 **e**, Bar chart plotting the quantification of the number of NE blebs per cell in the indicated

471 strains from micrographs in **b-d**. At least 50 cells of each genotype were quantified from

472 three independent experiments. Average and SD are shown. Statistics are from one-

473 way ANOVA where *ns* is $p > 0.05$, and **** $p \leq 0.0001$.

474

475 **Fig. 3** - **Atg39-containing NE-blebs are delivered to vacuoles by autophagy.**

476 **a**, The autophagic degradation of Heh1-GFP requires the Atg39 lumenal domain.

477 Western blot of proteins (anti-GFP.1) from whole cell extracts derived from cells

478 expressing Heh1-GFP in strains with the indicated *atg39* alleles treated with rapamycin.

479 GFP' is a stable fragment of GFP in vacuoles. Position of molecular weight standards

480 (in kD) at right. To assess relative protein loading, a portion of the blot is shown stained

481 with Ponceau.

22

482   **b**, Plot of mean and SD of the percentage degradation of Heh1-GFP from three

483   independent experiments as in **a**, Statistics from one-way ANOVA test and Tukey's

484   correction where *ns* is *p* > 0.05, and **** *p* ≤ 0.0001.

485   **c**, As in **a** but examining autophagic degradation of Heh2-GFP and Nup82-GFP.

486   **d**, As in **b** but plotting the percentage degradation of Heh2-GFP and Nup82-GFP.

487   **e**, Deconvolved fluorescence micrographs of a timecourse (30 min intervals) of

488   rapamycin or carrier-alone treated (DMSO) cells expressing Atg39-GFP (green) in a

489   *pep4Δ* strain; vacuoles are stained with FM 4-64 (magenta). Merged fluorescent images

490   shown. Scale bar is 3 μm.

491   **f**, Line plot of percentage of cells where Atg39-GFP is visible in vacuoles after treatment

492   with rapamycin (circles and magenta line) or carrier (squares and blue line) over the

493   timepoints indicated. 75 cells were evaluated each from three independent replicates.

494   SD from the mean percentage is indicated by the shaded area.

495   **g**, Violin plot of the fluorescence intensity of Atg39-GFP along the nuclear periphery

496   normalized to background fluorescence in the presence of rapamycin (+) or carrier (-) at

497   the indicated timepoints. 30 cells each from three independent replicates were

498   evaluated, each replicate was normalized to the mean NE fluorescence at 0 min. Solid

499   line denotes the median, width of the violin plot denotes relative frequency of data

500   points. Statistics are from Student's t-test with Welches correction where *ns* is *p* > 0.05,

501   ** is *p* ≤ 0.01 and **** is *p* ≤ 0.0001.

502

503   **Fig. 4 – INM is specifically captured in Atg39-containing NE blebs.**

504 **a**, Cartoon of protein and protein complexes at the NE including a spindle pole body

505 (SPB) and nuclear pore complex (NPC). Boxed region is magnified at bottom to show

506 integral INM proteins and truncation mutants. LEM is LAP2/emerin/MAN1, WH is

507 winged helix (WH). Numbers are amino acids.

508 **b**, Deconvolved fluorescent micrographs of the indicated GFP fusion proteins under

509 conditions where mCherry-Atg39 expression is repressed (+ glucose, OFF; left panels)

510 or induced (+ galactose, ON; right panels). Merged image of mCherry (magenta) and

511 GFP (green) is also shown. Arrowheads point to NE blebs containing mCherry-Atg39

512 and INM proteins. Scale bar is 5 µm.

513 **c**, Quantification of percentage of cells with Atg39 blebs colocalized with indicated NE

514 proteins. Error bars are SD from three independent replicates of 100 cells per replicate.

515

516 **Fig. 5 – CLEM of Atg39-GFP reveals expanded ONM with INM-derived vesicles in**

517 **the NE lumen.**

518 **a**, (i) Virtual slice of an electron tomogram of cell with "high" expression of Atg39-GFP

519 (fluorescence image overlaying EM in inset). Boxes represent magnified regions shown

520 in the following panels with corresponding colors. (ii) 3D model of continuous ONM with

521 arrowheads pointing to continuities between substructures. (iii) 3D model of likely INM-

522 derived vesicles within the ONM compartment with arrows pointing to constrictions. (iv)

523 Example virtual slice of tomogram with ONM and INM traced in blue and teal,

524 respectively. (v, vi) Two views of 3D model where ONM and INM are segmented. (vii,

525 viii and ix, x) Further magnifications of single tomographic slices (with and without

24

526   annotation) as defined by surrounding box color with key in (i). Arrowheads point to

527   continuity of ONM and arrows to INM constrictions. Scale bars are 200 nm.

528   **b**, Quantification of the diameter of INM-derived compartments from cells with "high

529   expression (4 cells), "low" expression (10 cells) of Atg39 from electron tomography or

530   "high" expression of Atg39 from FIB-SEM (52 cells). Total number of INM vesicle

531   diameters measured is indicated in the figure. Median and 95% confidence interval are

532   shown.

533   **c**, CLEM of "low" expressed GFP-Atg39. (i) Fluorescence micrograph of GFP-Atg39

534   with boxed region ultrastructure shown in (ii). Arrowhead points to a region of PMN. (ii)

535   Virtual slice from electron tomogram acquired at of region indicated by the box in panel

536   (i). (iii) Annotation of virtual slice from panel (ii) with the ONM in blue, the INM in teal.

537   (iv) 3D surface rendering of annotated structures in panel (iii). Scale bars: (i), 2 µm; (ii–

538   iv), 50 nm.

539   **d**, Quantification of indicated morphologies/substructures observed from 26 total GFP-

540   Atg39 NE foci.

541

542   **Extended Data Fig. 1** – **The split-GFP reconstitution of intranuclear GFP**

543   **fluorescence depends on the N-terminus of Atg39**.

544   **a-c**, Deconvolved fluorescence micrographs of the indicated GFP[1-10]-constructs co-

545   expressed with the nucleoplasmic split-GFP reporter (see inset and Fig. 1a). GFP[1-10]-

546   constructs are expressed behind a *GAL1* promoter that is induced by growth in

547   galactose for the indicated times (in hours). GFP (green), mCherry (magenta) and

548   merged images shown. Scale bars are 5 µm.

25

549

550 **Extended Data Fig. 2** – **Assessment of total levels of Atg39 fusion proteins**.

551 **a-e**, Western blot of proteins from whole cell extracts derived from cells expressing the

552 indicated GFP-fusions in indicated strains and drug treatments. GFP detected with anti-

553 GFP antibodies (anti-GFP for a, b; anti-GFP.2 for c, e), HRP-conjugated secondary

554 antibodies and ECL. Position of molecular weight standards (in kD) at right. To assess

555 relative protein loading, a portion of the blots are shown stained with Ponceau.

556

557 **Extended Data Fig. 3 – An Integral INM protein is enriched in NE blebs.**

558 **a**, Deconvolved fluorescence micrographs of a timelapse series showing cells

559 expressing Atg39-mCherry (magenta) and heh1-ΔL-GFP (green) with merge at the

560 indicated timepoints after addition of 2% galactose to induce Atg39-mCherry

561 expression. White arrowheads indicate position of NE blebs. Scale bar is 5 μm.

562

563 **Extended Data Fig. 4 – NE ultrastructure at sites of Atg39 accumulation.**

564 **a**, Western blot comparing relative levels of Atg39-GFP ("high" expression) and GFP-

565 Atg39 ("low" expression) to endogenous levels of the nucleoporin, Nup82-GFP (~1600

566 copies/cell). GFP detected with anti-GFP.1. Portion of blot stained with Ponceau shows

567 relative protein loads. Bar graph is quantification by densitometry of the anti-GFP signal

568 normalized to Nup82-GFP.

569 **b-f**, CLEM tomograms from cells expressing "low" or "high" levels of Atg39 as indicated.

570 (i) Fluorescence micrograph with arrows pointing to regions of interest similarly

571 annotated in corresponding EM tomogram.(ii) Virtual slice from electron tomogram with

26

572   the location of fluorescence from (i) indicated by similarly colored arrows. (iii)

573   Magnification of boxed view in (ii). (iv) Annotation of virtual slices from panel (iii) with the

574   ONM in blue, the INM in teal, vacuole in green and mitochondria in orange. Arrow points

575   to continuity of ONM. Asterisks denote nuclear pores. Scale bars are 100 nm.

576

577   **Extended Data Fig. 5. – FIB-SEM of cells expressing Atg39 with model of**

578   **nucleophagy.**

579   **a**, Total volume of FIB-SEM images of cells expressing Atg39-GFP.

580   **b**, Bar chart of the quantification of NE blebs and associated lipid droplets observed in a

581   total of 52 cells captured within the FIB-SEM volume shown in **a**.

582   **c**, (i) Orthogonal view of a block of FIB-SEM images (from **a**) of a single cell with

583   isotropic resolution of 7 x 7 x 7 nm voxels. (ii) surface rendering of NE (blue), lipid

584   droplets (LDs, yellow), mitochondria (magenta), and vacuole (grey); an SEM image of a

585   single Z-plane is also shown. (iii) top-down view of (ii). (iv) side view of surface

586   rendering of just NE and LDs. Arrowheads point to NE blebs. (v) Annotated SEM image

587   of a single z-slice with ONM blue, LD in yellow. (vi) top-down view of NE and LDs with

588   arrows pointing to NE blebs. (vii, viii) Zoom-view and alternate angles of region

589   containing NE blebs and LDs. Scale bars are 200 nm.

590   **d**, A proposed outside-in model of nucleophagy. Atg39 localizes to the ONM and

591   connects (directly or indirectly) to the INM through lumenal motifs. Evagination of the

592   INM and selective capture of INM cargo (Heh1) requires Atg39. INM derived vesicles

593   form after an INM scission event. Subsequent ONM scission would also be required to

27

594   liberate the NE bleb before its capture by the autophagosome through interactions with

595   the N-terminus of Atg39.

596

597   **Supplementary Table 1** – *S. cerevisiae* strains used in this study.

598   **Supplementary Table 2** – Plasmids used in this study.

599   **Supplementary Video 1 -** Related to Fig. 5a. "High" expression of Atg39-GFP results in

600   the formation of NE-blebs. Virtual slices from electron tomograph and 3D surface

601   rendering from tracing throughout the tomography with the ONM (blue) and INM (teal)

602   are shown. Scale bar is 100 nm.

603   **Supplementary Video 2** – Related to Extended Data Fig. 5c. Visualization of whole

604   volumes of cells expressing Atg39-GFP by FIB-SEM with 3D surface rendering of

605   membranes. The NE is shown in blue, lipid droplets in yellow, vacuole in grey, and

606   mitochondria in dark pink. Scale bar is 200 nm.

607   **Supplementary Video 3** – Related to Fig. 5d. "Low" expression of GFP-Atg39 leads to

608   formation of INM derived vesicles in the NE lumen. Virtual slices from an electron

609   tomogram and 3D surface rendering from tracing throughout the tomography with the

610   ONM in blue and INM in teal. Direct continuity between the INM and INM-derived

611   vesicle necks are visible. Scale bar is 50 nm.

612

613   **Materials and Methods**

614   ***Yeast strain construction and culturing conditions***

615   All strains used in this study are listed in Supplementary Table 1. Genomic integration of

616   sequences encoding fluorescent reporter genes, replacement of endogenous gene

28

617   promoters with *GAL1* promoter and gene deletions were generated using a PCR-based

618   homologous recombination approach using the pFA6a plasmid series[74,75] (as templates.

619

620   Yeast were cultured to mid-log phase in YP (1% Bacto-yeast extract (BD), 2% Bacto-

621   peptone (BD), 0.025% adenine hemi-sulfate (Sigma)) supplemented with 2% raffinose

622   (R; BD), 2% D-galactose (G; Alfa Aesar) 2% D-glucose (D; Sigma). To maintain

623   selection of plasmids, cells were cultured in Synthetic Complete (SC) medium (Sunrise

624   Science) that lacked the indicated amino acids. All experiments were performed at

625   30°C. Standard protocols for transformations, mating, sporulation, and dissection were

626   followed[76].

627

628   For induction of autophagy using rapamycin, rapamycin (in DMSO; Sigma-Aldrich) or an

629   equivalent volume of DMSO (carrier) was added to mid-log phase cultures to a final

630   concentration of 250 ng/mL. Samples collected at time points indicated in the figures

631   were prepared for imaging or immunoblotting as described below.

632

633   To induce autophagy by nitrogen starvation, mid-log phase cells were pelleted at ~375

634   *g*, washed twice with Synthetic Defined (SD) lacking nitrogen (SD-N) medium (0.17%

635   Difco Yeast Nitrogen Base without amino acids and ammonium sulfate (BD), and 2%

636   D), resuspended in SD-N, and returned to a shaking incubator at 30°C for the amount of

637   time indicated in figure legends.

638

29

639   To assess the localization of Atg39-GFP and GFP-Atg39 under the control of the *GAL1*

640   promoter, strains (SCCPL39, SCCPL40, SCCPL80, SCCPL82, SCCPL95, SCCPL131,

641   DTCPL911, PMCPL87, PMCPL112, PMCPL113, PMCPL114, PMCPL115, PMCPL28,

642   PMCPL29, PMCPL390, PMCPL392, PMCPL422, PMCPL424, and PMCPL471) were

643   grown in YPR to mid-log phase. Expression was induced by the addition of 2% G and

644   images were acquired at timepoints indicated in the figures.

645

646   To examine the subcellular localization of Atg39 in the split-GFP assay, strains

647   containing Atg39 split-GFP fusions under the control of the *GAL1* promoter (PMCPL21,

648   PMCPL34, PMCPL35, and PMCPL298) were transformed with plasmids containing

649   split-GFP-mCherry reporters for the nucleoplasm (pSJ1321), ONM/ER (pSJ1568) or

650   lumen (pSJ1602). Cells grown overnight in media lacking leucine were diluted into

651   YPAR. Expression of Atg39 fusions was induced by the addition of 2% G for 4 h or as

652   otherwise indicated in the figure legends.

653

654   To visualize Atg39-GFP within vacuoles upon induction of autophagy, strain SCCPL22

655   was treated with 2% G to induce Atg39 overexpression. In order to visualize the vacuole

656   membrane, 10 ml of culture was transferred to a foil wrapped tube and incubated with

657   FM 4-64 (1 µM in DMSO, Molecular Probes) for 30 min at 30°C. Cells were then

658   pelleted and resuspended in YPG. Atg39 expression was arrested after 4 h with the

659   addition of 2% D and samples were split. One culture was treated with rapamycin (final

660   concentration, 250 ng/ml, Sigma-Aldrich) to induce autophagy and the other with DMSO

661   (carrier).

30

662

Plasmid generation

664   All plasmids used in this study are listed in Supplementary Table 2.

665   To generate pPM1 (pFA6a-TRP1-GAL1-GFP[1-10]), the region encoding GFP[1-10] was

666   PCR amplified (KOD polymerase, EMD Millipore) from pSJ1256 using primers

667   containing the *Pac*I and *Asc*I (New England BioLabs) restriction sites. The ampliconwas

668   restriction digested with *Pac*I/*Asc*I (New England BioLabs), gel purified (Qiagen) and

669   ligated (T4 ligase, Invitrogen) into gel purified pFA6a-TRP1-GAL1 digested with

670   *Pac*I/*Asc*I (New England BioLabs). Successful subcloning was confirmed by

671   sequencing.

672

673   To generate pFA6a-3xHA-mCherry-natMX6, the 3xHA epitope sequence was PCR-

674   amplified with Q5 DNA polymerase using pFA6a-3xHA-his3MX6 (Longtine et al., 1998)

675   as a template. The PCR product was assembled into pFA6a-GFP-his3MX6 (Longtine et

676   al., 1998) digested with *Sal*I and *Pac*I (New England BioLabs) using the Gibson

677   Assembly Master Mix (New England BioLabs).

678

679   To generate pSC8, the 5' sequence of the native promoter and amino acids 1-443 of

680   *HEH1* were amplified via PCR (KOD polymerase, EMD Millipore) from genomic DNA

681   using primers encoding the restriction sites *BamH*I and *Hind*III. The subsequent

682   fragment was gel purified (Qiagen) and ligated using T4 ligase (Invitrogen) into pRS415-

683   GFP plasmid linearized with *BamH*1-HF and *Hind*III-HF. The C-terminal region of Heh1

684   was subsequently introduced by annealing oligos encoding *HEH1* amino acids 449-477

31

685   flanked with restriction sites *Hind*III and *Sal*I, followed by ligation using T7 ligase

686   (Invitrogen). The entire fragment GFP-heh1(1-443)-HindIII-heh1(449-477) was excised

687   by restriction digest with *BamH*I-HF and *Sal*I-HF. This purified fragment was ligated into

688   *pRS405* digested with *BamH*I-HF and *Sal*I-HF.

689

690   pSJ1602 (pRS315-NOP1pr-mCherry-SCS2TM-GFP11) was a gift from Sue Jaspersen

691   (Addgene plasmid # 86417; http://n2t.net/addgene:86417 ; RRID:Addgene_86417).

692   pSJ1321 (pRS315-NOP1pr-GFP11-mCherry-PUS1) was a gift from Sue Jaspersen

693   (Addgene plasmid # 86413; http://n2t.net/addgene:86413 ; RRID:Addgene_86413).

694   pSJ1568 (pRS315-NOP1pr-GFP11-mCherry-SCS2TM) was a gift from Sue Jaspersen

695   (Addgene plasmid # 86416; http://n2t.net/addgene:86416 ; RRID:Addgene_86416).

696   pSJ1256 (pFA6a-link-yGFP1-10-CaURA3MX) was a gift from Sue Jaspersen (Addgene

697   plasmid # 86419; http://n2t.net/addgene:86419 ; RRID:Addgene_86419).

698

699   ***Microscopy***

700   For all live-cell imaging, mid-log phase cells were gently pelleted and washed with SC

701   media contain 2% D and immediately imaged directly on cover glass. All images were

702   acquired on an Applied Precision DeltaVision microscope (GE Healthcare Life

703   Sciences) equipped with a 100x 1.4 NA oil immersion objective (Olympus), solid state

704   illumination, a CoolSnapHQ^2 CCD camera (Photometrics) or EMCCD (Photometrics).

705   The microscope stage was maintained at 30°C within an environmental chamber.

706

707   ***Image processing and analysis***

708 All presented micrographs were deconvolved using an iterative algorithm in SoftWoRx

709 (6.5.1; Applied Precision, GE Healthcare). Micrographs and immunoblots were analyzed

710 in FIJI/imageJ[77]. Unprocessed images were used for quantification of fluorescence

711 intensity.

712

713 Line profiles of fluorescence intensity were generated using the Plot Profile function in

714 FIJI/ImageJ[77]. The minimum and maximum measured values from individual fluorescent

715 channels were normalized to 0 and 1, respectively.

716

717 To quantify changes in Atg39-GFP fluorescence intensity at the NE, the integrated

718 density of a region of interest at the nuclear periphery comprising 4-pixels was

719 measured and normalized to mean background fluorescence.

720

721 ***Secondary Structure prediction***

722 The secondary structure of Atg39's lumenal domain was predicted by threading the

723 amino acid sequence of Atg39 through Jpred4[78].

724

725 ***Statistical methods***

726 Graphs were generated using Prism (GraphPad 9.0). Statistical significance tests were

727 used as indicated in figure legends. Significance values were calculated within Prism

728 (GraphPad 9.0) and p-values are indicated on the graph or in figure legends as: *ns*, $p >$

729 0.05; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Error bars are described in

730 figure legends.

731

Correlative light and electron microscopy

733   Correlative microscopy of resin-embedded cells was performed as previously

734   described[79]. Expression of Atg39 fusion proteins from cells (DTCPL688 (Atg39-GFP)

735   and PMCPL87 (GFP-Atg39)) cultured in YPAR, was induced by the addition of 2% G for

736   3 h. Cells were then collected by centrifugation for 2 min at 350 g. Yeast slurry was

737   transferred to the 200 μm recess of an aluminum platelet (Engineering Office M.

738   Wohlwend) and placed in an HPM100 (Leica Microsystems) for high pressure freezing.

739   Samples were freeze-substituted in 0.1% uranyl acetate in acetone and embedded in

740   Lowicryl HM20 (Polysciences) using the automated temperature control of an EM-AFS2

741   (Leica Microsystems) with manual agitation and solution exchange following the

742   published protocol[80]. Resin was polymerized under UV light, and the resin-embedded

743   cells were cut into 250 nm thick sections using an ultramicrotome (Leica Artos 3D)

744   equipped with a diamond knife (Diatome) and collected on 200 mesh copper grids with

745   carbon support (Ted Pella, Prod. # 01840).

746

747   Fluorescence and brightfield micrographs of resin-embedded sections were acquired as

748   described above. Several Z-sections were acquired every 200 or 250 nm at each grid

749   square of interest, and in-focus planes were selected for CLEM alignment and

750   presentation in figures.

751

752   Grids selected for tomography were post-stained with uranyl acetate and lead citrate. 15

753   nm protein A-coated gold beads (CMC UMC Utrecht) were adhered to the top and

34

754   bottom surfaces of grids and used as fiducial markers for the alignment and

755   reconstruction of the tilt series. Single (Fig. 5a, Extended Data Fig. 4b-f) or double (Fig.

756   5c) axis tilt series were collected on a FEI TF20 electron microscope operating at 200

757   kV using a high-tilt tomography holder (Fichione Instruments; Model 2020) from

758   approximately -65° to +65° with acquisition at 1° intervals. Images were acquired using

759   SerialEM software[81], at a 2 x 2 binned pixel size of 1.242 nm using a 4k x 4k Eagle CCD

760   (FEI) camera with a 150 μm C2 aperture and a 100 μm objective aperture. Subsequent

761   reconstruction and segmentation were completed in IMOD[82] in an automated fashion[83].

762   For all virtual slices presented, a Gaussian filter in IMOD was applied to reduce noise.

763   Alignment of fluorescence and electron microscopy data was completed using the ec-

764   CLEM Plugin[84] in the ICY imaging suite[85]. Low magnification EM was related to

765   fluorescence and brightfield micrographs by selecting ~6-8 points that corresponded to

766   features visible in both images.

767

768   ***FIB-SEM***

769   For visualization of entire cellular volumes using FIB-SEM, unfixed yeast slurries loaded

770   into 200 μm aluminum hats were frozen using a Leica HMP100 at 2000 psi. The frozen

771   samples were then freeze substituted using a Leica Freeze AFS unit starting at -95°C

772   using 0.1% uranyl acetate in acetone for 50 h to -600°C, then rinsed in 100% acetone

773   and infiltrated over 24 h at -600°C with Lowicryl HM20 resin (Electron Microscopy

774   Science). Samples were placed in gelatin capsules and UV hardened at -450°C for 48

775   h. The blocks were cured for a further few days before the resin block was trimmed to

776   rough area of interest and the surface cleanly cut using a Leica UltraCut UC7. The

777 entire block was carefully removed with a fine blade, and mounted on an aluminum stub

778 using conductive carbon adhesive and silver paint (Electron Microscopy Sciences,

779 Hatfield, PA, U.S.A,), then sputtered coated with approximately 20 nm Pt/Pd (80/20)

780 using Cressington HR equipment (Ted Pella,Inc. Redding CA) to reduce charging

781 effects.

782

783 A dual beam FIB-SEM (Zeiss CrossBeam 550) using a Gallium ion source was used to

784 mill and SE2 secondary electron detector was used to image the samples. SmartSEM

785 (Zeisss, Jenna Germany) was used to set up initial parameters and to find the regions

786 of interest (ROI) by SEM images at 10 kV, 50 μm width and 30 μm height. The actual

787 depth was 30 μm with 7 nm per pixel and 7 nm per slice. A Platinum- protective layer

788 was deposited at the ROI with the FIB (30 kV, 3 nA) to protect the structure and reduce

789 charging. Milling and highlight was done at 30 kV and 50 pA, with a carbon deposit 30

790 kV 3 nA. A course trench milled 30 kv 30 nA followed by fine milling at 30 kV 3 nA and

791 for final acquisition a cuboid the area of interest was milled at 30 kV and 300 pA. After

792 milling each slice an image was taken by detecting backscattered electrons of a primary

793 electron beam (acceleration voltage of 1.5 kV, imaging current of 2nA, and aperture

794 diameter of 100 μm) with a pixel dwell time of 3 μs. Atlas5 (Zeiss) was used for

795 preliminary SEM stack alignment and FIB/SEM image stacks were saved as TIFF and

796 MRC files. The images were imported into Dragonfly software (ORS, Montreal Canada)

797 for further alignment and segmentation. The total volume imaged was 19.25 μm x 35.8

798 μm x 8.9 μm. Segmentation of structures was done in IMOD[82] , approximately every 35

799 nm.

36

800

Immunoblotting

802   For immunoblotting, cells were harvested as previously described[86]. Briefly, ~1.5×10^8

803   cells were treated with 10% TCA for 1 h on ice and centrifuged at 15,000 *g* for 10 min at

804   4°C. The pellet was washed with ice-cold acetone, homogenized by sonication

805   (Bioruptor UCD-200) and pelleted by centrifugation. After two cycles of washing and

806   sonication, the pellet was vacuum-dried for 15 min. The dried cell pellet was then

807   mechanically disrupted with 100 μl glass beads (Sigma) and 100 μl urea cracking buffer

808   (50 mM Tris-HCl pH 7.5, 8 M Urea, 2% SDS, and 1 mM EDTA), followed by addition of

809   100 μl protein sample buffer (Tris-HCl pH 6.8, 7 M urea, 10% SDS, 24% glycerol,

810   bromophenol blue, and 10% β-mercaptoethanol).

811

812   To assess autophagy through GFP-fallout experiments, ~1.5×10^8 cells were harvested

813   and suspended in 0.2 M NaOH containing 0.1 M DTT, incubated on ice for 10 min,

814   followed by the addition of 10% TCA with incubation on ice for 15 min. After

815   centrifugation at 15,000 g for 5 min at 4°C, the pellet was washed with acetone, vacuum

816   dried, and then resuspended in SDS sample buffer (0.1M Tris-HCl pH 7.5, 2% SDS,

817   10% glycerol, 20 mM DTT) for 10 min at 65°C to dissolve the pellet, followed by 95°C

818   for 3 min.

819

820   Proteins from whole cell extracts were resolved on 4-20% SDS-polyacrylamide gels

821   (BioRad, #4561096), followed by transfer of the proteins to 0.2 μm nitrocellulose

822   membranes (Bio-Rad). The membranes were blocked in 5% non-fat milk in TBST for 1

37

823   h and immunoblotted with antibodies against GFP (anti-GFP.1, 4°C overnight, Takara

824   Bio Clontech, 632381 or anti-GFP.2, 1 h room temperature, anti-GFP gift from M. Rout,

825   as indicated in figure legends). Blots were incubated with HRP-conjugated secondary

826   antibodies (1 h room temperature; Sigma) and visualized by ECL (Thermo Fisher

827   Scientific) using a VersaDoc Imaging System (Bio-Rad). Relative protein loading was

828   visualized using Ponceau S Solution (Sigma).

829

830   ***Quantification of autophagic turnover***

831   To calculate the relative percent degradation of GFP-fusion proteins, ROIs were drawn

832   around immunoblot bands corresponding to cleaved GFP′ bands and GFP fusion

833   proteins in Fiji/ImageJ[77] and the total fluorescence intensity was measured. Measured

834   values for GFP' were divided by the sum of GFP' and related GFP-fused full-length

835   protein intensities.

836

837

38

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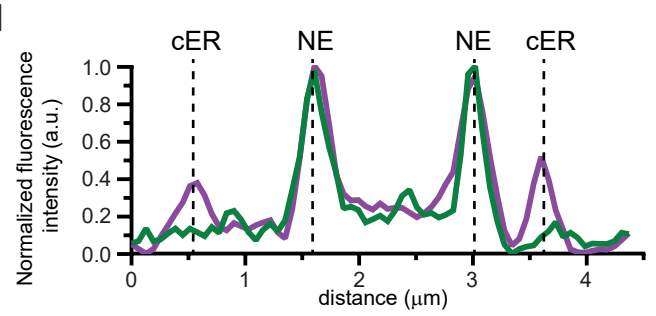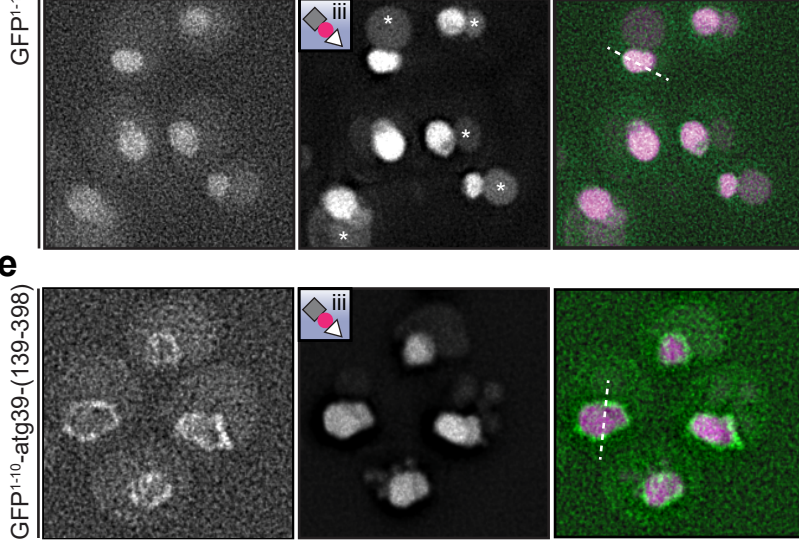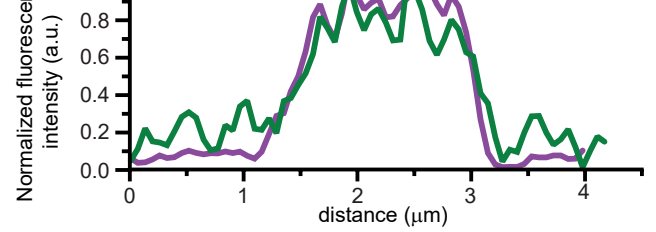
1050

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

a

i

ii ONM

iii INM

iv

v

vi 60°

vii

viii

ix

x

b

INM derived vesicle diameter (nm)

n=29 n=23 n=982

"high" "low" "high"
FIB-SEM

d

INM derived compartments

NVJ

PMN

Nucleus mitochondria association
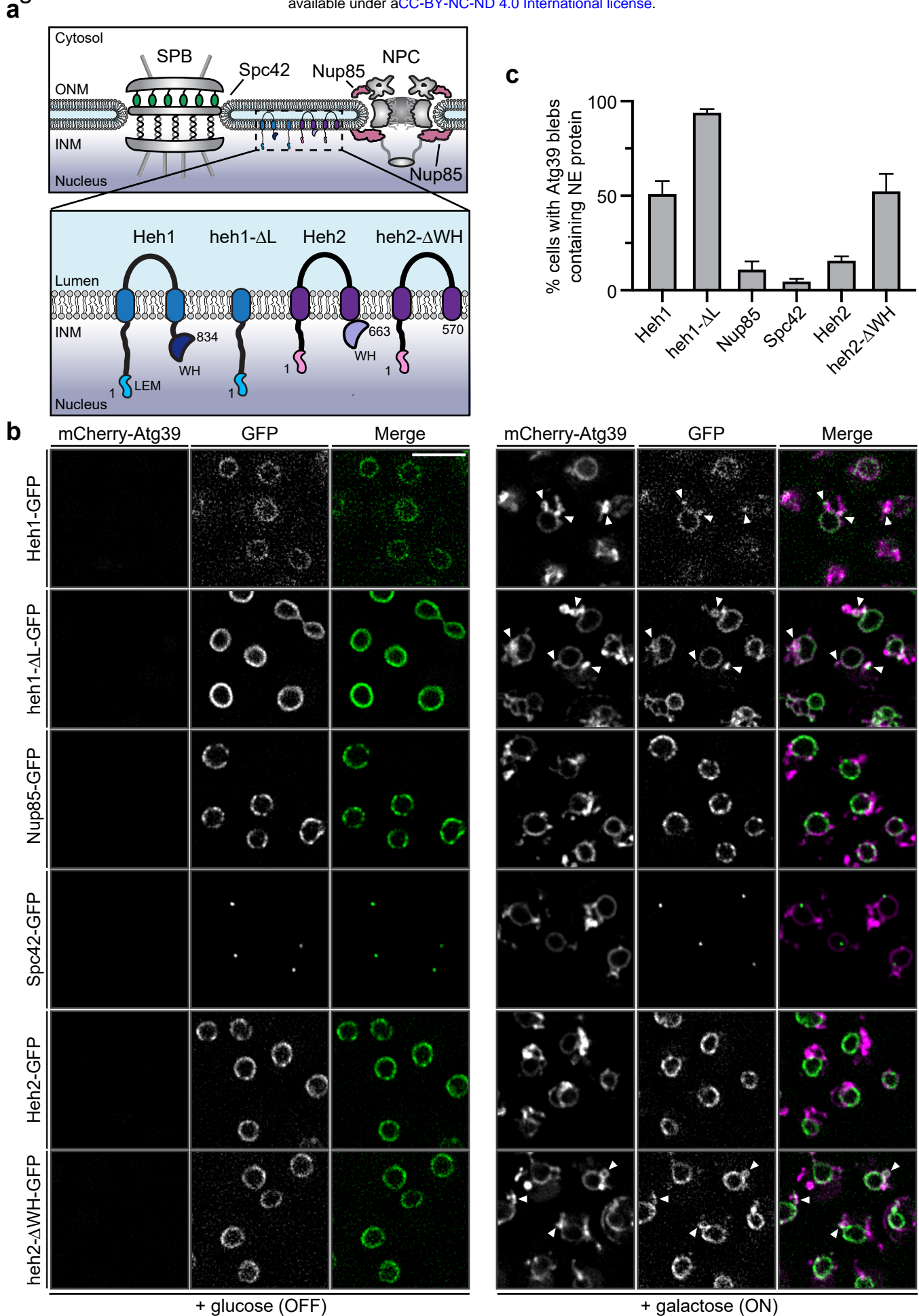
NE extensions with NPCs

No visible structure

observed

c

i ii iii iv ONM INM

Extended Data Fig. 1

a

	0 (h)	1	2	3	4

GFP

mCherry

Merge

+ galactose

GFP$^{1-10}$-Atg39

b

GFP

mCherry

Merge

+ galactose

GFP$^{1-10}$-atg39-(139-398)

c

GFP

mCherry

Merge

+ galactose

GFP$^{1-10}$-atg39-ΔL

Extended Data Fig. 2

a

GFP-Atg39
GFP-atg39-(1-333)
GFP-atg39-(1-312)
GFP-atg39-(1-275)
GFP-atg39-ΔL
GFP-atg39-(70-398)
GFP-atg39-(120-398)
GFP-atg39-(139-398)

(kD)

- 78
- 55
- 44
- 34

WB: α-GFP

Ponceau

b

WT   atg8Δ   atg11Δ   atg8Δatg11Δ

(kD)

GFP-Atg39 -

- 78
- 55
- 44
- 34

WB: a-GFP

Ponceau

c

WT   pep4Δ

rapamycin   +   -   +   -   (kD)

Atg39-GFP—

- 75
- 50
- 37

GFP'—

- 25

WB: a-GFP

Ponceau

d

ATG39-GFP   atg39-ΔL-GFP

rapamycin   -   +   -   +   (kD)

Atg39-GFP—
atg39ΔL-GFP—

- 75
- 50
- 37

GFP'—

- 25

WB: a-GFP

Ponceau

e

WT   atg8Δ   atg11Δ   atg8Δ atg11Δ

rapamycin   -   +   -   +   -   +   -   +   (kD)
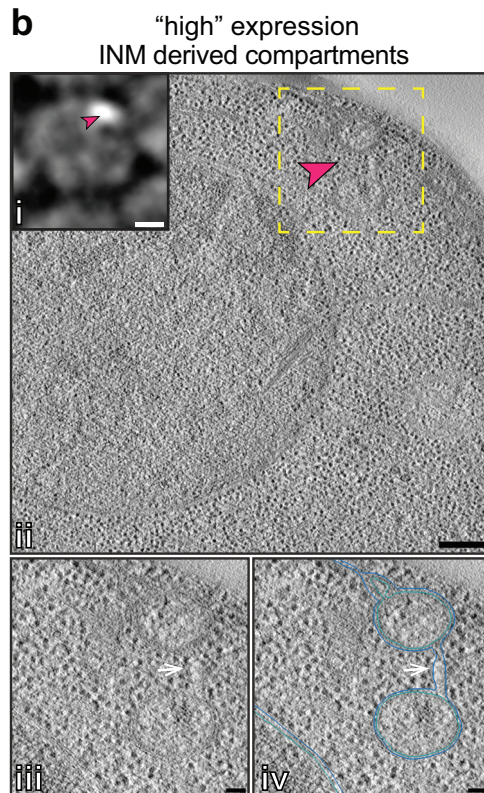
Atg39-GFP—

- 75
- 50
- 37

GFP'—

- 25

WB: a-GFP

Ponceau

Extended Data Fig. 3

Extended Data Fig. 4

a

b "high" expression
INM derived compartments

c "low" expression
nucleus-mitochondria association

d "low" expression
PMN + NVJ

e "low" expression
NE extensions containing NPCs

f "low" expression
INM derived compartments

Extended Data Fig. 5

a

8.9 mm

19.25 mm

38.5 mm

b

NE blebs — 47/52

No NE blebs — 5/52

Lipid droplets adjacent to blebs — 44/47

cells

c

Mitochondria   NE
Vacuole   LD

i   ii   iii   iv

v   vi   vii   viii

d

Nucleophagy

autophagosome engulfment

fusion with vacuole

Cytosol

Atg39 driven NE remodeling

Atg8 Atg11

NPC

Atg39

ONM scission

ONM

Lumen

INM

Heh2

Heh1

Heh1 sequestered into blebs
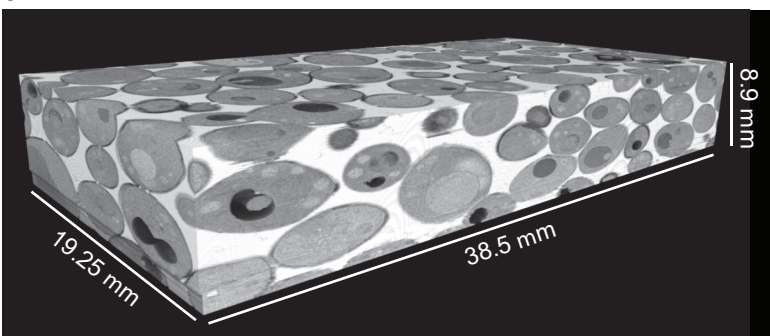
INM scission

INM derived vesicle generation

Nucleus

Supplementary Table 1: *S. cerevisiae* strains

Name	Genotype	Origin	Generation
W303a	*MATa, ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	EUROSCARF	
W303α	*MATα, ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	EUROSCARF	
FIGURE 1			
PMCPL21	W303, *natMX6::GAL1-ATG39-GFP$^{1-10}$::CaURA3MX*	This study	Transformation of DTCPL1589 with PCR product
PMCPL34	W303, *kanMX6::GAL1-GFP$^{1-10}$-ATG39*	This study	Transformation of W303 with PCR product
PMCPL35	W303, *kanMX6::GAL1-GFP$^{1-10}$-atg39-(139-398)*	This study	Transformation of W303 with PCR product
PMCPL298	W303, *kanMX6::GAL1-GFP$^{1-10}$-atg39-(1-169)::hphMX6*	This study	Transformation of PMCPL34 with PCR product
FIGURE 2			
PMCPL87	W303, *kanMX6::GAL1-GFP-ATG39*	This study	Transformation of W303 with PCR product
PMCPL112	W303, *kanMX6::GAL1-GFP-atg39-(1-169)::hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL113	W303, *kanMX6::GAL1-GFP-atg39-(1-275)::hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL114	W303, *kanMX6::GAL1-GFP-atg39-(1-312)::hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL115	W303, *kanMX6::GAL1-GFP-atg39-(1-333)::hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL29	W303, *kanMX6::GAL1-GFP-atg39-(139-398)*	This study	Transformation of W303 with PCR product
PMCPL390	W303, *TRP1::GAL1-GFP-atg39-(70-398)*	This study	Transformation of W303 with PCR product
PMCPL392	W303, *his3MX6::GAL1-GFP-atg39-(120-398)*	This study	Transformation of W303 with PCR product
PMCPL422	W303, *kanMX6::GAL1-GFP-Atg39 atg8Δ::natMX6*	This study	Progeny from cross between PMCPL86 and PMCPL16
PMCPL424	W303, *kanMX6::GAL1-GFP-Atg39 atg11Δ::hphMX6*	This study	Progeny from cross between BWCPL1377 and PMCPL87
PMCPL471	W303, *kanMX6::GAL1-GFP-Atg39 atg8Δ::natMX6 atg11Δ::hphMX6*	This study	Progeny from cross between PMCPL422 and BWCPL1378
FIGURE 3			
SCCPL113	W303, *ADE2* HEH1-GFP::*TRP1* ATG39-3xHA::*his3MX6*	This study	Transformation of DTCPL1221 with PCR product
SCCPL115	W303, *ADE2* HEH1-GFP::*TRP1* atg39-(1-169)::*his3MX6*	This study	Transformation of DTCPL1221 with PCR product
PMCPL410	W303, HEH1-GFP::*TRP1* atg39-(1-333)::*his3MX6*	This study	Progeny from cross between DTCPL999 and PMCPL195
PMCPL411	W303, HEH1-GFP::*TRP1* atg39-(1-312)::*his3MX6*	This study	Progeny from cross between DTCPL999 and PMCPL196
PMCPL412	W303, HEH1-GFP::*TRP1* atg39-(1-275)::*his3MX6*	This study	Progeny from cross between DTCPL999 and PMCPL197
PMCPL438	W303, HEH1-GFP::*TRP1* atg39Δ::*hphMX6*	This study	Progeny from cross between DTCPL1215 and PMCPL304
SCCPL114	W303, *ADE2* HEH2-GFP::*TRP1* ATG39-3xHA::*his3MX6*	This study	Transformation of DTCPL1219 with PCR product
SCCPL116	W303, *ADE2* HEH2-GFP::*TRP1* atg39-(1-169)-3xHA::*his3MX6*	This study	Transformation of DTCPL1219 with PCR product
PMCPL374	W303, *ADE2* HEH2-GFP::*TRP1* atg39Δ::*natMX6*	This study	Progeny from cross between DTCPL1219 and PMCPL359
PMCPL377	W303, *ADE2* NUP82-GFP::*TRP1* ATG39-3xHA::*his3MX6*	This study	Transformation of PMCPL371 with PCR product
PMCPL378	W303, *ADE2* NUP2-GFP::*TRP1* atg39-(1-169)-3xHA::*his3MX6*	This study	Transformation of PMCPL371 with PCR product
PMCPL372	W303, *ADE2* NUP82-GFP::*TRP1* atg39Δ::*natMX6*	This study	Progeny from cross between SBCPL74 and PMCPL359
SCCPL22	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1* pep4Δ::*hphMX6*	This study	Progeny from cross between DTCPL688 and BWCPL1374
FIGURE 4			
SCCPL39	W303, *ADE2 TRP1::GAL1*-3xHA-mCherry-ATG39 HEH1-GFP::*natMX6*	This study	Progeny from cross between SCCPL33 and CPL781
SCCPL95	W303, *ADE2 kanMX6::GAL1*-mCherry-ATG39 *heh1Δ::natMX6 pRS405-* GFP-heh1-(1-443)-*Hind*III-heh1-(449-477)::*LEU2*	This study	Transformation of linearized pSC8 into SCCPL44
SCCPL131	W303, *ADE2 kanMX6::GAL1*-mCherry-ATG39 NUP85-GFP::*his3MX6*	This study	Progeny from cross between SCCPL35 and SBCPL116
DTCPL911	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1* SPC42-mCherry::*natMX6*	This study	Progeny from cross between DTCPL689 and MCCPL25
SCCPL40	W303, *ADE2 TRP1::GAL1*-mCherry-3xHA-ATG39 HEH2-GFP::*natMX6*	This study	Progeny from cross between SCCPL33 and CPL782
SCCPL80	W303, *ADE2 kanMX6::GAL1*-mCherry-3xHA-ATG39 heh2-(1-570)-GFP::*his3MX6*	This study	Progeny from cross between SCCPL35 and SBCPL40
FIGURE 5			
PMCPL87	W303, *kanMX6::GAL1-GFP-ATG39*	This study	Transformation of W303 with PCR product
DTCPL688	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1*	This study	Transformation of DTMB05 with PCR product
FIGURE S1			
PMCPL34	W303, *kanMX6::GAL1*-GFP$^{1-10}$-ATG39	This study	Transformation of W303 with PCR product
PMCPL35	W303, *kanMX6::GAL1*-GFP$^{1-10}$-atg39-(139-398)	This study	Transformation of W303 with PCR product

PMCPL298	W303, *kanMX6::GAL1*-GFP[1-10]-atg39-(1-169)::*hphMX6*	This study	Transformation of PMCPL34 with PCR product
PMCPL29	W303, *kanMX6::GAL1*-GFP-atg39-(139-398)	This study	Transformation of W303 with PCR product
PMCPL28	W303, *kanMX6::GAL1*-GFP-atg39-(144-398)	This study	Transformation of W303 with PCR product
FIGURE S2			
PMCPL87	W303, *kanMX6::GAL1*-GFP-ATG39	This study	Transformation of W303 with PCR product
PMCPL115	W303, *kanMX6::GAL1*-GFP-atg39-(1-333)::*hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL114	W303, *kanMX6::GAL1*-GFP-atg39-(1-312)::*hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL113	W303, *kanMX6::GAL1*-GFP-atg39-(1-275)::*hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL112	W303, *kanMX6::GAL1*-GFP-atg39-(1-169)::*hphMX6*	This study	Transformation of PMCPL87 with PCR product
PMCPL390	W303, *TRP1::GAL1*-GFP-atg39-(70-398)	This study	Transformation of W303 with PCR product
PMCPL392	W303, *his3MX6::GAL1*-GFP-atg39-(120-398)	This study	Transformation of W303 with PCR product
PMCPL29	W303, *kanMX6::GAL1*-GFP-atg39-(139-398)	This study	Transformation of W303 with PCR product
DTCPL688	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1*	This study	Transformation of DTMB05 with PCR product
SCCPL22	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1 pep4Δ ::hphMX6*	This study	Progeny from cross between DTCPL688 and BWCPL1374
SCCPL01	W303, *hphMX6::GAL1*-ATG39-GFP::*TRP1*	This study	Transformation of DTCPL688 with PCR product
SCCPL03	W303, *hphMX6::GAL1*-atg39-(1-169)-GFP::*TRP1*	This study	Transformation of DTCPL980 with PCR product
PMCPL24	W303, *hphMX6::GAL1*-ATG39-GFP::*TRP1 atg8::natMX6*	This study	Progeny from cross between SCCPL01 and PMCPL15
PMCPL22	W303, *hphMX6::GAL1*-ATG39-GFP::*TRP1 atg11::hphMX6*	This study	Progeny from cross between SCCPL01 and PMCPL18
PMCPL23	W303, *hphMX6::GAL1*-ATG39-GFP::*TRP1 atg8::natMX6 atg11::hphMX6*	This study	Progeny from cross between SCCPL01 and PMCPL18
PMCPL422	W303, *kanMX6::GAL1*-GFP-Atg39 *atg8Δ::natMX6*	This study	Progeny from cross between PMCPL86 and PMCPL16
PMCPL424	W303, *kanMX6::GAL1*-GFP-Atg39 *atg11Δ::hphMX6*	This study	Progeny from cross between BWCPL1377 and PMCPL87
PMCPL471	W303, *kanMX6::GAL1*-GFP-Atg39 *atg8Δ::natMX6 atg11Δ::hphMX6*	This study	Progeny from cross between PMCPL422 and BWCPL1378
FIGURE S3			
PMCPL25	W303, *his3MX6::GAL1*-ATG39-mCherry::*kanMX6* heh1-(1-479)-GFP::*TRP1*	This study	Progeny from cross between DTCPL942 and PMCPL04
FIGURE S4			
SCCPL35	W303, *kanMX6::GAL1*-mCherry-ATG39	This study	Transformation of W303 with PCR product
PMCPL87	W303, *kanMX6::GAL1*-GFP-ATG39	This study	Transformation of W303 with PCR product
DTCPL688	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1*	This study	Transformation of DTMB05 with PCR product
FIGURE S5			
DTCPL688	W303, *his3MX6::GAL1*-ATG39-GFP::*TRP1*	This study	Transformation of DTMB05 with PCR product

Supplementary Table 2: Plasmids used in this study.

Name	Description	Origin
pFA6a-his3MX6-GAL1	Template for PCR based chromosomal integration of GAL1 promoter	[74]
pFA6a-kanMX6-GAL1	Template for PCR based chromosomal integration of GAL1 promoter	[74]
pFA6a-TRP1-GAL1	Template for PCR based chromosomal integration of GAL1 promoter	[74]
pFA6a-kanMX6-GAL1-mCherry	Template for PCR based chromosomal integration of GAL1 promoter and mCherry ORF	This study
pFA6a-TRP1-GAL1-mCherry-3xHA	Template for PCR based chromosomal integration of GAL1 promoter and mCherry-3xHA ORF	This study
pFa6a-GFP-hisMX6	Template for PCR based chromosomal integration of GFP ORF	[74]
pFa6a-GFP-TRP1	Template for PCR based chromosomal integration of GFP ORF	[74]
pFa6a-GFP-natMX6	Template for PCR based chromosomal integration of GFP ORF	[75]
pFA6a-mCherry-natMX6	Template for PCR based chromosomal integration of mCherry ORF	EUROSCARF
pFA6a-his3MX6	Template for PCR based chromosomal integration of his3MX6 cassette	[74]
pFA6a-hphMX6	Template for PCR based chromosomal integration of hphMX6 cassette	[74]
pFA6a-natMX6	Template for PCR based chromosomal integration of natMX6 cassette	[75]
pFA6a-3xHA-mCherry-natMX6	Template for PCR based chromosomal integration of 3xHA-mCherry ORF	This study
pFA6a-3xHA-hisMX6	Template for PCR based chromosomal integration of 3xHA	[74]
pRS405	YIP-*LEU2*	ATCC
pRS415	*CEN6, LEU2*	ATCC
pSC8	pRS405-GFP-heh1-(1-443)-HindIII-heh1-(449-477)::LEU2	This study
pPM1	pFA6a-kanMX6-GAL1-GFP$^{1-10}$	This study
pSJ1602	pRS315-NOP1pr-mCherry-SCS2TM-GFP[11]	Gift from Sue Jaspersen, Addgene plasmid # 86417 ; http://n2t.net/addgene:86417 ; RRID:Addgene_86417
pSJ1321	pRS315-NOP1pr-GFP[11]-mCherry-PUS1	Gift from Sue Jaspersen, Addgene plasmid # 86413 ; http://n2t.net/addgene:86413 ; RRID:Addgene_86413
pSJ1568	pRS315-NOP1pr-GFP[11]-mCherry-SCS2TM	Gift from Sue Jaspersen, Addgene plasmid # 86416 ; http://n2t.net/addgene:86416 ; RRID:Addgene_86416
pSJ1256	pFA6a-link-yGFP$^{1-10}$-CaURA3MX	Gift from Sue Jaspersen, Addgene plasmid # 86419 ; http://n2t.net/addgene:86419 ; RRID:Addgene_86419