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1 **LGG-1/GABARAP lipidation is not required for autophagy**
2 **and development in *Caenorhabditis elegans***

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20
21
22 **Abstract**

23 The ubiquitin-like proteins Atg8/LC3/GABARAP are required for multiple steps of autophagy,
24 such as initiation, cargo recognition and engulfment, vesicle closure and degradation. Most
25 of LC3/GABARAP functions are considered dependent on their post-translational
26 modifications and their association with the autophagosome membrane through a
27 conjugation to a lipid, the phosphatidyl-ethanolamine. Contrarily to mammals, *C. elegans*
28 possesses single homologs of LC3 and GABARAP families, named LGG-2 and LGG-1. Using
29 site-directed mutagenesis, we inhibited the conjugation of LGG-1 to the autophagosome
30 membrane and generated mutants that express only cytosolic forms, either the precursor or
31 the cleaved protein. LGG-1 is an essential gene for autophagy and development in *C.*
32 *elegans*, but we discovered that its functions could be fully achieved independently of its
33 localization to the membrane. This study reveals an essential role for the cleaved form of
34 LGG-1 in autophagy but also in an autophagy-independent embryonic function. Our data
35 question the use of lipidated GABARAP/LC3 as the main marker of autophagic flux and
36 highlight the high plasticity of autophagy.

37
38
39 **Keywords:** Atg8, LC3, GABARAP, lipidation, ubiquitin-like, di-glycine, *C. elegans*,
40 development, CRISPR-Cas9, mitophagy, aggrephagy

41

42 **Introduction**

43

44 Macroautophagy is a highly dynamic vesicular degradation system that sequesters
45 intracellular components in double membrane autophagosomes and delivers them to the
46 lysosome (Klionsky et al., 2021). Upon induction, the successive recruitment of protein
47 complexes triggers the phosphorylation of lipids, the transfer of lipids from various
48 reservoirs, the recognition of cargoes, the tethering and the fusion (Galluzzi *et al*, 2017;
49 Nakatogawa, 2020). One of the key players is the ubiquitin-like protein Atg8, which in yeast
50 is required for several steps during autophagy, such as initiation, cargo recognition and
51 engulfment, and vesicle closure (Kirisako et al., 2000; Knorr et al., 2014; Kraft et al., 2012;
52 Nakatogawa et al., 2007; Xie et al., 2008). There are seven isoforms of Atg8 homologs in
53 humans defining two families, the MAP-LC3 (abbreviated as LC3A-a, LC3A-b, LC3B, LC3C) and
54 the GABARAP (GABARAP, GABARAPL1, GABARAPL2) (Shpilka et al., 2011). LC3/GABARAP
55 proteins could have both similar and very specific functions during the autophagic flux
56 (Alemu et al., 2012; Grunwald et al., 2020; Joachim et al., 2015; Lystad et al., 2014; Pankiv et
57 al., 2007; Weidberg et al., 2010). LC3/GABARAP proteins can bind numerous proteins
58 through specific motifs (LIR, LC3 interacting Region) and their interactomes are only partially
59 overlapping (Behrends et al., 2010).

60 The pleiotropy of Atg8/LC3/GABARAP proteins in multiple cellular processes (Galluzzi and
61 Green, 2019; Schaaf et al., 2016) entangles the study of their specific functions in human
62 (Nguyen et al., 2016). Moreover, a series of post-translational modifications, similar to the
63 ubiquitin conjugation, is involved in the membrane targeting of Atg8/LC3/GABARAP
64 proteins. These proteins are initially synthesized as a precursor (P), then cleaved at their C-
65 terminus after the invariant Glycine 116 (form I), and eventually conjugated to
66 phosphatidylethanolamine (form II) at the membrane of autophagosomes (Figure 1A)
67 (Kabeya et al., 2004, 2000; Scherz-Shouval et al., 2003). Structural analyses have shown that
68 LC3/ GABARAP can adopt an open or close configuration (Coyle et al., 2002). In addition,
69 several other post-translational modifications have been reported, like phosphorylation
70 (Cherra et al., 2010; Herhaus et al., 2020; Wilkinson et al., 2015), deacetylation (Huang et al.,
71 2015) ubiquitination (Joachim et al., 2017) and oligomerization (Chen et al., 2007; Coyle et
72 al., 2002), whose effects on LC3/GABARAP function and localization are largely unknown.
73 The subcellular localization of Atg8/LC3/GABARAP proteins is either diffuse in the cytosol
74 and nucleus, or associated to the membrane of various compartments or the cytoskeleton
75 (Schaaf et al., 2016).

76 Due to such a versatile and pleiotropic repertoire, it is of particular interest to address the
77 level of redundancy and specificity, including tissue specificity, of the various LC3/GABARAP
78 members, and the possible functions of the forms P and I. In the nematode *Caenorhabditis*
79 *elegans*, the presence of single homologs of LC3 and GABARAP, called respectively LGG-2
80 and LGG-1, represents an ideal situation to characterize their multiple functions (Chen et al.,
81 2017; Leboutet et al., 2020).

82 The structure of LGG-1/GABARAP and LGG-2/LC3 is highly conserved (Wu et al., 2015) and
83 both proteins are involved in autophagy processes during development, longevity and stress
84 (Alberti et al., 2010; Chang et al., 2017; Chen et al., 2021; Meléndez et al., 2003;
85 Samokhvalov et al., 2008). In particular, the elimination of paternal mitochondria upon
86 fertilization, also called allophagy (Al Rawi et al., 2011; Sato and Sato, 2011), has become a
87 paradigm for dissecting the molecular mechanisms of selective autophagy (Djeddi et al.,
88 2015; Zhou et al., 2016). Genetic analyses indicated that LGG-1 and LGG-2 do not have
89 similar functions in autophagy (Alberti et al., 2010; Jenzer et al., 2019; Manil-Ségalen et al.,
90 2014; Wu et al., 2015). During allophagy, LGG-1 is important for the recognition of
91 ubiquitinated cargoes through interaction with the specific receptor ALLO-1 (Sato et al.,
92 2018) and the formation of autophagosomes, whereas LGG-2 is involved in their maturation
93 into autolysosomes and trafficking (Djeddi et al., 2015; Manil-Ségalen et al., 2014). LGG-1
94 and LGG-2 are also differentially involved during physiological autophagy in embryo, with
95 temporal-specific and cargo-specific functions (Wu et al., 2015). Based on the presence of
96 LGG-1 and LGG-2, three populations of autophagosomes have been described in *C. elegans*
97 embryo: the major part are LGG-1 only, but LGG-2 only and double positives
98 autophagosomes are also present (Manil-Ségalen et al., 2014; Wu et al., 2015). Moreover,
99 LGG-1 is essential for embryonic and larval development, while LGG-2 is dispensable.
100 Using CRISPR-Cas9 editing, we investigated the functions of the non-lipidated cytosolic forms
101 of LGG-1/GABARAP for bulk autophagy, mitophagy and autophagy, but also during
102 starvation and longevity as well as apoptotic cell engulfment and morphogenesis. Here, we
103 demonstrate that the non-lipidated form (LGG-1 I), but not the precursor form (LGG-1 P), is
104 sufficient to maintain LGG-1 functions during development and aging. The cleavage of LGG-1
105 into form I is essential for autophagosome initiation and biogenesis while form II is involved
106 in cargo recognition and autophagosome degradation.

107
108

109 **Results**

110

111 **The G116G117 di-Glycine motif is a substrate for cleavage of LGG-1 precursor**

112 The LGG-1 protein is highly conserved from residue 1 to residue 116, sharing 92% and 74%
113 similarity with the human GABARAP and the yeast Atg8, respectively (Manil-Ségalen et al.,
114 2014). However, the GEVEKKE C-terminus of LGG-1 is unusual by its length and the presence
115 of a non-conserved glycine residue in position 117 (Figure 1B, Figure 1- figure supplement
116 1). As consistent with other *Caenorhabditis* species as well as several nematodes and
117 arthropods, the presence of a C-terminal di-glycine is reminiscent of other ubiquitin-like
118 proteins such as SUMO and Nedd8 (Cappadocia and Lima, 2018; Jentsch and Pyrowolakis,
119 2000). These specificities raise the possibility that the C-terminus could confer particular
120 functions to the precursor and the cleaved form.

121 To analyze the functions of LGG-1 P and LGG-1 I, a CRISPR-Cas9 approach was used to
122 substitute the conserved glycine 116 by an alanine, and to generate three specific *lgg-1*

123 mutants with various C-terminus (Figure 1B). In theory, both *lgg-1(G116A)* and *lgg-*
124 *1(G116AG117A)* mutants were expected to accumulate a P form due to the blockage of its
125 cleavage by ATG-4 (Wu et al., 2012). Alternatively, the *lgg-1(G116AG117*)* mutant should
126 produce a form I. Five supplementary *lgg-1* frameshift mutants were isolated during the
127 CRISPR experiments, resulting in deletion/insertion at the C-terminus (Figure 1B and Figure
128 1- figure supplement 1). Among them, *lgg-1(ΔC100-123)* and *lgg-1(ΔC112-123)* have been
129 used in the present study. The allele *lgg-1(tm3489)*, which deletes 48% of the open reading
130 frame, was used as a negative control (Manil-Ségalen et al., 2014) as it is considered as a null
131 mutant, and thereafter noted *lgg-1(Δ)*.

132 To assess whether *lgg-1(G116A)*, *lgg-1(G116AG117A)* and *lgg-1(G116AG117*)* alleles code
133 for a precursor and form I, respectively, we performed a western blot analysis with two
134 different LGG-1 antibodies (Al Rawi et al., 2011; Springhorn and Hoppe, 2019)(Figure 1C). In
135 basal conditions the wild-type LGG-1 was mainly present as form I (13.9 kDa) with a low
136 amount of the faster migrating form II and no detectable precursor (14,8 kDa)(Figure 1C),
137 while no band was observed in the allele *lgg-1(tm3489)* confirming that it is a *bona fide* null
138 mutant. While the *lgg-1(G116AG117*)* mutant presented a major form I, the *lgg-1(G116A)*
139 mutant accumulated both the expected precursor form and form I. This indicated that the
140 cleavage of the LGG-1(G116A) precursor was still present although less efficient. In both
141 mutants, an unexpected minor form was observed migrating differently from the lipidated
142 form II, which was no longer detected. The *lgg-1(G116AG117A)* mutant accumulated the
143 precursor form (96% of the protein) indicating that the cleavage observed in the LGG-
144 1(G116A) was dependent on the presence of a second glycine in position 117.

145 The respective protein substitutions were further confirmed by mass spectrometry analyses
146 after affinity purification of LGG-1(G116A) and LGG-1(G116AG117*) (Figure 1- figure
147 supplement 2). The identification of C-terminal peptides validated the expected precursor
148 form in LGG-1(G116A) and its cleavage after A116, and confirmed A116 as the last residue in
149 LGG-1(G116AG117*). These latter forms are called hereafter “cleaved form” and “truncated
150 form”, respectively.

151

152 **Glycine 116 is essential for lipidation of LGG-1 after cleavage**

153 To confirm western blot analyses, we next performed immunofluorescence in the embryo to
154 analyze the subcellular localization of LGG-1 protein from the various alleles. At the 1 cell-
155 stage and around 100 cells-stage, two selective autophagy processes have been well
156 characterized, removing paternal mitochondria and maternal aggregates, respectively (Al
157 Rawi et al., 2011; Sato and Sato, 2011; Zhang et al., 2009). The punctate staining, observed
158 in the wild-type animals (Figure 1D) with two independent anti-LGG-1 antibodies, was
159 characteristic for the autophagosomes formed during each process, and was absent in the
160 *lgg-1(Δ)* mutant (Figure 1E). The five mutants *lgg-1(G116A)*, *lgg-1(G116AG117*)*, *lgg-*
161 *1(G116AG117A)*, *lgg-1(ΔC100-123)* and *lgg-1(ΔC112-123)* presented no puncta but a diffuse
162 cytosolic staining. (Figure 1F-K), indicating that neither the precursor nor the form I are able
163 to conjugate to the autophagosome membrane. The increase of the diffuse signal in *lgg-*

164 *1(G116A)* and *lgg-1(G116AG117*)* embryos (Figure 1L) suggests that the protein is less
165 degraded in these mutants. Moreover, no LGG-1(G116A) puncta were observed after
166 depleting the tethering factor EPG-5 compared to the strong accumulation of puncta in LGG-
167 1(wt) (Figure 1- figure supplement 2) (Tian et al., 2010).

168 We performed cellular fractionation of membrane vesicles to test whether LGG-1(G116A)
169 and LGG-1(G116AG117*) are associated with autophagosomes. Compared with ER resident
170 SEL-1 or ER-associated CDC-48, the LGG-1(wt) protein was detected in both the cytosolic
171 fraction and the membrane pellet and could only be extracted with high salt or the
172 detergent Triton X-100. In contrast to LGG-1(wt), both LGG-1(G116A) and LGG-
173 1(G116AG117*) were absent in the membrane pellet fraction (Figure 1M), suggesting
174 defective lipidation of both LGG-1 mutant proteins. In an alternative approach, we observed
175 the localization of overexpressed GFP::LGG-1 and GFP::LGG-1(G116A) (Manil-Ségalen et al.,
176 2014) after induction of autophagic flux by acute heat stress (aHS) (Chen et al., 2021; Kumsta
177 et al., 2017). After aHS, GFP::LGG-1 formed numerous puncta that further accumulated
178 when autolysosome formation was impaired by depletion of RAB-7 or EPG-5 (Figure 1- figure
179 supplement 3). In contrast, in GFP::LGG-1(G116A), puncta were not reduced under any
180 condition. Electron microscopy and immunogold labeling confirmed that GFP::LGG-1 was
181 frequently detected to autophagosome membranes (Manil-Ségalen et al., 2014), whereas
182 GFP::LGG-1(G116A) was rarely detected in association with autophagosomes and in these
183 rare cases was predominantly localized in the lumen (Figure 1- figure supplement 3). Taken
184 together, these results suggest that the G116A mutation does not allow conjugation of LGG-
185 1 to the autophagosome membrane despite its cleavage. LGG-1(G116AG117A) represents
186 only a precursor form and LGG-1(G116AG117*) only a truncated form, whereas LGG-
187 1(G116A) produces both a precursor and a cleaved form. This allele series provides an ideal
188 situation to study the respective roles of the precursor and form I in absence of lipidated
189 form II.

190

191 **The essential function of LGG-1 during development is dependent of its cleavage but not** 192 **its conjugation**

193 The developmental phenotypes of the mutants *lgg-1(G116A)*, *lgg-1(G116AG117A)* and *lgg-1(G116AG117*)*
194 were explored in embryo, larvae and adults and compared with *lgg-1(Δ)* and
195 wild-type animals (Figure 2). We confirmed that *lgg-1(Δ)* homozygous animals present a
196 massive lethality during late embryogenesis or first larval stage (Figure 2B, H) (Manil-Ségalen
197 et al., 2014). However, few escapers, circa 8% of the progeny, were able to reach adulthood
198 and reproduce, allowing to maintain a *lgg-1(Δ)* homozygous population.

199 Neither *lgg-1(G116A)* nor *lgg-1(G116AG117*)* homozygous animals presented any
200 observable defect in development (Figure 2C, D, H) or adulthood and they reproduced at a
201 similar rate compared to wild-type animals (Figure 2I). In contrast, *lgg-1(G116AG117A)* and
202 the five independent mutants harboring various deletions and frameshifts of the C-terminus
203 presented a very strong lethality with the characteristic embryonic phenotype of *lgg-1(Δ)*
204 animals (Figure 2E-H). Among them *lgg-1(Δ112-123)* presented a premature stop codon at

205 position 112 and two others a frameshift in position 114 leading to an extension of the C-
206 terminus (Figure 2 and Figure 1- figure supplement 1).

207 These data indicate that the cleaved LGG-1(G116A) and the truncated LGG-1(G116AG117*)
208 forms, but not the precursor, are sufficient to recapitulate the normal development and
209 viability, independently of membrane conjugation. These data suggest that cleavage of the
210 C-terminus is necessary for LGG-1 developmental functions.

211

212 **Autophagy is functional in LGG-1(G116A)**

213 To address the functionality of LGG-1 precursor and form I, we analyzed autophagy-related
214 processes that have been well characterized during *C. elegans* life cycle (Leboutet *et al.*,
215 2020). Selective autophagy was studied in the early embryo, where a stereotyped mitophagy
216 process occurs. The degradation of selective cargos was observed in live embryos using
217 specific labeling of the paternal mitochondria (HSP-6::GFP and mitoTracker, Figure 3A-F and
218 Figure 3- figure supplement 1). In *lgg-1(Δ)* animals, the cargos accumulated while they were
219 degraded in the wild-type situation. In *lgg-1(G116A)*, but neither in *lgg-1(G116AG117*)* nor
220 in *lgg-1(G116AG117A)* mutants, paternal mitochondria were degraded, suggesting that the
221 LGG-1(G116A) protein maintained autophagic activity.

222 Bulk autophagy was then studied by starvation of the first stage larvae (Figure 3G). While
223 *lgg-1(G116AG117*)* and *lgg-1(G116AG117A)* mutants displayed a marked decrease of
224 survival, *lgg-1(G116A)* mutants showed no difference compared the wild-type animals.
225 Moreover, the longevity of adults, which depends on bulk autophagy, was similar for *lgg-*
226 *1(G116A)* and wild-type animals (Figure 3H), but strongly reduced for *lgg-1(G116AG117*)*
227 mutants.

228 The autophagic capacity of LGG-1(G116A) protein, but not LGG-1(G116AG117*) or LGG-
229 1(G116AG117A), was further documented by the elimination of apoptotic corpses in the
230 embryo (Figure 3I, and Figure 3- figure supplement 1) (Jenzer *et al.*, 2019).

231 Overall, these data demonstrate that, despite its defect to localize to autophagosomes, LGG-
232 1(G116A) achieves both selective and bulk autophagy during physiological and stress
233 conditions. This is the first *in vivo* evidence that the autophagy functions of LGG-1/GABARAP
234 can be uncoupled from its membrane conjugation. The non-functionality of LGG-
235 1(G116AG117A) suggests that the precursor form is not responsible of LGG-1(G116A)
236 autophagy activity. Despite an identical protein sequence, the truncated LGG-
237 1(G116AG117*) is not functional in autophagy, indicating that the cleavage of the C-
238 terminus from the precursor is essential for the functionality of LGG-1(G116A). Moreover,
239 the normal development of *lgg-1(G116AG117*)* animals demonstrates that the
240 developmental functions of LGG-1 are independent of its autophagic functions. Interestingly,
241 the expression in *S. cerevisiae* of LGG-1(wt) and LGG-1(G116A), but not LGG-1(G116AG117*),
242 slightly improved the nitrogen starvation survival of *atg8Δ* mutant (Supplementary data and
243 Figure 3- figure supplement 2), suggesting that the LGG-1(G116A) retains a partial autophagy
244 functionality in the yeast.

245

246 **Autophagy but not developmental functions of LGG-1(G116A) partially depends on LGG-2**

247 Our previous study has shown a partial redundancy of LGG-1 and LGG-2 during starvation
248 survival, and longevity (Alberti et al., 2010), which raises the possibility of functional
249 compensation of *lgg-1(G116A)* by LGG-2. To test this possibility, we used the large deletion
250 mutant *lgg-2(tm5755)*, which is considered as a null (Manil-Ségalen et al., 2014), and
251 constructed the double mutant strains *lgg-1(G116A); lgg-2(tm5755)* and *lgg-1(G116AG117*); lgg-2(tm5755)*.
252

253 Similar to the single mutants *lgg-1(G116A)* and *lgg-2(tm5755)*, the double mutant *lgg-1(G116A); lgg-2(tm5755)*
254 animals were viable and presented no morphological defect (Figure
255 4A-F). These data indicate that the correct development of *lgg-1(G116A)* is not due to a
256 compensative mechanism involving *lgg-2*.

257 Next, we analyzed the autophagy functions in *lgg-1(G116A); lgg-2(tm5755)* animals. If LGG-2
258 compensates for LGG-1(G116A) in autophagy, *lgg-1(G116A); lgg-2(tm5755)* animals should
259 behave similarly to *lgg-1(G116AG117*); lgg-2(tm5755)* (of note *lgg-1(Δ); lgg-2(tm5755)*
260 animals are not viable). The *lgg-1(G116A); lgg-2(tm5755)* animals presented a decrease for
261 both survival to starvation and longevity compared to *lgg-1(G116A)* single mutant. However,
262 they survived better than *lgg-1(G116AG117*); lgg-2(tm5755)* animals (Figure 4G, H). These
263 results indicate that the functionality of LGG-1(G116A) in bulk autophagy partially relies on
264 LGG-2. Selective autophagy during early embryogenesis was then quantitatively analyzed in
265 the double mutant strains (Figure 4I-K). Surprisingly, paternal mitochondria were eliminated
266 in the *lgg-1(G116A); lgg-2(tm5755)* animals indicating that LGG-1(G116A) was sufficient for
267 the allophagy process. This suggests that paternal mitochondria could be degraded by
268 autophagosomes devoid of both LGG-1 and LGG-2. However, a delay in the degradation was
269 observed compared to *lgg-1(G116A)* animals suggesting that the autophagy flux is reduced.
270 These results revealed a partial redundancy between LGG-1 and LGG-2 in autophagy, but
271 demonstrated at the same time that LGG-1(G116A) fulfills developmental functions and
272 maintains some autophagy activity independent of LGG-2.

273 Interestingly, this detailed analysis also revealed a slight delay in the elimination of paternal
274 mitochondria in *lgg-1(G116A)* animals compared to wild-type (Figure 4K). Although the
275 cleaved LGG-1 is sufficient for autophagy, this observation suggests that loss of membrane
276 targeting could affect the dynamics of autophagy flux.

277

278 **The degradation of autophagosomes is delayed in LGG-1(G116A)**

279 The autophagic flux and the dynamics of autophagosome formation were compared
280 between *lgg-1(G116A)*, *lgg-1(G116AG117*)* and *lgg-1(Δ)* animals. We first focused on the
281 early embryo where the autophagy process is stereotyped and the nature of the cargos and
282 the timing of degradation are well characterized. Moreover, the autophagosomes
283 sequestering the paternal mitochondria were clustered and positive for LGG-2 (Figure
284 5A)(Manil-Ségalen et al., 2014). In *lgg-1(Δ)* mutant, LGG-2 autophagosomes were not
285 detected as a cluster but were spread out in the whole embryo as single puncta that
286 persisted after the 15-cells stage (Figure 5B, E). This indicated that individual LGG-2

287 structures could be formed in absence of LGG-1, but were not correctly localized and not
288 degraded properly, presumably because of the role of LGG-1 in cargo recognition (Sato et al.,
289 2018) and of its latter involvement in the maturation of autophagosomes, respectively. The
290 pattern of LGG-2 was somehow different in *lgg-1(G116A)* and *lgg-1(G116AG117*)* mutants,
291 forming sparse structures of heterogeneous size, which persisted longer (Figure 5C-F). These
292 data suggested that the cleaved and the truncated LGG-1 could both promote the
293 recruitment of LGG-2 to autophagic structures, but display an altered autophagic flux. The
294 analysis of the colocalization between paternal mitochondria and LGG-2 did not reveal an
295 increase in *lgg-1(G116A)* or *lgg-1(G116AG117*)* mutants (Figure 5G-J and Figure 5- figure
296 supplement 1). These data suggested that the elimination of paternal mitochondria in *lgg-*
297 *1(G116A)* animals was not due to the enhanced recruitment of LGG-2. A western blot
298 analysis of worm lysates indicated that there was no increase of LGG-2 expression in *lgg-*
299 *1(Δ)*, *lgg-1(G116A)* and *lgg-1(G116AG117*)* mutants (Figure 5K).

300 The autophagic structures in *lgg-1(G116A)* and *lgg-1(G116AG117*)* embryos were further
301 characterized by electron microscopy and compared with wild-type and *lgg-1(Δ)* mutant
302 embryos (Figure 6). In wild-type animals, autophagosomes containing cytoplasmic materials
303 (referred as type 1) and the characteristic paternal mitochondria (Zhou et al., 2016) were
304 observed in early embryos (Figure 6A). At that stage, rare autophagosomes containing
305 partially degraded material were present (referred as type 2). As expected, almost no
306 autophagosome was observed in *lgg-1(Δ)* embryos and paternal mitochondria were non-
307 sequestered (Figure 6B). In *lgg-1(G116A)* embryos, the numbers of type 1 and type 2
308 autophagosomal structures increased. The autophagosomes appeared to be closed and
309 contained various cellular materials and membrane compartments (Figure 6C-E). This
310 confirmed that LGG-1(G116A) was sufficient to form functional autophagosomes but with
311 delayed degradation. On the other hand, *lgg-1(G116AG117*)* embryos presented non-
312 sequestered paternal mitochondria (Figure 6F) and multi-lamellar structures containing
313 cytoplasm but no membrane organelles (type 3 Figure 6G, K). The analysis of the double
314 mutant strains *lgg-1(G116A); lgg-2(tm5755)* and *lgg-1(G116AG117*); lgg-2(tm5755)*
315 revealed the presence of types 1 and 2 autophagosomes, but less frequent than in the single
316 *lgg-1* mutants (Figure 6H-K). This data confirmed that LGG-1(G116A) alone was able to
317 initiate the formation of autophagosomes but less efficiently in absence of LGG-2. Type 3
318 structures were only observed in *lgg-1(G116AG117*)* and *lgg-1(G116AG117*); lgg-*
319 *2(tm5755)*, suggesting a neomorphic function of the truncated LGG-1(G116AG117*) protein
320 that induced a non-functional compartment.

321 Altogether, these data indicate that the cleaved, but not the truncated, LGG-1 form I is able
322 to form functional autophagosomes with a delayed degradation.

323

324 **The lipidated LGG-1 is involved in the coordination between cargo recognition and** 325 **autophagosome biogenesis.**

326 To better understand the function of LGG-1 form I during autophagy flux, we next analyzed a
327 developmental autophagy process (Figure 7). The Zhang lab has demonstrated that

328 aggregate-prone proteins are degraded through autophagy in *C. elegans* embryo through
329 liquid-liquid phase separation promoted by the receptor SEPA-1 and regulated by the
330 scaffolding protein EPG-2 (Lu et al., 2011; Tian et al., 2010; Wu et al., 2015; Zhang et al.,
331 2018, 2009). Initiation and elongation of autophagosomes were analyzed by quantifying the
332 colocalization between ATG-18/WIPI2 and LGG-2 (Figure 7A-E) during autophagosome
333 formation. ATG-18, the worm homolog of the omegasome marker WIPI2 (Polson et al.,
334 2010), acts at an early step of biogenesis (Lu et al., 2011). Puncta labelled with ATG-18 only,
335 both ATG-18 and LGG-2, or LGG-2 only were considered as omegasomes, phagophores, and
336 autophagosomes, respectively. In *lgg-1(RNAi)* animals the number of omegasomes increased
337 while the proportion of phagophore decreased compared to the wild-type embryos (Figure
338 7A, B, E). This indicates that the initiation of autophagy was triggered in absence of LGG-1,
339 but the biogenesis of autophagosome was defective. *lgg-1(G116A)* animals showed no
340 difference with the wild-type (Figure 7C, E) supporting that both initiation and phagophore
341 extension are normal with the cleaved LGG-1. Similar to the *lgg-1(RNAi)*, *lgg-1(G116AG117*)*
342 animals were defective in the phagophore extension (Figure 7D, E). These data confirmed
343 that the cleaved LGG-1(G116A), but not the truncated LGG-1(G116AG117*), is functional for
344 the early step of autophagosome biogenesis. Moreover, RNAi depletion demonstrated that
345 the function of LGG-1(G116A) in aggrephagy pathway was dependent on UNC-51/Ulk1 and
346 the scaffolding protein EPG-2 (Supplementary data and Figure 7- figure supplement 1).
347 Quantification of SEPA-1::GFP in late embryo showed that *lgg-1(G116A)* mutant was able to
348 perform aggrephagy but not *lgg-1(G116AG117*)* or *lgg-1(G116AG117A)* mutants (Figure 7F-
349 K). However, the elimination was decreased compared to wild-type confirming that LGG-
350 1(G116A) was less efficient for selective cargo degradation.
351 Finally, the interactions between cargoes and autophagosomes were studied in *lgg-1(G116A)*
352 mutant and wild-type embryos by analyzing the colocalization between SEPA-1
353 and LGG-1 or LGG-2. In wild-type embryos, immunofluorescence analyses showed the
354 presence of LGG-1 and LGG-2 autophagosomes in contact with SEPA-1 aggregates (Figure 7L,
355 N). In *lgg-1(G116A)* embryos, LGG-2 positive autophagosomes were observed but no LGG-1
356 dots, in line with the absence of lipidation (Figure 7M, O). A part of LGG-2 puncta was
357 present close to SEPA-1 aggregates, however, they were less numerous and the overlap
358 between LGG-2 and SEPA-1 signals was weaker (Figure 7P). These data suggested that LGG-
359 1(G116A) was able to maintain the function of LGG-1 for initiation and extension of
360 autophagosomes but was partially deficient for cargo sequestering.

361 Altogether, the analyses of LGG-1(G116A) indicate that many of the functions of LGG-1 in
362 autophagy can be achieved by the cleaved, non-lipidated form I. However, the lipidation of
363 LGG-1 appears to be important for the coordination between cargo recognition and
364 autophagosome biogenesis and for the correct degradation of the autophagosome.

365

366

367 **Discussion**

368

369 The most surprising result of this study is the discovery that LGG-1(G116A) is functional for
370 many autophagy processes, covering physiological or stress conditions and selective or bulk
371 autophagy. To our knowledge, it is the first report demonstrating that different autophagy
372 processes are fully achieved *in vivo* in a non-lipidated LC3/GABARAP mutant. In cultured
373 cells, an elegant CRISPR strategy allowed to knock out together the six LC3/GABARAP
374 homologs, but point mutations of the conserved glycine have not been reported (Nguyen et
375 al., 2016). Most of the studies on the terminal glycine used transgenic overexpression
376 constructs (Chen et al., 2007; Kabeya et al., 2004). Interestingly, one study reported that part
377 of the autophagy functions of GABARAPL1 is independent of its lipidation (Poillet-Perez et
378 al., 2017). Several studies have used mutations in the conjugation machinery (Atg3, Atg5,
379 Atg7) or the Atg4 protease to analyze the role of the form I (Hill et al., 2019; Hirata et al.,
380 2017; Nishida et al., 2009; Ohnstad et al., 2020; Vujić et al., 2021). A non-canonical
381 autophagy has been reported in *Atg5*, *Atg7* mutants (Nishida et al., 2009), but blocking the
382 conjugation system presumably affects all LC3/GABARAP homologs. Moreover, the presence
383 of four homologs of Atg4 in mammals, which specificity versus LC3/GABARAP is unknown,
384 and the dual role in the cleavage of the precursor and the delipidation entangle the analysis
385 of the phenotypes.

386 Our data show no evidence for an intrinsic function of the LGG-1 precursor but the
387 importance of its active cleavage. This finding is not surprising because in many species the
388 Atg8 precursor is not detected, suggesting that the cleavage occurs very soon after or even
389 during translation. Moreover, phylogenetic analyses of LC3/GABARAP show no conservation
390 in sequence and length of the C-terminus but the presence of at least one residue after the
391 conserved G116. The hypothesis of a selective constraint on the cleavage but not on the C-
392 terminus sequence could explain the persistence of a precursor form. Further studies are
393 necessary to clarify the precise implication of the di-glycine G116G117 in the process.

394 Albeit a similar sequence, the difference of functionality between the cleaved LGG-1(G116A)
395 and the truncated LGG-1(G116AG117*) suggests that the cleavage allows a first level of
396 specificity for LGG-1 functions. The normal development of *lgg-1(G116AG117*)* animals is
397 the first evidence that LGG-1 function in development relies on the cleavage but is
398 independent of autophagy and conjugation. Our results could explain the embryonic
399 lethality reported upon depletion of the two Atg4 homologs precursors in *C. elegans* (Wu et
400 al., 2012). While the cleavage is sufficient for developmental functions, autophagy functions
401 of LGG-1 form I seem to require a further modification to be efficient. Our data suggest that
402 this modification is dependent on and possibly associated to the cleavage. The presence of a
403 new minority band for LGG-1(G116A) could reflect an intermediary transient processing
404 state but should not correspond to a functional form because it was also detected for LGG-
405 1(G116AG117*) and LGG-1(G116AG117A).

406 Our observations in yeast also support an autophagy independent function of Atg8 form I in
407 vacuolar shaping. Non-autophagic functions for LC3/GABARAP have been identified in yeast
408 and higher eukaryotes (Ishii et al., 2019; Liu et al., 2018; Schaaf et al., 2016; Wesch et al.,
409 2020), but the roles of the cytosolic forms are poorly documented especially in the context

410 of the development. The two Atg8 homologs of drosophila are involved in several
411 developmental processes independently of canonical lipidation (Chang et al., 2013) or
412 autophagy (Jipa et al., 2020). They are highly similar and both correspond to GABARAP
413 homologs (Manil-Ségalen et al., 2014). It is possible that duplication of Atg8 during evolution
414 allowed the acquisition of specific developmental functions by GABARAP proteins but
415 reports in apicomplex parasites (Lévêque et al., 2015; Mizushima and Sahani, 2014) rather
416 support a non-autophagy ancestral function of Atg8.

417 The major goal of this study was to bring new insights concerning the implication of LGG-1
418 form I in various steps of autophagy. Numerous studies identified interacting partners of
419 Atg8/LC3/GABARAP family during autophagy but its mechanistic function for
420 autophagosome biogenesis is still debated. In yeast, the amount of Atg8 regulates the level
421 of autophagy and controls phagophore expansion, but is mainly released from the
422 phagophore assembly site during autophagosome formation (Xie et al., 2008). *In vitro*
423 studies using liposomes or nanodiscs suggested that Atg8 is a membrane-tethering factor
424 and promotes hemifusion (Nakatogawa et al., 2007), membrane tubulation (Knorr et al.,
425 2014), or membrane-area expansion and fragmentation (Maruyama et al., 2021). Another
426 study showed that Atg8-PE assembles with Atg12-Atg5-Atg16 into a membrane scaffold
427 that is recycled by Atg4 (Kaufmann et al., 2014). A similar approach with LGG-1 supports a
428 role in tethering and fusion activity (Wu et al., 2015). *In vivo*, the functions of these proteins
429 could depend on their amount, their posttranslational modifications, and the local
430 composition of the membrane. For instance, an excess of lipidation of the overexpressed
431 LGG-1 form I mediates the formation of enlarged protein aggregates and impedes the
432 degradation process (Wu et al., 2015). A recent report showed that the phosphorylation of
433 LC3C and GABARAP-L2 impedes their binding to ATG4 and influences their conjugation and
434 de-conjugation (Herhaus et al., 2020).

435 Our genetic data suggest that form I of LGG-1 is sufficient for initiation, elongation and
436 closure of autophagosomes but that lipidated LGG-1 is important for the cargo sequestering
437 and the dynamics of degradation. However, the partial redundancy with LGG-2 is
438 presumably an important factor during these processes. If the main functions of LGG-1
439 reside in its capacity to bind multiple proteins, the localization to autophagosome
440 membrane through lipidation is an efficient but not unique way to gather cargoes and
441 autophagy complexes. Furthermore, the possibility that non-positive LGG-1/LGG-2
442 autophagosomes could mediate cargo degradation questions the use of Atg8/GABARAP/LC3
443 family as a universal marker for autophagosomes. Overall, our results confirm the high level
444 of plasticity and robustness of autophagosome biogenesis.

445

446

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459

460

461 **Declaration of interests**

462 The authors declare no competing interests

463

464

465 **Materials and Methods**

466

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
gene (<i>C. elegans</i>)	<i>lgg-1</i>	Wormbase	WBGene0002980	
strain, strain background (<i>C. elegans</i>)	N2	CGC		<i>Wild-type strain</i>
genetic reagent (<i>C. elegans</i>)	DA2123	CGC		<i>adls2122[gfp::lgg-1;rol-6(su1006)]</i>
genetic reagent (<i>C. elegans</i>)	GK1057	Sato and Sato, 2011		<i>Pspe-11-hsp-6::GFP</i>
genetic reagent (<i>C. elegans</i>)	HZ455	CGC		<i>him-5(e1490) V; bpls131[sepa-1::gfp]</i>
genetic reagent (<i>C. elegans</i>)	HZ1685	CGC		<i>atg-4.1(bp501)</i>
genetic reagent (<i>C. elegans</i>)	MAH247	CGC		<i>sqls25[atg-18p::atg-18::gfp+rol-6(su1006)]</i>
genetic reagent (<i>C. elegans</i>)	RD202	Legouis lab		<i>Is202[unc-119(ed3)III;plgg-1::GFP::LGG-1 G->A]</i>
genetic reagent (<i>C. elegans</i>)	<i>lgg-1(Δ)</i>	Mitani lab	NBRP: tm3489	<i>lgg-1(tm3489)</i>
genetic reagent (<i>C. elegans</i>)	<i>lgg-2(tm5755)</i>	Mitani lab	NBRP: tm5755	<i>lgg-2(tm5755)</i>
genetic reagent (<i>C. elegans</i>)	RD363 ; <i>lgg-1(Δ112-123)</i>	This paper		<i>lgg-1(pp22)dpy-10(pp157)</i> Legouis lab
genetic reagent	RD367 ;	This paper		<i>lgg-1(pp65[G116A])</i> Legouis lab

(<i>C. elegans</i>)	lgg-1(G116A)			
genetic reagent (<i>C. elegans</i>)	RD368 ; lgg-1(Δ 100-123)	This paper		<i>lgg-1(pp66)</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD420 ; lgg-1(G116AG117*)	This paper		<i>lgg-1(pp141[G116AG117stop])</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD421 ; lgg-1(G116AG117A)	This paper		<i>dpy-10(pp163)lgg-1(pp142[G116AG117A])</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD425	This paper		<i>dpy-10(pp163)lgg1(pp142)/+; SEPA-1::gfp</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD435	This paper		<i>lgg-1(pp141[G116AG117stop]) ; atg-18p::atg-18::gfp+rol-6(su1006)</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD436	This paper		<i>lgg-1(pp65[G116A]); atg-18p::atg-18::gfp+rol-6(su1006)</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD440	This paper		<i>lgg-1(pp141[G116AG117stop]) ; lgg-2(tm5755)</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD446	This paper		<i>lgg-1(pp65[G116A]); lgg-2(tm5755)</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD447	This paper		<i>lgg-1(tm3489) ; atg-18p::atg-18::gfp+rol-6(su1006)</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD448	This paper		<i>lgg-1(pp65[G116A]); SEPA-1::gfp</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD449	This paper		<i>lgg-1(pp141[G116AG117stop]); SEPA-1::gfp</i> Legouis lab
genetic reagent (<i>C. elegans</i>)	RD450	This paper		<i>lgg-1(tm3489)II; SEPA-1::gfp</i> Legouis lab
strain, strain background (<i>S. cerevisiae</i>)	BY4742	Euroscarf		<i>Mat alpha ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0</i>
genetic reagent (<i>S. cerevisiae</i>)	OC513	YKO collection		<i>BY4742, atg1::KanMX4</i>
genetic reagent (<i>S. cerevisiae</i>)	OC612	YKO collection		<i>BY4742, atg8::KanMX4</i>
genetic reagent (<i>S. cerevisiae</i>)	OC608-OC609	This paper		<i>BY4742, atg8G116A</i> Legouis lab
genetic reagent (<i>S. cerevisiae</i>)	OC610-OC611	This paper		<i>BY4742, atg8G116A-R117*</i> Legouis lab
genetic reagent (<i>S. cerevisiae</i>)	OC613	This paper		<i>BY4742, pho8::pho8Δ60-URA3KL</i> Legouis lab
genetic reagent (<i>S. cerevisiae</i>)	OC614	This paper		<i>BY4742, atg1::KanMX4, pho8::pho8Δ60-URA3KL</i> Legouis lab

genetic reagent (<i>S. cerevisiae</i>)	OC615	This paper		BY4742, <i>atg8::KanMX4, pho8::pho8Δ60-URA3KL</i> Legouis lab
genetic reagent (<i>S. cerevisiae</i>)	OC616-OC617	This paper		BY4742, <i>atg8G116A, pho8::pho8Δ60-URA3KL</i> Legouis lab
genetic reagent (<i>S. cerevisiae</i>)	OC618-OC619	This paper		BY4742, <i>atg8G116A-R117*, pho8::pho8Δ60-URA3KL</i> Legouis lab
Strain strain background (<i>E. coli</i>)	OP50	CGC		see Material and Methods
genetic reagent (<i>E. coli</i>)	JA-C32D5.9	Open Biosystem		<i>lgg-1</i> RNAi feeding bacterial clone
genetic reagent (<i>E. coli</i>)	JA-C56C10.12	Open Biosystem		<i>epg-5</i> RNAi feeding bacterial clone
genetic reagent (<i>E. coli</i>)	JA-Y55F3AM.4	Open Biosystem		<i>atg-3</i> RNAi feeding bacterial clone;
genetic reagent (<i>E. coli</i>)	JA-M7.5	Open Biosystem		<i>atg-7</i> RNAi feeding bacterial clone
genetic reagent (<i>E. coli</i>)	JA-W03C9.3	Open Biosystem		<i>rab-7</i> RNAi feeding bacterial clone
genetic reagent (<i>E. coli</i>)	JA-Y39G10AR.10	Open Biosystem		<i>epg-2</i> RNAi feeding bacterial clone
sequenced-based reagent	CrRNA(s)	Paix et al 2015		<i>dpy-10</i> : 5'GCUACCAUAGGCACCACGAGGUUUUAGAG CUAUGCUGUUUUG3'
sequenced-based reagent	CrRNA(s)	This paper		<i>lgg-1</i> Legouis lab 5'UACAGUGACGAAAGUGUGUAGUUUAGA GCUAUGCUGUUUUG3'
sequenced-based reagent	Repair template	Paix et al., 2015;		<i>dpy-10</i> : 5'CACTTGAACCTCAATACGGCAAGATGAGAAT GACTGGAACCGTACCGCATGCGGTGCCTATG GTAGCGGAGCTTCACATGGCTTCAGACCAACA GCCTAT3'
sequenced-based reagent	Repair template	This paper		<i>lgg-1</i> (G116A): Legouis lab 5'CTTTACATCGCGTACAGTGACGAAAGTGTCT ACGCCGAGAGGTCGAAAAGAAGGAATAAAG TGTCATGTAT3'
sequenced-based reagent	Repair template	This paper		<i>lgg-1</i> (G116AG117 *): Legouis lab 5'TTCCTTACATCGCTACAGTGACGAAAGTGT GTACGCCTAAGAATTCGAAAAGAAGGAATAAA GTGTCATGTATTATCCG3'

sequenced-based reagent	Repair template	This paper		<i>lgg-1</i> (G116AG117A): Legouis lab 5'TTCCTTTACATCGCCTACAGTGACGAAAGTGT GTACGCCGAGAGGTCGAAAAGAAGGAATAA GAATTCAGTGTTCATGTATTATCCGCCGACGAAT GTGTATAC3'
sequenced-based reagent	Universal tracrRNA	Dharmacon GE	U-002000-05	5'AACAGCAUAGCAAGUUAAAAUAAGGCUAG UCCGUUAUCAACUUGAAAAAGUGGCACCGAG UCGGUGCUUUUUUU3'
peptide, recombinant protein	<i>S. pyogenes</i> Cas9	Dharmacon	CAS11201	Edit-R Cas9 Nuclease Protein, 1000 pmol
Antibody	anti-LGG-1 (rabbit polyclonal)	Springhorn & Hoppe, 2019		Ab#3 WB (1:3000)
Antibody	anti-LGG-1 (rabbit polyclonal)	Al Rawi <i>et al.</i> , 2011		Ab#1 WB (1:200) IF(1:100)
Antibody	anti-LGG-2 (rabbit polyclonal)	Manil-Ségalen <i>et al.</i> , 2014		WB (1:200) IF (1:200)
Antibody	anti-Tubulin (mouse monoclonal)	Sigma	078K4763	WB (1:1000)
Antibody	anti-SEL-1 (rabbit polyclonal)	Hoppe's lab		WB (1:8000)
Antibody	anti-CDC-48.1 (rabbit polyclonal)	Hoppe's lab		WB (1:5000)
Antibody	Anti-Rabbit HRP (goat polyclonal)	Promega	W401B	WB (1:5000)
Antibody	Anti-mouse HRP (goat polyclonal)	Promega	W4021	WB (1:10000)
Antibody	anti-GABARAP (rabbit polyclonal)	Chemicon	AB15278	IF (1:200)
Antibody	anti-GFP (mouse monoclonal)	Roche	1814460	IF (1:250)
Antibody	anti-mouse IgG Alexa Fluor®488 (goat polyclonal)	Molecular Probes	A11029	IF (1:500 to 1:1000)
Antibody	anti-rabbit IgG Alexa Fluor®488	Molecular Probes	A110034	IF (1:500 to 1:1000)

	(goat polyclonal)			
Antibody	anti-rabbit IgG Alexa Fluor®568 (goat polyclonal)	Sigma-Aldrich	A11036	IF (1:500 to 1:1000)
Antibody	anti-GFP (rabbit polyclonal)	Abcam	ab6556	(Immunogold 1:10)
Antibody	anti-rabbit IgG goat polyclonal)	Biovalley	810.011	Coupled to 10-nm colloidal gold particles (Immunogold 1:20)
chemical compound, drug	EPON	Agar Scientific	R1165	see Material and Methods
chemical compound, drug	lead citrate	Sigma-Aldrich	15326	see Material and Methods
chemical compound, drug	LRWHITE	Electron Microscopy Sciences	14381	see Material and Methods
peptide, recombinant protein	LC3 traps	Quinet <i>et al.</i> , 2022		Molecular traps for LGG-1
commercial assay or kit	Super Signal Pico Chemiluminescent Substrate	Thermo Scientific	34579	see Material and Methods
commercial assay or kit	NuPAGE 4%-12% Bis- Tris gel	Life Technologies	NP0321 BOX	see Material and Methods
software, algorithm	ImageJ	http://imagej.nih.gov/ij		see Material and Methods
software, algorithm	Fidji	https://fiji.sc/		see Material and Methods
software, algorithm	Prism	GraphPad		see Material and Methods
software, algorithm	R software	www.r-project.org		see Material and Methods
software, algorithm	Crispr	http://Crispr.mit.edu		see Material and Methods
software, algorithm	Crispor	http://crispor.org		see Material and Methods
other	MitoTracker Red CMXRos	Molecular Probes	M7512	see Material and Methods

467 Further information and requests for resources and reagents should be directed to the
468 corresponding author, Renaud Legouis (renaud.legouis@i2bc.paris-saclay.fr).

469

470

471 ***C. elegans* culture and strains**

472 Nematode strains were grown on nematode growth media [for 500ml H₂O: 1.5g NaCl
473 (Sigma-Aldrich, 60142), 1.5g bactopectone (Becton-Dickinson, 211677), 0.5ml cholesterol
474 (Sigma-Aldrich, C8667) 5mg/ml, 10g bacto agar (Becton-Dickinson, 214010) supplemented
475 with 500µl CaCl₂ (Sigma-Aldrich, C3306) 1M, 500µl MgSO₄ (Sigma-Aldrich, M5921) 1M, 10ml
476 KH₂PO₄ (Sigma-Aldrich, P5655) 1M, 1650µl K₂HPO₄ (Sigma-Aldrich, 60356) 1M] and fed
477 with *Escherichia coli* strain OP50.

478

479 **CRISPR-Cas9**

480 A CRISPR-Cas9 approach optimized for *C. elegans* was used, based on a *dpy-10* co-CRISPR
481 protocol (Paix et al., 2015). All reagents are in 5mM Tris-HCl pH 7.5. Crispr.mit.edu and
482 CRISPOR (<http://crispor.org>) web tools were used to choose a Cas9 cleavage site (NGG)
483 close to the edit site, the best sequence of the crRNAs (50 to 75% of GC content) , and for
484 off-target prediction. 1µL of CrRNA(s) (8µg/µL or 0.6nmole/µL) and repair template(s)
485 (1µg/µL) designed for *lgg-1* and *dpy-10* genes were mixed with 4.1 µL of *S. pyrogenes* Cas9
486 (20 pmole/µL) and 5 µL of universal tracrRNA (4µg/µL 4µg/µL or 0.17 nmol/µL molarity) in
487 0.75 µL Hepes (200 mM) 0.5µL KCl (1M) and water up to 20 µL. The mix was heated for 10
488 min at 37°C and injected in the gonad of young adult hermaphrodites. Progenies of injected
489 animals were cloned and genotyped by PCR. Mutants were outcrossed three times and *lgg-1*
490 gene was sequenced to check for the specific mutations.

491

492 **Nematode starvation and lifespan**

493 For starvation experiments, adult hermaphrodites were bleached to obtain synchronized L1
494 larvae. L1 were incubated in 0.5 mL sterilized M9 at 20°C on spinning wheel. At each time
495 point, an aliquot from each sample tube was placed on a plate seeded with *E. coli* OP50. The
496 number of worms surviving to adulthood was counted 2 or 4 days after. Life span was
497 performed on more than 100 animals for each genotype with independent duplicates and
498 analyzes using Kaplan-Meier method and Log-Rank (Mantel-Cox) test.

499

500 **RNA mediated interference**

501 RNAi by feeding was performed as described (Kamath et al., 2003). Fourth-larval stage (L4)
502 animals or embryos were raised onto 1 mM isopropyl-D-β-thiogalactopyranoside (IPTG)-
503 containing nematode growth media (NGM) plates seeded with bacteria (*E. coli* HT115[DE3])
504 carrying the empty vector L4440 (pPD129.36) as a control or the bacterial clones from the J.
505 Ahringer library, Open Biosystem).

506

507 **Western blot and cellular fractionation**

508 The worms were collected after centrifugation in M9 and then mixed with the lysis buffer
509 described previously (Springhorn and Hoppe, 2019) (25mM tris-HCl, pH7.6 ; 150mM NaCl ;
510 1mM ethylenediaminetetraacetic acid (EDTA) 1% Triton X-100 ; 1% sodium deoxycholate
511 (w/v) ; 1% SDS (w/v)) containing glass beads (Sigma-Aldrich 425-600um G8772100G). They
512 were then denatured using Precellys 24 machine at 6000 rpm with incubation for about 5

513 min twice to cool down the sample. The protein extracts are then centrifuged at 15000 rpm
514 and separated on a NuPAGE 4%-12% Bis-Tris gel (Life Technologies, NP0321BOX). The non-
515 specific sites are then blocked after the incubation for one hour with PBS Tween 0.1% (Tris
516 Base NaCl, Tween20) BSA 2%. Blots were probed with anti-LGG-1 (1:3000 rabbit Ab#3
517 (Springhorn and Hoppe, 2019) or 1:200 Ab#1 (Al Rawi et al., 2011)), anti-LGG-2 (1:200
518 rabbit), anti-Tubulin (1:1000 mouse; Sigma, 078K4763), anti-SEL-1 (1:8000, rabbit), anti-CDC-
519 48.1 (1:5000, rabbit) and revealed using HRP-conjugated antibodies (1: 5000 promega
520 W401B and 1:10000 promega W4021) and the Super Signal Pico Chemiluminescent
521 Substrate (Thermo Scientific, 34579). Signals were revealed on a Las3000 photoimager (Fuji)
522 and quantified with Image Lab software. For cellular fractionation, 4000 age-synchronized
523 worms (day 1 of adulthood) were collected from NGM/OP-50 plates, washed three times
524 with M9 buffer and transferred to NGM plates without OP-50 to induce starvation. Worms
525 were starved at 20°C for 7 hours, and then transferred to fractionation buffer (50 mM Tris-
526 HCl pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail). For cell
527 lysis, worms were homogenized 50 times using a Dounce homogenizer and sonicated for 20
528 seconds at 60% amplitude. Cell lysates were centrifuged at 500 RCF and 4°C for 5 minutes to
529 remove cell debris and the nuclear fraction. The supernatant was centrifuged again at
530 20,000 RCF and 4°C for 90 minutes to separate soluble (cytosolic) and insoluble (membrane)
531 fractions. Supernatant and pellet were separated and the pellet was resuspended in 150 µL
532 of fractionation buffer. From this step, input samples were prepared for Western blot
533 analysis. Subsequently, 30 µL of the pellet sample was mixed with 3 µL each of fractionation
534 buffer, 3 µL 5 M NaCl, and 3 µL Triton X-100. Treated pellet samples were incubated on ice
535 for 1 hour and then centrifuged at 20,000 RCF and 4 °C for 60 minutes. The resulting
536 supernatants and pellets were again separated and analyzed by Western blotting.

537

538 **Immunofluorescence and light microscopy**

539 Fifty adult hermaphrodites were cut to release the early embryos on a previously poly-L-
540 lysinated slide (0.1%). Late embryos were deposited using a flattened platinum wire and
541 bacteria as glue. Embryos were prepared for immunofluorescence staining by freeze-
542 fracture and methanol fixation 30 min at -20°C, incubated 40 min in 0.5% Tween, 3% BSA,
543 PBS solution, and washed twice 30 min in 0.5% Tween PBS solution. Incubation overnight at
544 4°C overnight with the primary antibodies anti-LGG-1(rabbit 1:100) anti-GABARAP (rabbit
545 1:200) (1: 200), anti-LGG-2 (rabbit 1:100) was followed by 2 washes, 2 hours incubation at
546 room temperature with the secondary antibodies, Alexa488 and Alexa 568 (1: 1000), and
547 two washes. Embryos were mounted in DABCO and imaged on an AxioImagerM2
548 microscope (Zeiss) equipped with Nomarski optics, coupled to a camera (AxioCam506mono)
549 or a confocal Leica TCS SP8 microscope with serial z sections of 0.5 to 1 µm. Images were
550 analyzed, quantified and processed using ImageJ or Fiji softwares.

551 For live imaging samples were mounted on a 2% agarose pad and larvae were immobilized
552 by 40 mM sodium azide. For MitoTracker staining, adult worms were transferred to NGM
553 agar plates containing 3.7 µM of Red CMXRos (Molecular Probes, Invitrogen) and incubated
554 for overnight in the dark.

555

556 **Electronic microscopy**

557 One-day adults were transferred to M9 20% BSA (Sigma-Aldrich, A7030) on 1%
558 phosphatidylcholine (Sigma-Aldrich) pre-coated 200 µm deep flat carriers (Leica Biosystems),
559 followed by cryo-immobilization in the EMPACT-2 HPF apparatus (Leica Microsystems;
560 Vienna Austria) as described (Largeau & Legouis, 2019). Cryo-substitution was performed
561 using an Automated Freeze-substitution System (AFS2) with integrated binocular lens, and
562 incubating chamber (Leica Microsystems; Wetzlar, Germany) with acetone. Blocks were
563 infiltrated with 100% EPON, and embedded in fresh EPON (Agar Scientific, R1165). Ultrathin
564 sections of 80 nm were cut on an ultramicrotome (Leica Microsystems, EM UC7) and
565 collected on a formvar and carbon-coated copper slot grid (LFG, FCF-2010-CU-50). Sections
566 were contrasted with 0,05% Oolong tea extract (OTE) for 30 minutes and 0.08 M lead citrate
567 (Sigma-Aldrich, 15326) for 8 minutes. Sections were observed with a Jeol 1400 TEM at 120
568 kV and images acquired with a Gatan 11 Mpixels SC1000 Orius CCD camera.

569

570 **Affinity purification of LGG-1**

571 1 mg of total proteins from *C. elegans* lysate were incubated on ice 10 minutes in 800 µL of
572 TUBE lysis buffer [50 mM sodium fluoride, 5 mM tetra-sodium pyrophosphate, 10 mM β-
573 glyceropyrophosphate, 1% Igepal CA-630, 2 mM EDTA, 20 mM Na₂HPO₄, 20 mM NaH₂PO₄,
574 and 1.2 mg/ml complete protease inhibitor cocktail (Roche, Basel, Switzerland)]
575 supplemented with 200 µg of purified LC3 traps or GST control (Quinet et al., 2022). After
576 cold centrifugation at 16200 g for 30 minutes, supernatant was harvested and added to 400
577 µl of prewashed glutathione-agarose beads (Sigma), and incubated for 6 hours rotating at
578 4°C. Beads were centrifugated at 1000 g for 5 min at 4°C (Beckman Coulter Microfuge 22R,
579 Fullerton, CA, USA), washed five times using 10 column volumes of PBS-tween 0.05%. Elution
580 was done in 100 µL of [Tris pH7.5, 150mM NaCl, 1% Triton, 1% SDS] at 95°C during 10
581 minutes, and supernatant was harvested.

582

583 **Mass spectrometry**

584 Protein samples affinity purification were prepared using the single-pot, solid-phase-
585 enhanced sample-preparation (SP3) approach as described (Hughes et al., 2019). Samples
586 were mixed with 10 µl of 10 µg/µl solution of Sera-Mag SpeedBeads™ hydrophilic and
587 hydrophobic magnetic beads (GE healthcare, ref 45152105050250 and 65152105050250)
588 with a bead to sample ratio of 10:1. After a binding step in 50% ethanol in water, and three
589 successive washes with 80% ethanol in water, sample were digested with 100 µl of a 5 ng/ul
590 sequencing grade modified trypsin solution (PROMEGA). 50 µl of Trypsin-generated peptides
591 were vacuum dried, resuspended in 10 µl of loading buffer (2% acetonitrile and 0.05%
592 Trifluoroacetic acid in water) and analyzed by nanoLC-MSMS using a nanoElute liquid
593 chromatography system (Bruker) coupled to a timsTOF Pro mass spectrometer (Bruker).
594 Briefly, peptides were loaded on an Aurora analytical column (ION OPTIK, 25cm x75µm, C18,
595 1.6µm) and eluted with a gradient of 0-35% of solvent B for 100 min. Solvent A was 0.1 %

596 formic acid and 2% acetonitrile in water, and solvent B was 99.9% acetonitrile with 0.1%
597 formic acid. MS and MS/MS spectra were recorded and converted into mgf files. Proteins
598 identification were performed with Mascot search engine (Matrix science, London, UK)
599 against a database composed of all LGG-1 sequences including the wild-type and mutant
600 sequences. Database searches were performed using semi-trypsin cleavage specificity with
601 five possible miscleavages. Methionine oxidation was set as variable modification. Peptide
602 and fragment tolerances were set at 15 ppm and 0.05 Da, respectively. A peptide mascot
603 score threshold of 13 was set for peptide identification. C-terminal peptides were further
604 validated manually.

605

606 **Quantification and statistical analysis**

607 All experiments were done at least three times. All data summarization and statistical
608 analyses were performed by using either the GraphPad-Prism or the R software (www.r-project.org). The Shapiro-Wilk's test was used to evaluate the normal distribution of the
609 values and the Hartley Fmax test for similar variance analysis. Data derived from different
610 genetic backgrounds were compared by Student t test, Anova, Kruskal-Wallis or Wilcoxon-
611 Mann-Whitney tests. The Fisher's exact test was used for nominal variables. Longevity was
612 assessed using Log-Rank (Mantel-Cox) test. Error bars are standard deviations and boxplot
613 representations indicate the minimum and maximum, the first (Q1/25th percentile), median
614 (Q2/50th percentile) and the third (Q3/75th percentile) quartiles. NS (Not Significant $p > 0.05$;
615 * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $0.001 > p > 0.0001$ and **** $p < 0.0001$. Exact values of n
616 and statistical tests used can be found in the figure legends.

618

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858
 859
 860

861 Figure legends

862

863 **Figure 1 - G116A abolishes the conjugation of LGG-1 to the membrane but not its cleavage**

864 (A) Schematic representation of the various isoforms of Atg8s proteins after cleavage of the
 865 precursor and reversible conjugation to a phosphatidylethanolamine (PE).

866 (B) Diagram of the theoretical proteins produced by the allelic *lgg-1* series used in this study.
 867 LGG-1(Δ) protein corresponds to the reference allele *lgg-1(tm3489)*, considered as a null, all
 868 others mutants have been generated using CRISP-Cas9. Black arrows point to the di-glycine
 869 residues which are mutated in alanine or stop codon (*). Other deletion mutants of the C-
 870 terminus result from non-homologous end joining. The mapping of the epitopes recognized
 871 by the LGG-1 antibodies (Ab#1, 2, 3) used in this study are indicated by horizontal grey
 872 arrows.

873 (C) Western blot analysis of endogenous LGG-1 from total protein extracts of *wild-type*, *lgg-*
 874 *1(G116A)*, *lgg-1(G116AG117*)*, *lgg-1(G116AG117A)*, *lgg-1(Δ)* young adults. The data shown
 875 is representative of three experiments using Ab#3 and was confirmed with Ab#1. The
 876 theoretic molecular mass of the precursor, and the form I are 14.8kDa and 14.0 kDa,
 877 respectively, while the lipidated form II migrates faster. The asterisk indicates an unknown
 878 band. The quantification of each LGG-1 isoforms was normalized using tubulin.

879 (D-L) Immunofluorescence analysis of endogenous LGG-1 (Ab#1 or Ab#2) in early and late
 880 embryos in *wild-type* (D), *lgg-1(Δ)* (E), *lgg-1(G116A)* (F), *lgg-1(Δ 112-123)* (G), *lgg-*
 881 *1(G116AG117*)* (H), *lgg-1(G116AG117A)* (I), *lgg-1(Δ 100-123)* (J). Inset in E shows the
 882 corresponding DAPI staining of nuclei. Box-plots quantification showing the absence of
 883 puncta in all *lgg-1* mutants (K, left n= 19, 13, 11, 10, 6, 7, 6; right n= 18, 14, 12, 10, 10, 9, 12)
 884 and the increase of cytosolic staining in *lgg-1(G116A)* and *lgg-1(G116AG117*)* (L, n= 19, 13,
 885 11, 10). Kruskal Wallis test, p-value *<0.05, **<0.01, ****<0.0001, NS non-significant. Scale
 886 bar is 10 μ m.

887 (M) Cellular fractionation of membrane vesicles. Western blot analysis for detection of LGG-
888 1 together with LGG-2 (autophagosome marker), SEL-1 (ER marker), and CDC-48 (ER-
889 associated and cytosol) using supernatant (S) and pellet (P) fractions of *lgg-1 wild-type*, *lgg-1(G116A)*,
890 and *lgg-1(G116AG117*)* worm lysates treated with fractionation buffer (-),
891 sodium chloride (NaCl) or Triton X-100 (TX-100) after subcellular fractionation. Proteins
892 associated with membranes are solubilized by NaCl, and resident proteins in membrane-
893 bound organelles are released only by dissolving the membrane with detergents. While wild-
894 type LGG-1 is detected in the cytosolic fraction (input S) and in the various membrane
895 fractions, mutant LGG-1 is almost exclusively present in the cytosolic fraction in *lgg-1(G116A)*
896 and *lgg-1(G116AG117*)*.

897

898 **Figure 2 - *lgg-1(G116A)* and *lgg-1(G116AG117*)* mutants are viable with no developmental**
899 **defect**

900 (A-G) DIC images of embryos after morphogenesis in *wild-type* (A), *lgg-1(Δ)* (B), *lgg-1(G116A)*
901 (C), *lgg-1(G116AG117*)* (D), *lgg-1(G116AG117A)* (E), *lgg-1(Δ112-123)* (F), *lgg-1(Δ100-123)*
902 (G). *lgg-1(G116AG117A)*, *lgg-1(Δ112-123)*, *lgg-1(Δ100-123)*, *lgg-1(Δ)* mutant embryos
903 present severe developmental defects. Short and long white arrows point to the anterior (a)
904 and posterior (p) part of the pharynx, respectively. Scale bar is 10 μm.

905 (H) The viability, expressed as the percentage of embryos reaching adulthood, is not affected
906 in *lgg-1(G116A)* and *lgg-1(G116AG117*)* mutants (42<n<103).

907 (I) The fertility, total number of progenies, of *lgg-1(G116A)* and *lgg-1(G116AG117*)* adults is
908 similar to *wild-type* (n=20).

909

910 **Figure 3 - Autophagy is functional in *lgg-1(G116A)* but not in *lgg-1(G116AG117*)* and *lgg-1(G116AG117A)***
911

912 (A-E) *In vivo* epifluorescence images of paternal mitochondria (HSP-6::GFP) at the 1-cell and
913 15-cells stages in *wild-type* (A), *lgg-1(Δ)* (B), *lgg-1(G116A)* (C), *lgg-1(G116AG117*)*(D), *lgg-1(G116AG117A)*(E)
914 embryos showing an effective degradation of paternal mitochondria in wt
915 and *lgg-1(G116A)* but not in *lgg-1(Δ)* *lgg-1(G116AG117*)* and *lgg-1(G116AG117A)*.
916 Quantification are shown in (F).

917 (G, H) Bulk autophagy during aging and stress was assessed by worm longevity (G, log rank
918 test n > 100 animals, p-value ****<0.001) and starvation survival (H, Chi-square test at day
919 15 p-value ****<0.001). The survival is significantly reduced in *lgg-1(Δ)*, *lgg-1(G116AG117*)*
920 and *lgg-1(G116AG117A)* compared to wt and *lgg-1(G116A)*. NS non-significant.

921 (I) Box-plots quantification of apoptotic corpses showing a defective degradation in *lgg-1(G116AG117*)*
922 and *lgg-1(G116AG117A)* but not in *lgg-1(G116A)* (n=22, 40, 46, 14, 21
923 Kruskal Wallis test ***<0.001, ****<0.0001, NS non-significant).

924

925 **Figure 4 - Autophagy but not developmental function of LGG-1(G116A) partially depends**
926 **on LGG-2**

927 (A-F) DIC images of embryos and bright field images of adults in *wild-type* (A), *lgg-2(tm5755)*
928 (B, E), *lgg-1(G116A); lgg-2(tm5755)* (C, F). The double mutant *lgg-1(G116A); lgg-2(tm5755)*
929 animals have no morphogenetic defects and no decrease in viability compare to single
930 mutants or the *lgg-1(Δ)* (quantification in D).
931 (G-H) Bulk autophagy during stress and aging was assessed by starvation survival (G, Chi-
932 square test at day 9 ***p-value<0.001) and worm longevity (H, log rank test n>100 animals,
933 ***p-value<0.001, ****p-value<0.0001). The survival of double mutants *lgg-1(G116A); lgg-2(tm5755)*
934 and *lgg-1(G116AG117*); lgg-2(5755)* is reduced compared to *wild-type* and single
935 mutant *lgg-1(G116A)* and *lgg-2(tm5755)*. *lgg-1(G116A); lgg-2(tm5755)* animals survive to
936 starvation better than *lgg-1(G116AG117*); lgg-2(5755)* and present a slightly higher lifespan.
937 (I-K) *In vivo* epifluorescence imaging of paternal mitochondria (HSP-6::GFP) at the 1-cell, 15-
938 cells and 30-cells stages in *lgg-2(tm5755)*, (I) *lgg-1(G116A); lgg-2(tm5755)* (J) embryos and
939 quantification (n=50, 39, 35, 45, 46 Chi-square test ****<0.0001) (K). Elimination of
940 mitochondria is efficient but delayed in *lgg-1(G116A); lgg-2(5755)* compared to *lgg-1(G116A)*.
941 Insets show the corresponding DIC pictures.
942 Scale bar is 10 μm (A-C, I, J) or 100 μm (E, F).

943

944 **Figure 5 - The degradation of autophagosomes is delayed in *lgg-1(G116A)***

945 (A-F) Confocal images of LGG-2 immunofluorescence in 2 cells, 4 cells and 15 cells in *wild-*
946 *type* (A), *lgg-1(Δ)* (B), *lgg-1(G116A)* (C), *lgg-1(G116AG117*)* (D) and quantification of the
947 number (E) and size of puncta (F) (embryo analyzed 19, 37, 28, 14; Mann-Whitney test, p-
948 value ****<0.0001). In *lgg-1(G116A)* and *lgg-1(G116AG117*)* mutants LGG-2 is detected as
949 heterogeneous sparse structures that persist.

950 (G-J) Colocalization analysis of paternal mitochondria (HSP-6::GFP) and LGG-2 puncta (H)
951 from confocal images of *wild-type* (H), *lgg-1(G116A)* (I) and *lgg-1(G116AG117*)* (J) early
952 embryos. (Mean + SD, n = 16, 20, 12, Kruskal Wallis test p-value*<0.05). The clustering of
953 paternal mitochondria and LGG-2 autophagosomes are absent in *lgg-1(G116A)* and *lgg-1(G116AG117*)*
954 where HSP-6::GFP and LGG-2 puncta are mainly separated with rare
955 colocalization events (yellow arrows).

956 (K) Western blot analysis of endogenous LGG-2 from total protein extracts from *wild-type*,
957 *lgg-1(G116A)*, *lgg-1(G116AG117*)*, *lgg-1(Δ)* young adults. The quantification of LGG-2 upper
958 and lower bands was normalized using tubulin.

959

960 **Figure 6 - The cleaved LGG-1 is sufficient for autophagosome biogenesis**

961 (A-J) Electron microscopy images of autophagosomes in *wild-type* (A), *lgg-1(Δ)* (B), *lgg-1(G116A)*
962 (C-E), *lgg-1(G116AG117*)* (F-G), *lgg-1(G116A); lgg-2(tm5755)* (H), *lgg-1(G116AG117*) lgg-2(tm5755)*
963 (I) and *lgg-2(tm5755)* (J) early embryos. Type 1
964 autophagosomes (A, C, D) appear as closed structures containing various membrane
965 organelles. Among those, sequestered paternal mitochondria (black arrows) are observed in
966 *wild-type* and *lgg-1(G116A)* embryos but remain unsequestered in *lgg-1(Δ)* and *lgg-1(G116AG117*)*
967 embryos. Type 2 autophagosomes (E, white arrow in H, J) appear as closed

968 structures containing unidentified or degraded materials. Type 3 structures (G, I) are multi-
969 lamellar structures only detected in *lgg-1(G116AG117*)* embryos. Scale bar is 200 nm.
970 (K) Quantification of type 1, type 2 and type 3 structures in early embryo (1 to 12 cells). In
971 *lgg-1(G116A)* embryos, the numbers of type 1 and type 2 autophagosomal structures
972 increase supporting a retarded degradation. The formation of autophagosomes in *lgg-*
973 *1(G116A)* and *lgg-1(G116AG117*)* embryos is partially dependent of LGG-2 (n sections= 32,
974 62, 32, 19, 32, 26, 52).

975

976 **Figure 7 - The lipidated LGG-1 is involved in the coordination between cargo recognition**
977 **and autophagosome biogenesis.**

978 (A-E) Confocal images of ATG-18::GFP (green) and LGG-2 (red) immunofluorescence in *wild-*
979 *type* (H), *lgg-1(RNAi)* (I), *lgg-1(G116A)* (J), *lgg-1(G116AG117*)* (K) 100-cells embryos. Insets
980 are 2-fold magnification of the white boxed regions. (E) Compared to ATG-18 puncta the
981 number of colocalization is decreased in *lgg-1(RNAi)* (p-value <0.05) and *lgg-1(G116AG117*)*
982 (p-value* <0.001) but not *lgg-1(G116A)* embryos (mean + SD, n= 10, 10, 10, 10; Kruskal Wallis
983 p-value* <0.05 ** <0.01).

984 (F-K) Quantification (F) and maximum projections of epifluorescence images of the
985 aggrephagy cargo SEPA-1::GFP in 1.5 fold embryos for *wild-type* (G), *lgg-1(Δ)* (H), *lgg-*
986 *1(G116A)* (I), *lgg-1(G116AG117*)* (J) and *lgg-1(G116AG117A)* (K). Boxplots of SEPA-1::GFP
987 dots (n=10) (F) indicate that the degradation is stronger in *lgg-1(G116A)* embryos than in *lgg-*
988 *1(RNAi)*, *lgg-1(G116AG117*)* and *lgg-1(G116AG117A)* but weaker than in *wt*.

989 (L-P) Confocal images of SEPA-1::GFP (green) and LGG-1 (L, M) or LGG-2 (N, O) (red)
990 immunofluorescence in *wild-type* (L, N) and *lgg-1(G116A)* (M, O) 100-cells embryos. Insets
991 are 2.5-fold magnification of the white boxed regions. In *lgg-1(G116A)* embryos LGG-2-
992 positive/ LGG-1-negative autophagosomes are detected close to SEPA-1::GFP cargos but with
993 a decreased overlap. P) Box-plots of the overlap between green and red pixels (Manders
994 coefficient) in *wild-type* and *lgg-1(G116A)* (n=11, 13; Mann-Whitney test ** <0.01).

995 Scale bar is 10 μm (A-K) or 5 μm (L- O).

996

997

998 **Supplementary figures**

999

1000 **Figure 1-figure supplement 1 - Description of *lgg-1* alleles**

1001 (A) Schematic representation of *lgg-1* locus indicating the exon/intron composition and the
1002 localization of the PAM used for CRISPR-Cas9. The names of the alleles and the theoretical
1003 sequences of the proteins series are indicated. Residues in red are the results of the point
1004 mutations and residues in blue italics are the theoretical consequence of frameshifts. The di-
1005 glycine motif in position 116-117 is shown in green and the red arrow points to the
1006 conserved glycine 116. The grey box shows the aligned sequence of *S. cerevisiae* Atg8

1007 (B) Comparison of Atg8/GABARAP homologs in worm (Ce), fly (Dm), human (Hs), mouse
1008 (Mm), zebrafish (Dr), plants (At, Gm), yeasts (Sp, Sc), amoeba (Dd) and parasite protozoa

1009 (Ld), identified in the eukaryotic proteomes from NCBI landmark Blast database. The
1010 conserved core sequence (blue box) is represented using a LOGO analysis (after removal of a
1011 small insertion in At Atg8D and in two Gm uncharacterized homologs) and the variable N-
1012 and C-terminus sequences are indicated in light beige. Green arrows points to the invariable
1013 glycine 116.

1014

1015 **Figure 1-figure supplement 2 - Identification of LGG-1(G116A) and LGG-1(G116AG117*) forms**

1016 **(A)** Western blot analysis of affinity purified samples using LC3 traps. Molecular weight
1017 markers (kDa) are indicated on the right.

1018 **(B)** Protein sequence, and peptides coverage of LGG-1(G116AG117*) identified by mass
1019 spectrometry analyses after trypsin treatment (blue underline). The MS/MS fragmentation of
1020 the C-terminal peptide 89-116 (green) identifying the truncated form is shown below.

1021 **(C)** Protein sequence and peptides coverage of LGG-1(G116A) identified by mass
1022 spectrometry analyses after trypsin treatment (blue underline). The yellow underline
1023 indicates a C-terminal 111-123 peptide identified after trypsin and AspN digestion. The
1024 MS/MS fragmentation of the C-terminal peptide 107-121 (red box) identifying the precursor
1025 form and peptide 89-116 (green) identifying the cleaved form are shown below.

1026 **(D-G)** Mutant for the protease *atg-4.1(bp501)* (D) still presents few small puncta (arrows) in
1027 wild-type due to the presence of a paralog, *atg-4.2*. Autophagosome maturation defective
1028 *epg-5(RNAi)* embryos accumulate LGG-1 positive autophagosomes (E) but do not show
1029 puncta in *lgg-1(G116A)* and *lgg-1(G116AG117*)* embryos (F, G). Scale bar is 10 μ m.

1030 **(H)** Schematic representation of the forms present in the wild-type and *lgg-1* mutants based
1031 on western-blot, immunofluorescence and mass spectrometry analyses.

1032

1033 **Figure 1-figure supplement 3 - GFP::LGG-1(G116A) does not localize to autophagosomes**

1034 **(A)** Western blot analysis of GFP::LGG-1 and GFP::LGG-1(G116A) using Ab#3, from total
1035 protein extracts from young adults. Accumulation of the precursor but absence of form II is
1036 observed. Molecular weight markers (kDa) are indicated on the left.

1037 **(B)** Schema of the experimental approach indicating the GFP reporters and the autophagy
1038 steps affected upon specific RNAi depletion. A 60 minutes heat-stress at 37°C induces an
1039 autophagy flux.

1040 **(C-D)** *In vivo* epifluorescence images of GFP::LGG-1(wt) (left columns) and GFP::LGG-
1041 1(G116A) (right columns) in the epidermis of *control*, *atg-3(RNAi)*, *atg-7(RNAi)*, *epg-
1042 5(RNAi)*, and *rab-7(RNAi)* 4th stage larvae. GFP::LGG-1(wt) puncta indicate the
1043 autophagosomes formed upon autophagy induction. The alteration of the conjugation
1044 process (*atg-3*, *atg-7*) or the fusion with the lysosome (*epg-5*, *rab-7*) results in a decrease or
1045 an accumulation of the puncta, respectively. GFP::LGG-1(G116A) does not localize to
1046 autophagosomes even in the conditions of strong accumulation. The zoomed images are 3
1047 fold magnifications of the dotted rectangles. Scale bar is 50 μ m. Quantifications are shown in
1048 (D) (n=10, 10, 6, 7, 10, 10, 4, 8, 10, 10, 7, 10, 10, 10, 4, 6, 10, 10, 5, 6).

1049 (E-F) Electron microscopy images of GFP-immunolabeled GFP::LGG-1(wt) and GFP::LGG-
1050 1(G116A) in the epidermis of 4th stage larvae after heat stress. Arrows indicates gold beads
1051 present on autophagosomes and quantifications are shown in (F). In GFP::LGG-1(G116A)
1052 animals labelled autophagosomes are very rare and beads are located in the lumen (n=110,
1053 116, from 4 independent immunostainings). Scale bar is 200 nm.

1054

1055 **Figure 3-figure supplement 1 - Autophagy is functional in *lgg-1(G116A)* but not in *lgg-*
1056 **1(G116AG117*)****

1057 (A-B) *In vivo* epifluorescence images of paternal mitochondria (left, mitotracker) and
1058 corresponding DIC images (right) at the 1-4 cells and 20-50 cells stages in *wild-type*, *lgg-1(Δ)*,
1059 *lgg-1(G116A)*, *lgg-1(G116AG117*)* embryos showing an effective degradation of paternal
1060 mitochondria in *wt* and *lgg-1(G116A)* but not in *lgg-1(Δ)* *lgg-1(G116AG117*)*. Quantifications
1061 are shown in (B).

1062 (C) DIC images of 1.5 fold stage *wild-type*, *lgg-1(Δ)*, *lgg-1(G116A)*, *lgg-1(G116AG117*)* and
1063 *lgg-1(G116AG117A)* embryos. Apoptotic corpses in the head region are indicated by yellow
1064 arrows. The quantification of apoptotic corpses is shown in Figure 3I. Scale bar is 10 μm.

1065

1066 **Figure 3-figure supplement 2 - Atg8(G116A) and Atg8(G116AR117*) are functional for**
1067 **vacuolar shaping but not for autophagy in *S. cerevisiae***

1068 (A) Schematic representation of wild-type and mutant Atg8 proteins.

1069 (B) Autophagic activity is abolished in *S. cerevisiae* mutant strains *atg8G116A* or *atg8G116A-*
1070 *R117Δ* forms of Atg8. Autophagic activity of wild-type, *atg1Δ*, *atg8Δ*, *atg8G116A* and
1071 *atg8G116AR117** strains was measured using the quantitative Pho8Δ60 assay, before (+N)
1072 and after (-N) 4 hours of nitrogen starvation. The data shown are the mean ± s.e.m. of 3
1073 independent experiments (Mann-Whitney test, p-value****<0.0001).

1074 (C) *S. cerevisiae* mutants *atg8Δ*, *atg8G116A* or *atg8G116AR117** have a similar cell viability
1075 defect upon nitrogen starvation.

1076 (D) Epifluorescence image of FM4-64 staining of the vacuole of *wild-type* and *atg8* mutant
1077 strains (single focal plan).

1078 (E) Quantification of vacuoles in wild-type and *atg8* mutants. *atg8G116A* and
1079 *atg8G116AR117** mutants present a less severe vacuolar phenotype than *atg8Δ* mutant (n >
1080 200 cells, Chi-square test, p-value *<0.05, ***<0.001, ****<0.0001).

1081 (F) Rescue assays of *atg8Δ* by *lgg-1(wt)*, *lgg-1(G116A)* and *lgg-1(G116AG117*)*. In the upper
1082 part, the survival in nitrogen starvation of *atg8Δ* cells transformed with *lgg-1(wt)* or *ATG8* is
1083 compared with *atg8Δ* and *wild-type*. In the lower part, the survival in nitrogen starvation of
1084 *atg8Δ* cells transformed with *lgg-1(wt)*, *lgg-1(G116A)*, *lgg-1(G116AG117*)* are compared
1085 with *atg8Δ*. The percentage of surviving cells was calculated in comparison with day 0. *lgg-*
1086 *1(G116A)* expression improves the survival but less efficiently than *lgg-1(wt)*. Black and red
1087 stars show statistical difference with wild-type and *atg8Δ*, respectively, Mann-Whitney test,
1088 p-value **<0.001).

1089

1090 **Figure 5- figure supplement 1 - Colocalization quantification of HSP-6::GFP and LGG-2**
1091 Quantification of puncta positive for the paternal mitochondria marker HSP-6::GFP, the
1092 autophagosomal marker LGG-2 and both, in early embryos *wild-type*, *lgg-1(G116A)* and *lgg-*
1093 *1(G116AG117*)* (Mean + SD, n= 16, 20, 12 Kruskal Wallis p-value* <0.05 , ** <0.01).

1094

1095 **Figure 7- figure supplement 1 - LGG-1(G116A) function in aggrephagy is dependent on**
1096 **UNC-51 and EPG-2**

1097 (A-H) *In vivo* confocal images of ATG-18::GFP, showing the initiation of autophagosome
1098 biogenesis, in 50-100 cells embryos for *wild-type* (A), *lgg-1(Δ)*(B), *lgg-1(G116A)* (C), *lgg-*
1099 *1(G116AG117*)* (D), *unc-51 (RNAi)*(E), *unc-51(RNAi);lgg-1(Δ)*(F), *unc-51(RNAi);lgg-*
1100 *1(G116A)*(G) and *unc-51(RNAi);lgg-1(G116AG117*)*(H).

1101 (I, J) Boxplots of the number of ATG-18::GFP puncta (P) (n=13, 24, 10, 14, 28, 36, 27, 20) and
1102 the mean fluorescent intensity (Q)(n= 42, 45, 55, 140, 145, 130,115, 100) (Kruskal-Wallis test
1103 p-value * <0.05 , ** $p<0.01$ *** $p<0.001$, **** $p<0.0001$, NS non-significant)

1104 (K-O) Maximum projections of epifluorescence images of the aggrephagy cargo SEPA-1::GFP
1105 in 1.5 fold embryos for *epg-2(RNAi)* (K), *lgg-1(G116A) epg-2(RNAi)* (L), *lgg-1(G116AG117*)*
1106 *epg-2(RNAi)* (M) and *lgg-1(G116AG117A) epg-2(RNAi)* (N). Boxplots of SEPA-1::GFP dots
1107 (n=10, Kruskal Wallis p-value*** <0.001 , NS non-significant) (O). The degradation of SEPA-
1108 1::GFP in *lgg-1(G116A)* animals is dependent on EPG-2. Scale bar is 10 μ m.

1109

1110 **Source data**

1111

1112 **Figure1-Source_Data1**

1113 Folder containing original microscopy pictures, quantification data and western blots shown
1114 in Figure 1

1115 **Figure2-Source_Data1**

1116 Folder containing original microscopy pictures and quantification data shown in Figure 2

1117 **Figure3-Source_Data1**

1118 Folder containing original microscopy pictures and quantification data shown in Figure 3

1119 **Figure4-Source_Data1**

1120 Folder containing original microscopy pictures and quantification data shown in Figure 4

1121 **Figure5-Source_Data1**

1122 Folder containing original microscopy pictures, quantification data and western blots shown
1123 in Figure 5

1124 **Figure6-Source_Data1**

1125 Folder containing original microscopy pictures and quantification data shown in Figure 6

1126 **Figure7-Source_Data1**

1127 Folder containing original microscopy pictures and quantification data shown in Figure 7

1128 **Figure1-figure-supplement2-Source_Data1**

1129 Folder containing original microscopy pictures and quantification data shown in Figure1-
1130 figure-supplement2

1131 **Figure1-figure-supplement3-Source_Data1**
1132 Folder containing original microscopy pictures and quantification data shown in Figure1-
1133 figure-supplement3
1134 **Figure3-figure-supplement1-Source_Data1**
1135 Folder containing original microscopy pictures and quantification data shown in Figure3-
1136 figure-supplement1
1137 **Figure3-figure-supplement2-Source_Data1**
1138 Folder containing original microscopy pictures and quantification data shown in Figure3-
1139 figure-supplement2
1140 **Figure5-figure-supplement1-Source_Data1**
1141 Folder containing quantification data shown in Figure5-figure-supplement1
1142 **Figure7-figure-supplement1-Source_Data1**
1143 Folder containing original microscopy pictures and quantification data shown in Figure7-
1144 figure-supplement1
1145

Appendix 1

LGG-1(wt) and LGG-1(G116A) partially restore the survival to nitrogen starvation of yeast *atg8*(Δ)

Our results demonstrate that the localization of LGG-1 to the membrane is dispensable for autophagy and other functions. To address whether it is a particularity of *C. elegans*, a similar strategy was performed for Atg8 in the yeast *S. cerevisiae*. Atg8 precursor ends with an arginine at position 117 (Figure 1-figure supplement 3A) and using mutant proteins expressed from centromeric plasmids the Oshumi lab (Kirisako et al., 2000; Nakatogawa et al., 2012) has shown that G116 is essential for autophagy. The endogenous *ATG8* was modified by an homologous recombination strategy to generate *atg8(G116A)* and *atg8(G116AR117*)* alleles, and the autophagy flux was assessed using the Pho8 Δ 60 reporter (Noda and Klionsky, 2008). Both *atg8(G116A)* and *atg8(G116AR117*)* mutants were unable to achieve a functional autophagy and behave similarly to *atg8*(Δ) or *atg1*(Δ) null mutants (Figure 1-figure supplement 3B). The analysis of nitrogen starvation survival showed that both *atg8(G116A)* and *atg8(G116AR117*)* strains were unable to recover after a 4 days starvation, similarly to *atg1* Δ and *atg8* Δ (Figure 1-figure supplement 3C).

The non-autophagy function of *atg8(G116A)* and *atg8(G116AR117*)* mutants was assessed by analyzing the shape of the vacuole (Banta et al., 1988). During exponential growth, *atg8*(Δ) cells frequently presented multiple small vacuoles compared to wild-type cells which harbor usually less than 4 vacuoles (Figure 1-figure supplement 3D, E). The incidence of defective vacuolar shape decreased in *atg8(G116A)* and *atg8(G116AR117*)* cells indicating that the non autophagy functions were partially maintained. These data suggest that in *S. cerevisiae* the non-autophagy functions of Atg8 are partially independent of its cleavage and conjugation. *Atg8* mutant was then used to investigate whether the functionality of LGG-1(G116A) in autophagy was restricted to *C. elegans*. The capacity of LGG-1(G116A) to restore nitrogen starvation survival to *atg8*(Δ) mutant cells was compared with LGG-1(wt) and LGG-1(G116AG117*). The corresponding cDNAs were cloned in a centromeric vector and the proteins were expressed in *atg8*(Δ) mutants. The expression of LGG-1(wt) and LGG-1(G116A), but not LGG-1(G116AG117*), improved weakly the nitrogen starvation survival indicating a partial complementation (Figure 1-figure supplement 3F). This suggests that the functionality of LGG-1(G116A) is not restricted to *C. elegans*, supporting an intrinsic property of LGG-1 form I.

LGG-1(G116A) function in aggrephagy is dependent on UNC-51 and EPG-2

Data in yeast and mammals have revealed that Atg8/LC3/GABARAP can interact with Atg1/ULK1 and modify the kinase activity of the ULK1 complex (Alemu et al., 2012; Grunwald et al., 2020; Kraft et al., 2012; Nakatogawa et al., 2012). In *C. elegans*, LGG-1 can directly binds

41 UNC-51/ULK1 (Wu et al., 2015) and the cargo SEPA-1 (Zhang et al., 2009) and could have an
42 early function for initiating aggrephagy (Lu et al., 2011). To decipher the function of LGG-1
43 form I in the induction of autophagy we performed a genetic approach. Using RNAi we
44 depleted UNC-51 and quantified the initiation events *in vivo* in wild-type, *lgg-1(Δ)*, *lgg-*
45 *1(G116A)*, and *lgg-1(G116AG117*)* embryos (Figure 7-figure supplement 1A-J). As expected,
46 depleting UNC-51 resulted in the decrease of ATG-18 puncta while depleting LGG-1 lead to
47 the increase of ATG-18 intensity and number of puncta. The decrease of ATG-18::GFP puncta
48 after co-depletion of UNC-51 and LGG-1 indicated that LGG-1 functions depends on UNC-51.
49 For *lgg-1(G116A)* animals, a small decrease was observed in the number of puncta compared
50 to the wild-type animals but no change in the total signal of ATG-18::GFP. Moreover, the
51 depletion of UNC-51 further decreased ATG-18::GFP puncta and intensity confirming that
52 LGG-1(G116A) almost behaves like the wild-type LGG-1 for the initiation (Figure 7-figure
53 supplement 1C, G, I, J). In *lgg-1(G116AG117*)* embryos, a marked increase of ATG-18 puncta
54 was observed, but contrarily to *lgg-1(Δ)*, the number did not decrease when UNC-51 was
55 depleted (Figure 7-figure supplement 1D, H, I, J). These data confirm electron microscopy
56 observations and support a neomorphic function for the truncated LGG-1(G116AG117*),
57 independent of ULK1 complex.

58 Finally, we analyzed whether the cargoes degradation by LGG-1(G116A) was dependent of the
59 scaffolding protein EPG-2 (Figure 7-figure supplement 1K-O). Aggregate-prone proteins are
60 degraded through autophagy in *C. elegans* embryo through liquid-liquid phase separation
61 promoted by the receptor SEPA-1 and regulated by the scaffolding protein EPG-2 (Zhang et
62 al., 2018). The depletion of EPG-2 induced the persistence of SEPA-1::GFP aggregates in wild-
63 type and in *lgg-1(G116A)* embryos. This data indicates that the function of LGG-1(G116A) for
64 degrading SEPA-1 is dependent of EPG-2.

65

66 **Supplementary Material and Methods**

67

68 **Immunostaining and electron microscopy**

69 200 μm-deep flat carriers (Leica Biosystems) were incubated few minutes in 1%
70 phosphatidylcholine (Sigma-Aldrich,61755) in chloroform. Young adults were transferred to
71 the carriers containing 20% BSA (Sigma-Aldrich, A7030) in M9 buffer, followed by cryo-
72 immobilization in the EMPACT-2 HPF apparatus (Leica Microsystems) and cryo-substitution
73 with Automated Freeze-substitution System (AFS2, Leica Microsystems). Cryosubstitution
74 medium, composed by 0.1% acetate uranyl in acetone, for 3 days with a slow increase of the
75 temperature from -90°C to -15°C. After several washes of acetone and ethanol at -15°C,
76 samples were incubated successively in 25% to 100% LRWHITE resin (Electron Microscopy
77 Sciences, 14381) in ethanol, then UV-polymerized 24 hours at -15°C. 80 nm thin sections
78 were collected on a Nickel 100-mesh grids (Electron Microscopy Sciences, FCF-100-Ni) and
79 immunostained with the immunogold labelling system (IGL, Leica microsystem). Samples were
80 labelled during 1 h with the primary rabbit anti-GFP antibody (Abcam, ab6556; 1:10 dilution
81 in 0.1% BSA in PBS), washed 4 times for 2min with PBS, and twice for 5 min with 0.1% BSA in

82 PBS. Samples were then labelled during 30 min with the secondary goat anti-rabbit antibody
83 coupled to 10-nm colloidal gold particles (Biovalley, 810.011) at 1:20 dilution in 0.1% BSA in
84 PBS. Sections were contrasted with 2% uranyl acetate (Merck, 8473) for 8 min and 0.08 M lead
85 citrate (Sigma-Aldrich, 15326) for 2 min, and were observed with a Jeol 1400 TEM at 120 kV
86 equipped with a Gatan SC1000 Orius CCD camera (Roper Industries).

87

88 ***S. cerevisiae* culture and strains**

89 Yeast cells were grown to log phase in YPD (1% yeast extract, 2% bactopectone and 2%
90 glucose) or complete synthetic medium (CSM) without uracil or leucine. The reference strain
91 is BY4742. Other strain and genotypes are listed in the Key Resources Table.

92

93 ***S. cerevisiae* culture and autophagy assays**

94 The quantitative Pho8Δ60 assay for bulk autophagy, was performed as described (Noda and
95 Klionsky, 2008). Cells were grown to log phase in YPD medium then were transferred to
96 nitrogen starvation medium for 4h. At different time point, 5 OD₆₀₀ units of cells were
97 collected, washed and resuspended in ice-cold assay buffer (250mM Tris-HCl, pH 9; 10mM
98 MgSO₄ and 10 μM ZnSO₄) with 1mM PMSF. Then cells were broken using glass beads. For the
99 assay, 10 μl of lysed cells are added to 500 μl of ice-cold assay buffer, placed at 30°C for 5 min
100 before adding 50 μl of 55 mM α-naphthyl phosphate disodium salt for 20 min at 30°C. The
101 reaction was stopped with 500 μl of 2M glycine-NaOH, pH 11 and the fluorescence measured
102 (345 nm excitation /472 nm emission). The Pho8Δ60 activity corresponds to light emission per
103 amount of protein in the reaction (mg) and reaction time (min).

104 The number of vacuoles was counted after incubation of exponentially growing cells with
105 FM4-64 (33 μM) in YPD medium at 30°C for one hour, washing and imaging.

106 For survival to nitrogen starvation cells were grown to log phase in appropriate complete
107 synthetic medium (CSM) and transferred to nitrogen starvation medium (0.17% yeast nitrogen
108 base and 2% glucose). After 0 to 6 days of starvation, cells were spread on YPD plates and
109 colonies were counted after 2 days at 30°C. For LGG-1 rescue assays, LGG-1(G116A) and LGG-
110 1(G116AG117*) were generated by PCR amplification from cDNA LGG-1 and cloned in pRS416
111 vector under the control of GPD promoter.

112

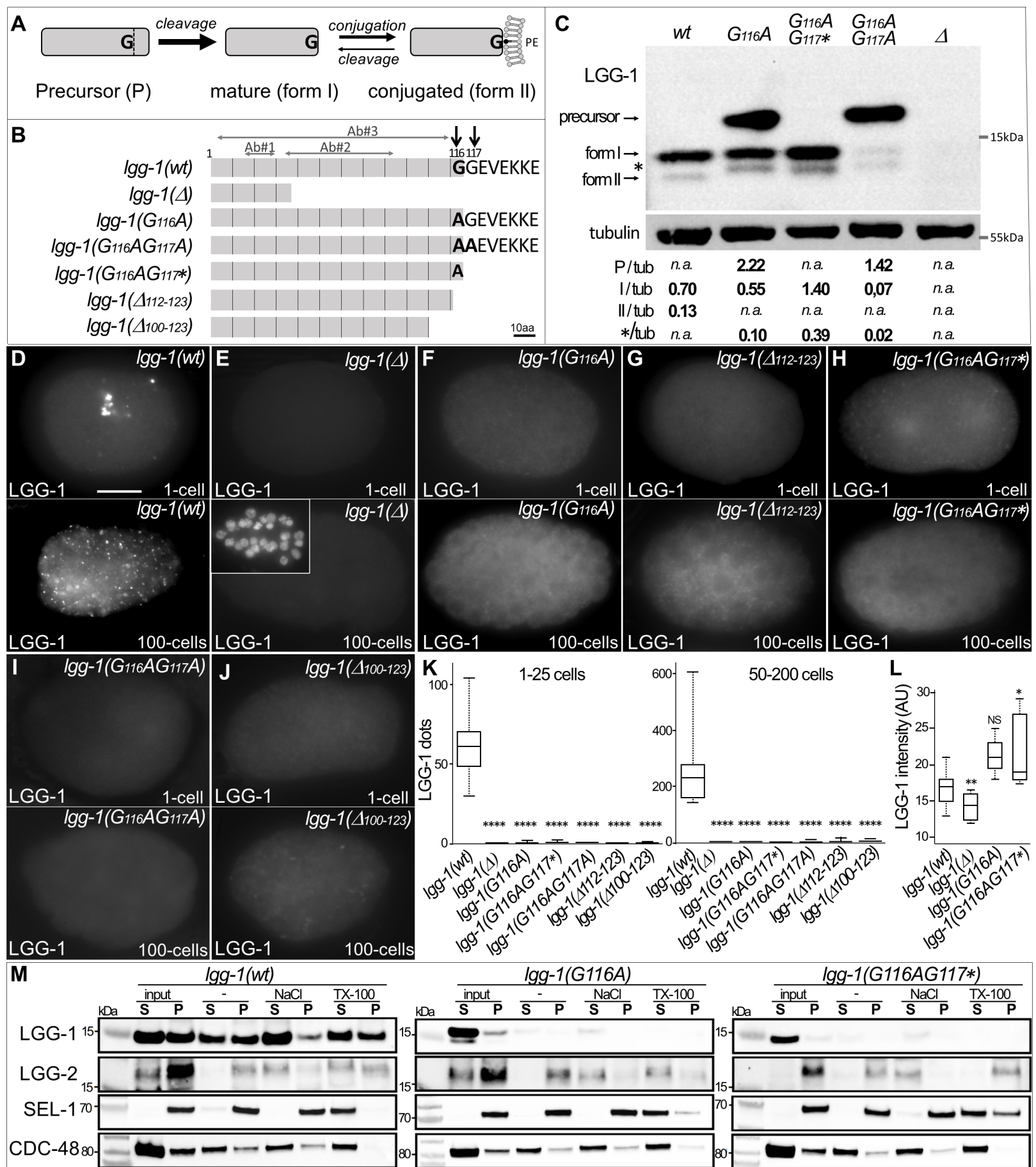


Figure 1 Leboutet et al.

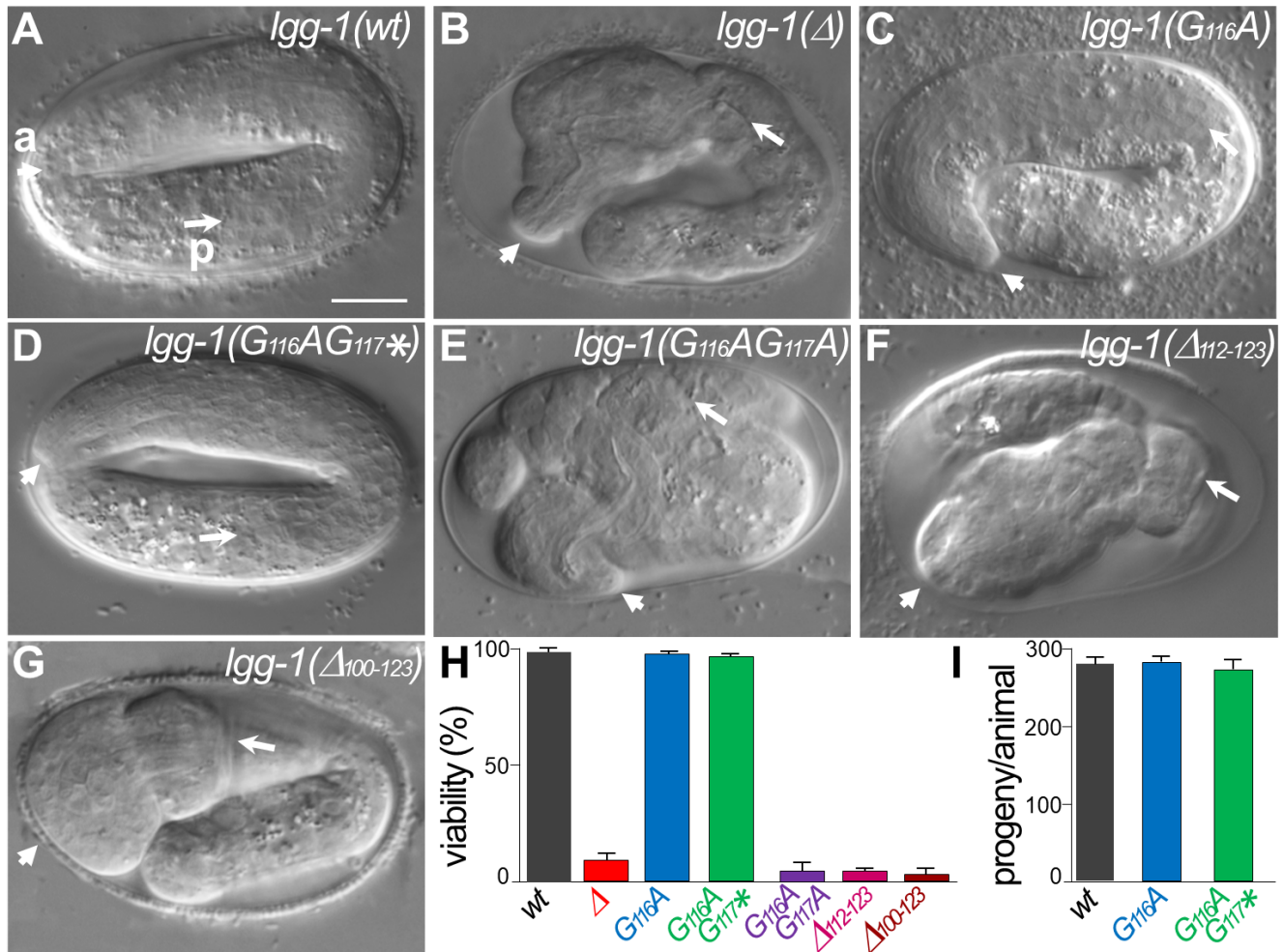


Figure 2 Leboutet et al.

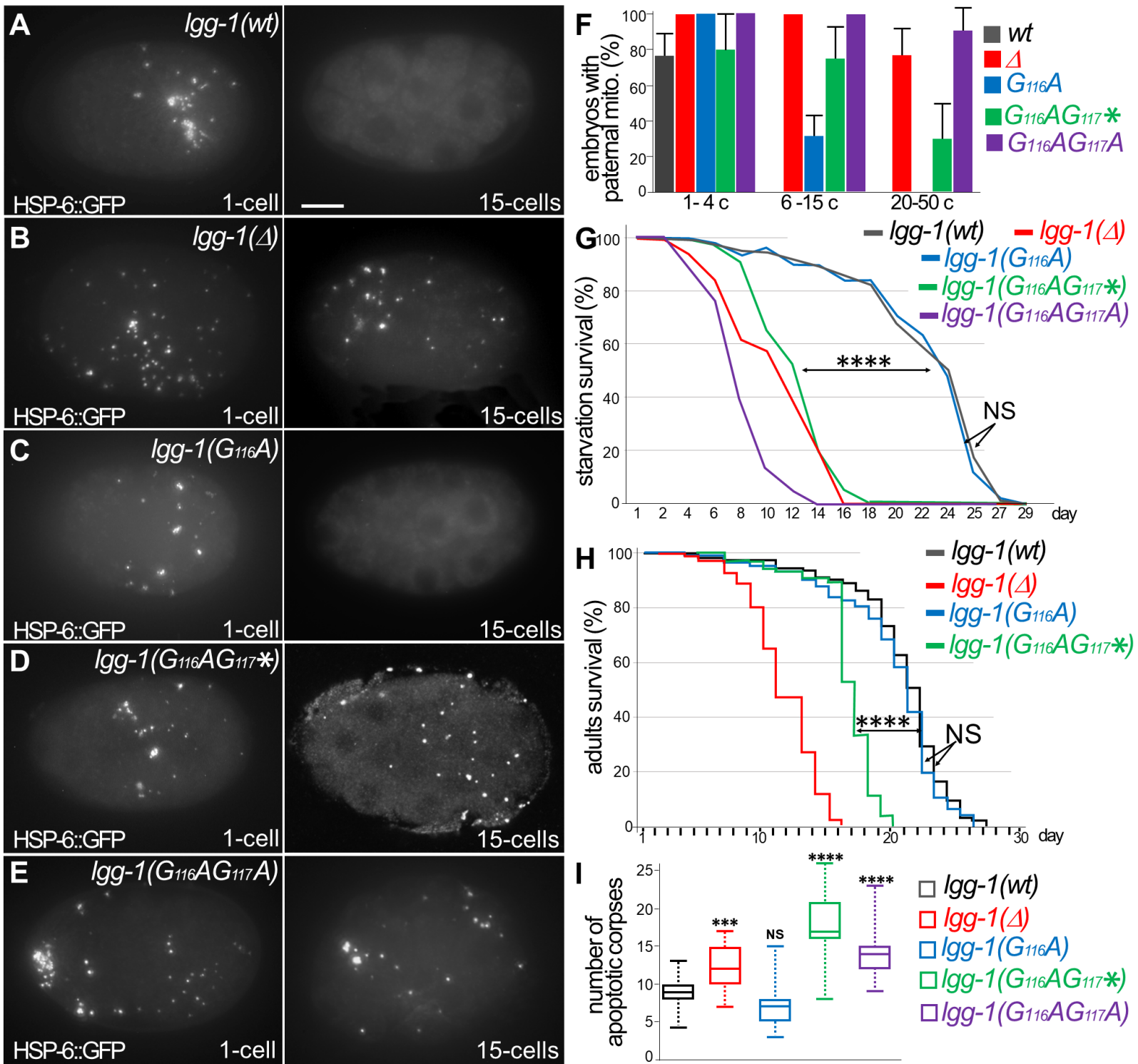


Figure 3 Leboutet et al

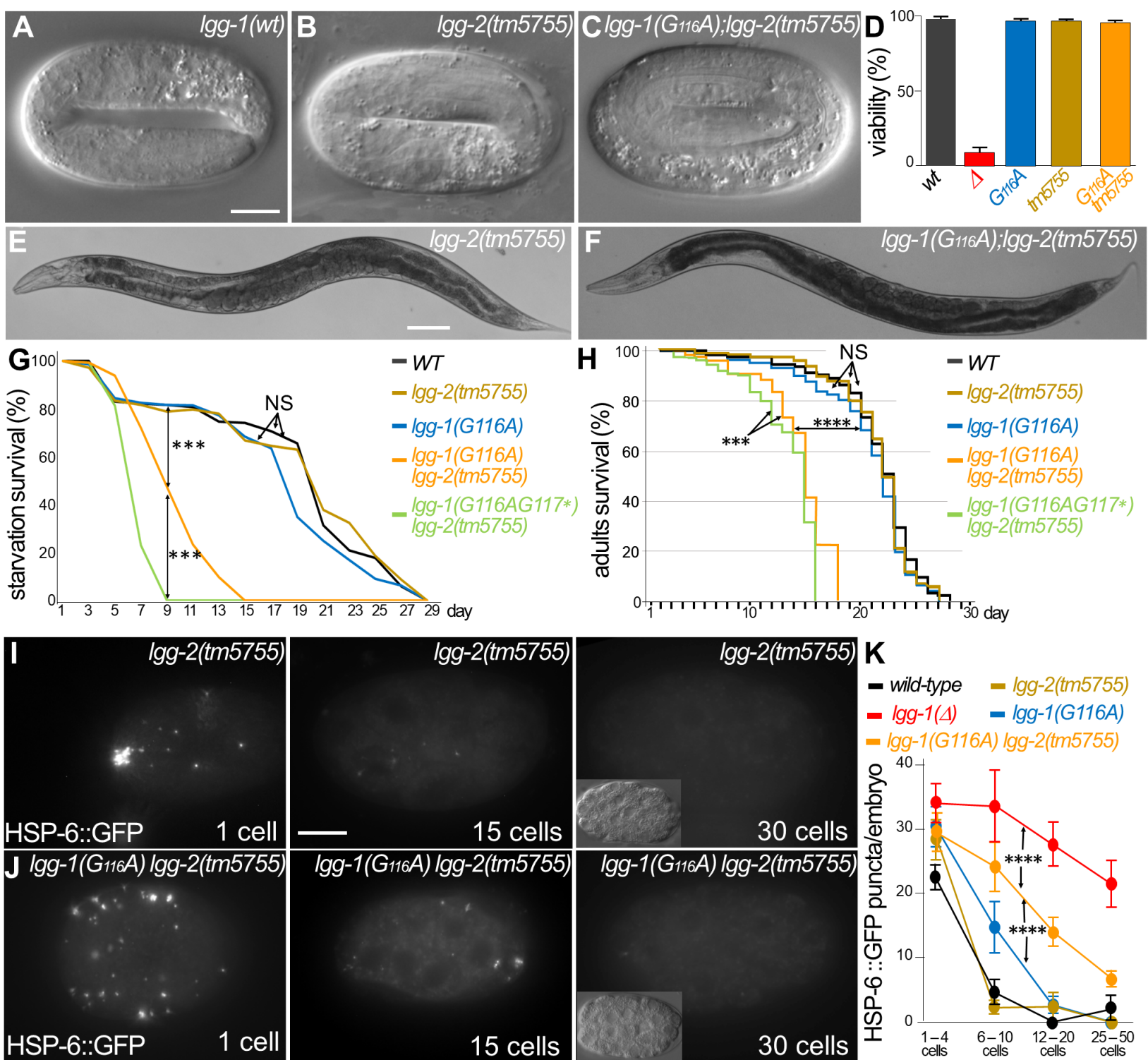


Figure 4 Leboutet et al.

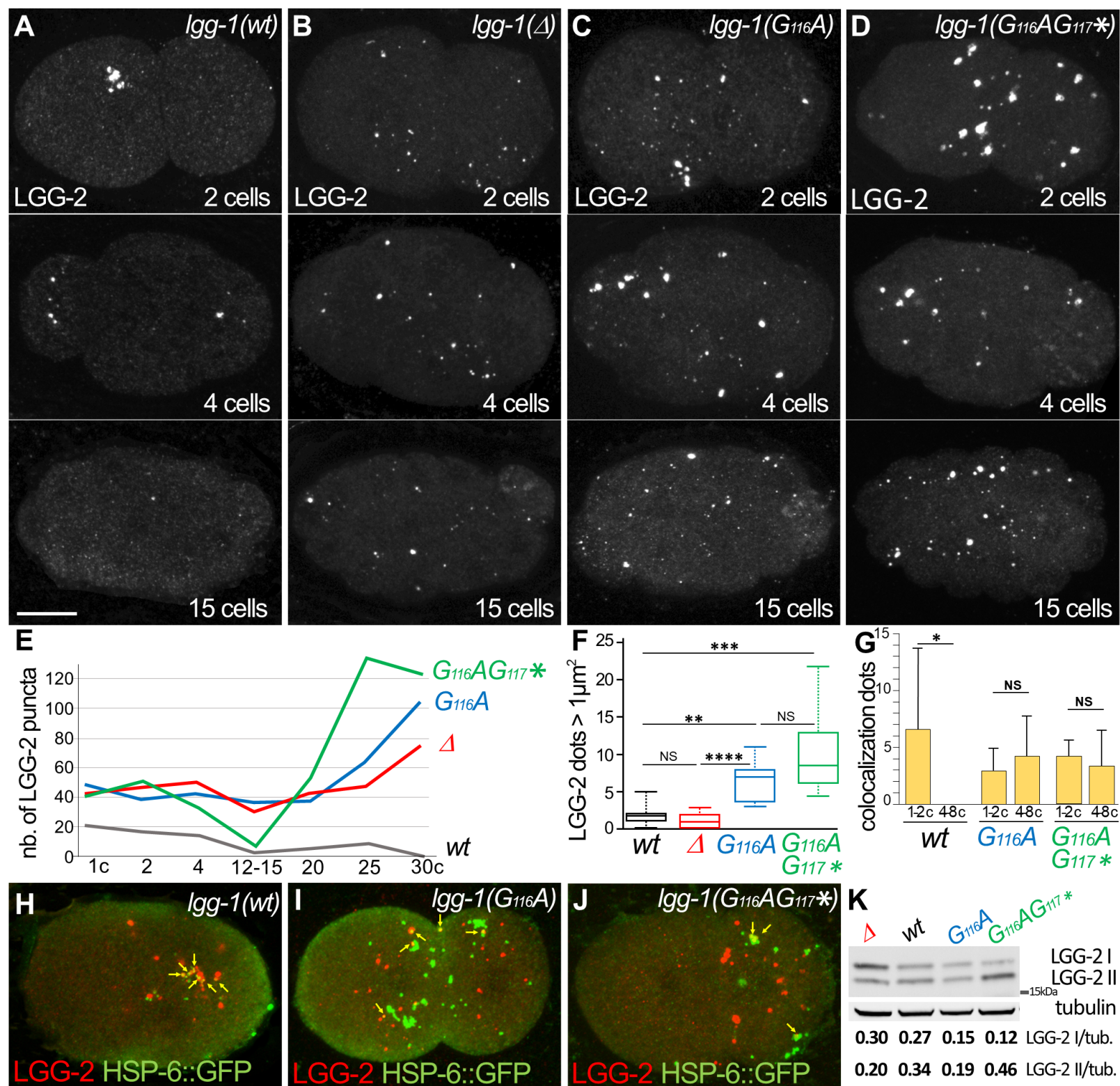


Figure 5 Leboutet et al.

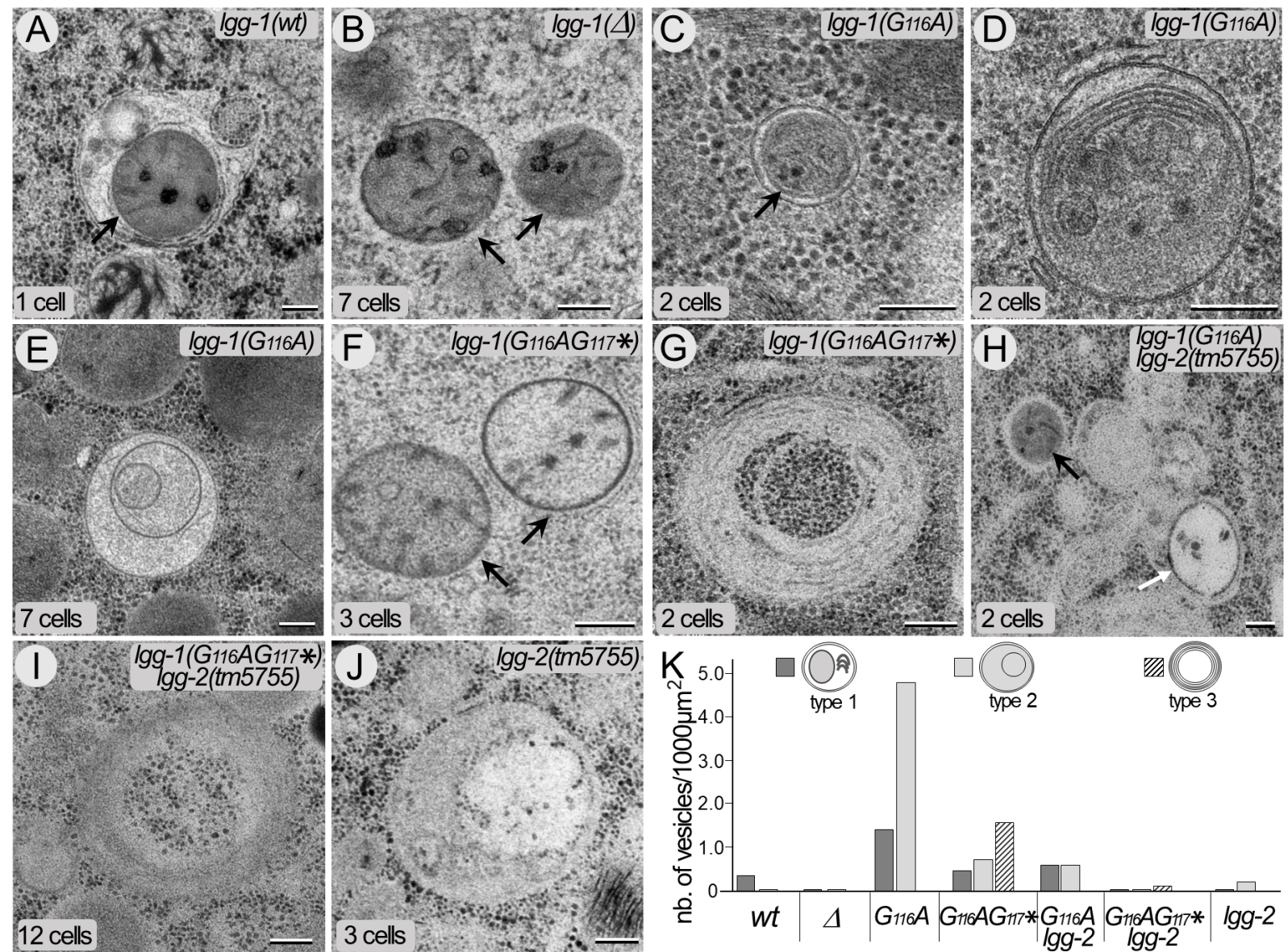


Figure 6 Leboutet et al.

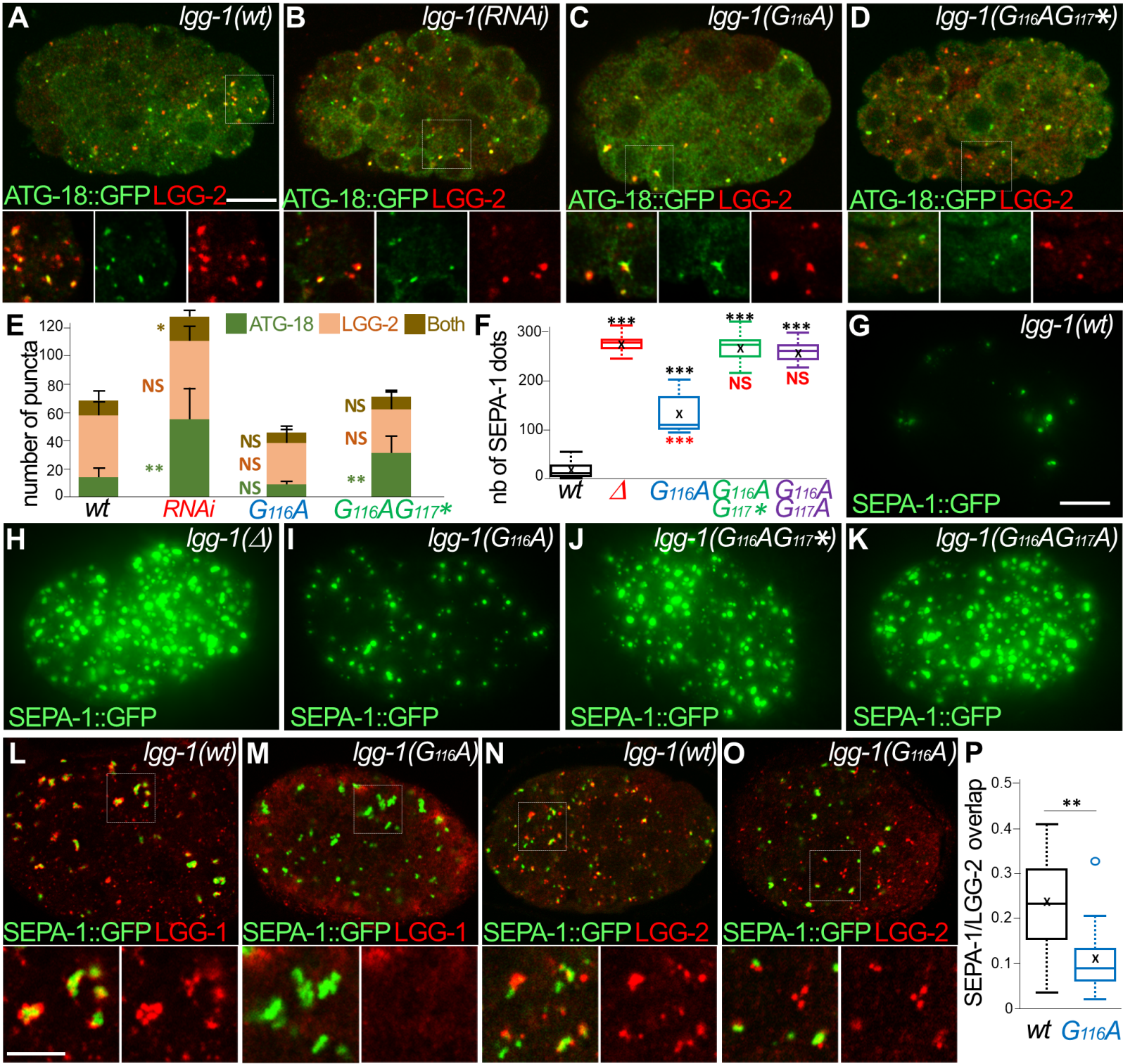


Figure 7 Leboutet et al.

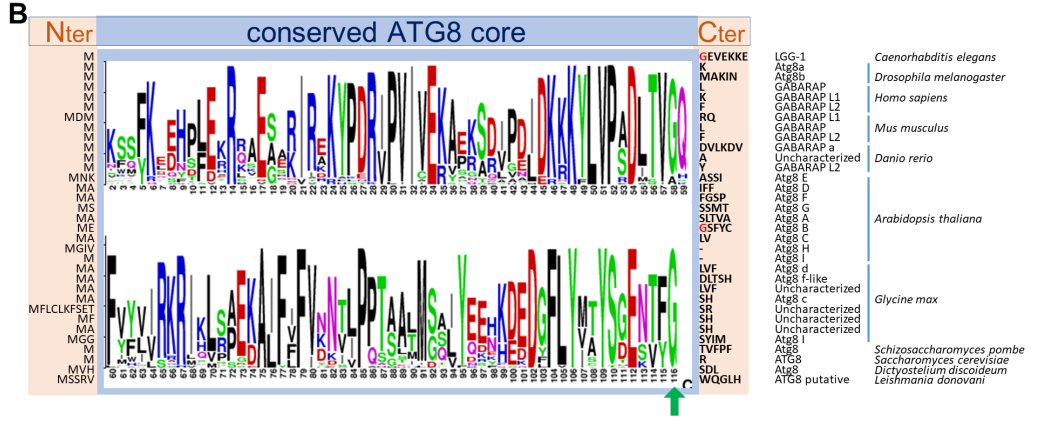
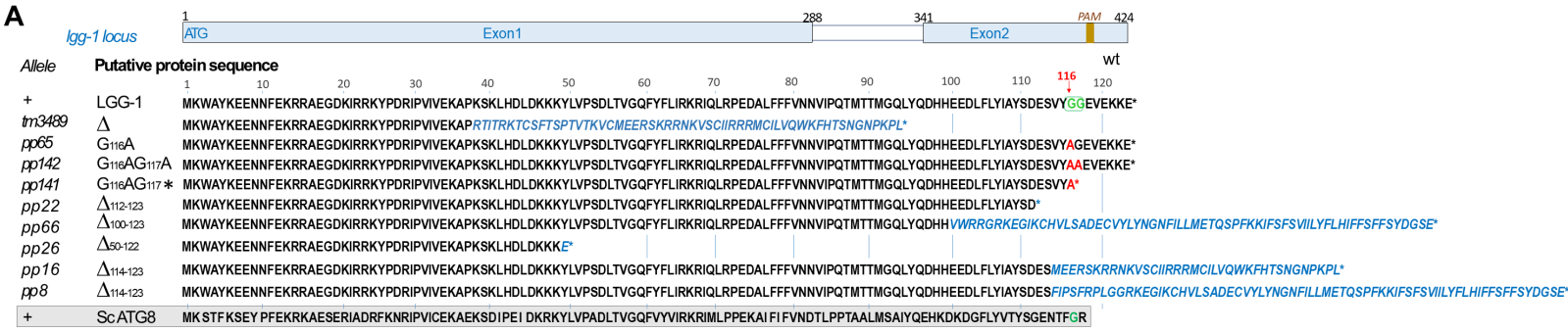


Figure 1-figure supplement 1 Leboutet et al.

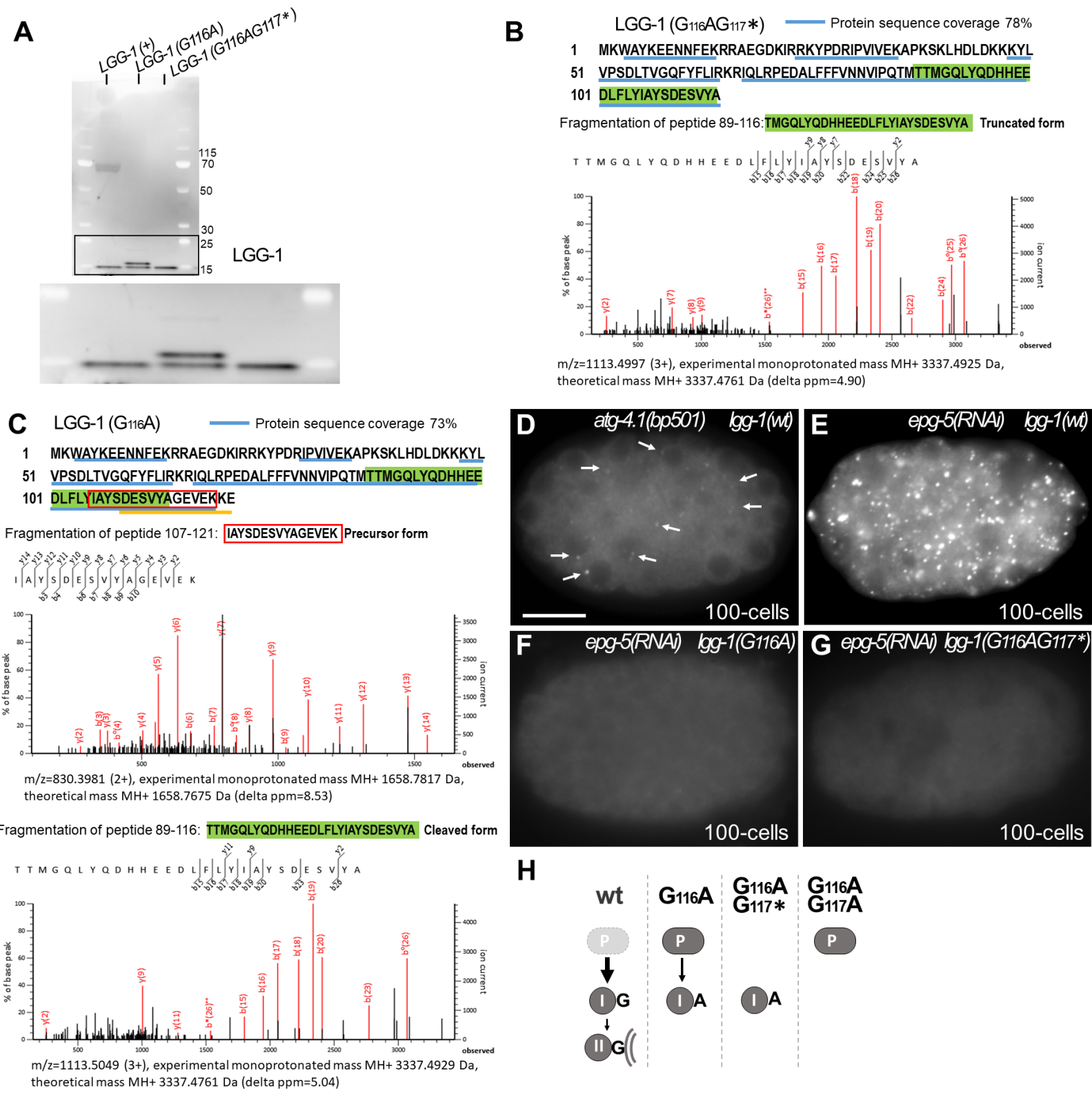


Figure 1-figure supplement 2 Leboutet et al.

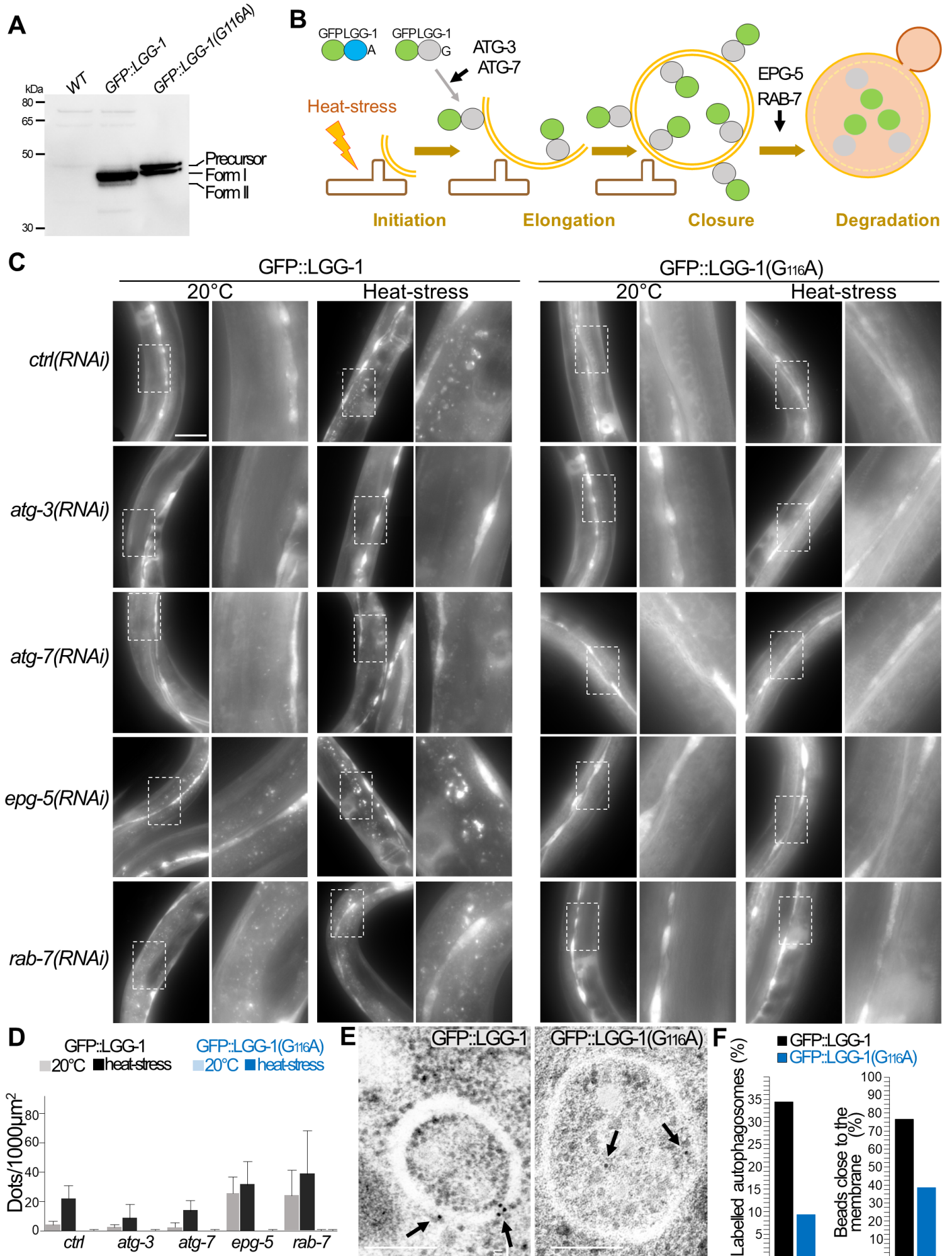


Figure 1-figure supplement 3 Leboutet et al.

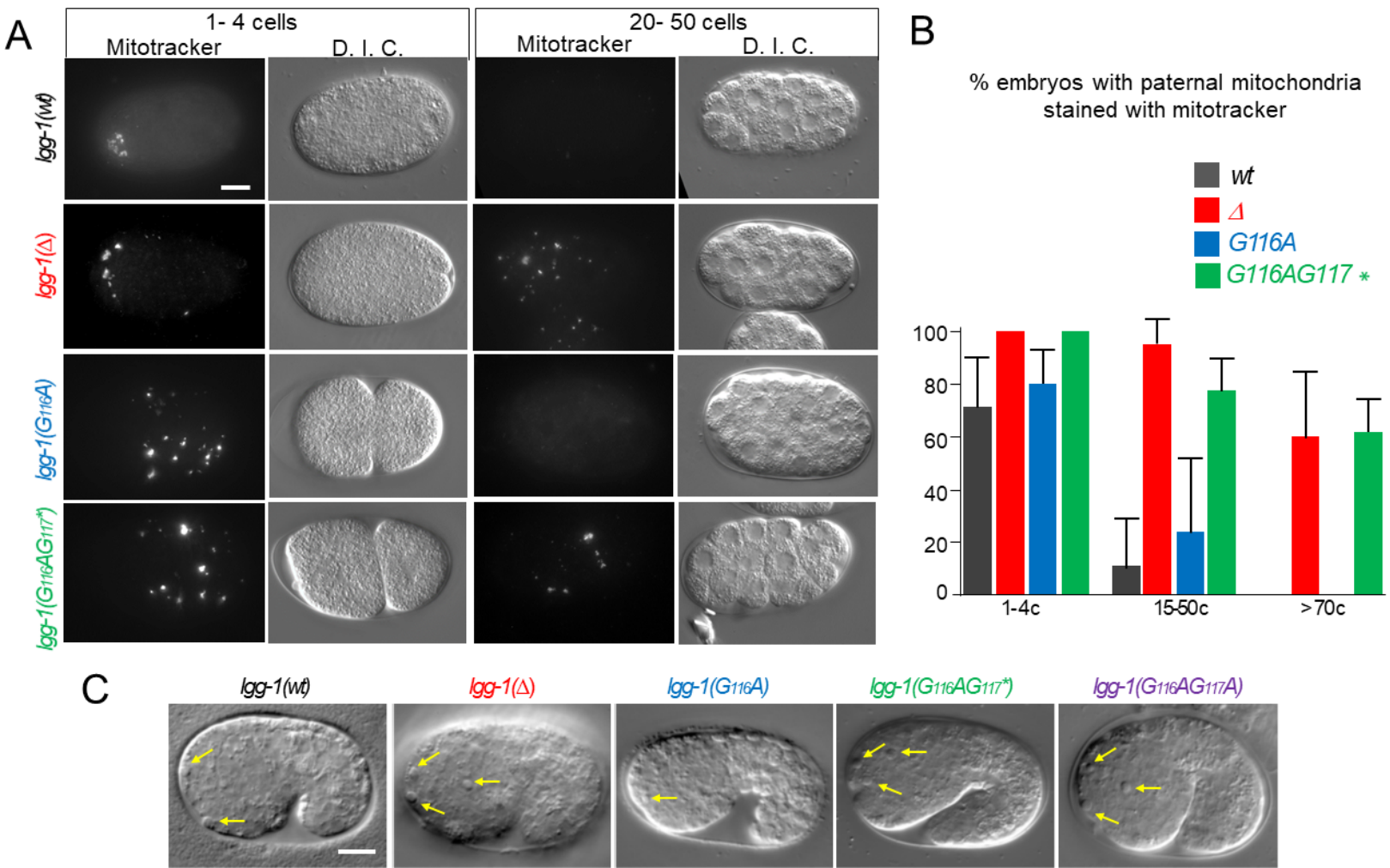


Figure 3-figure supplement 1 Leboutet et al.

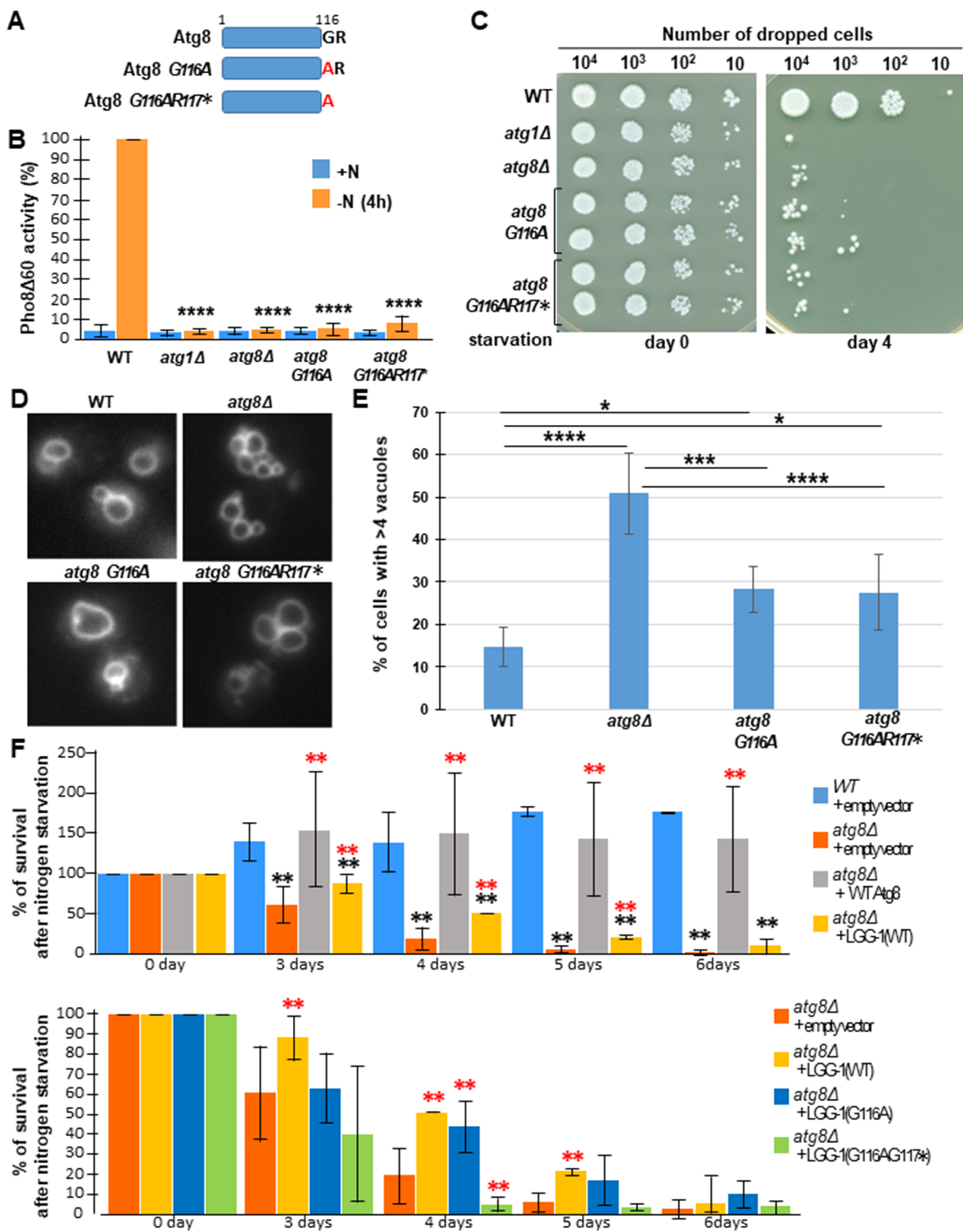


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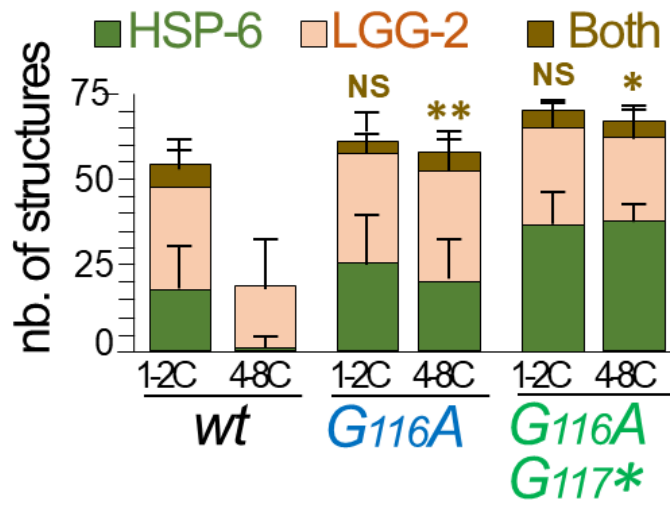


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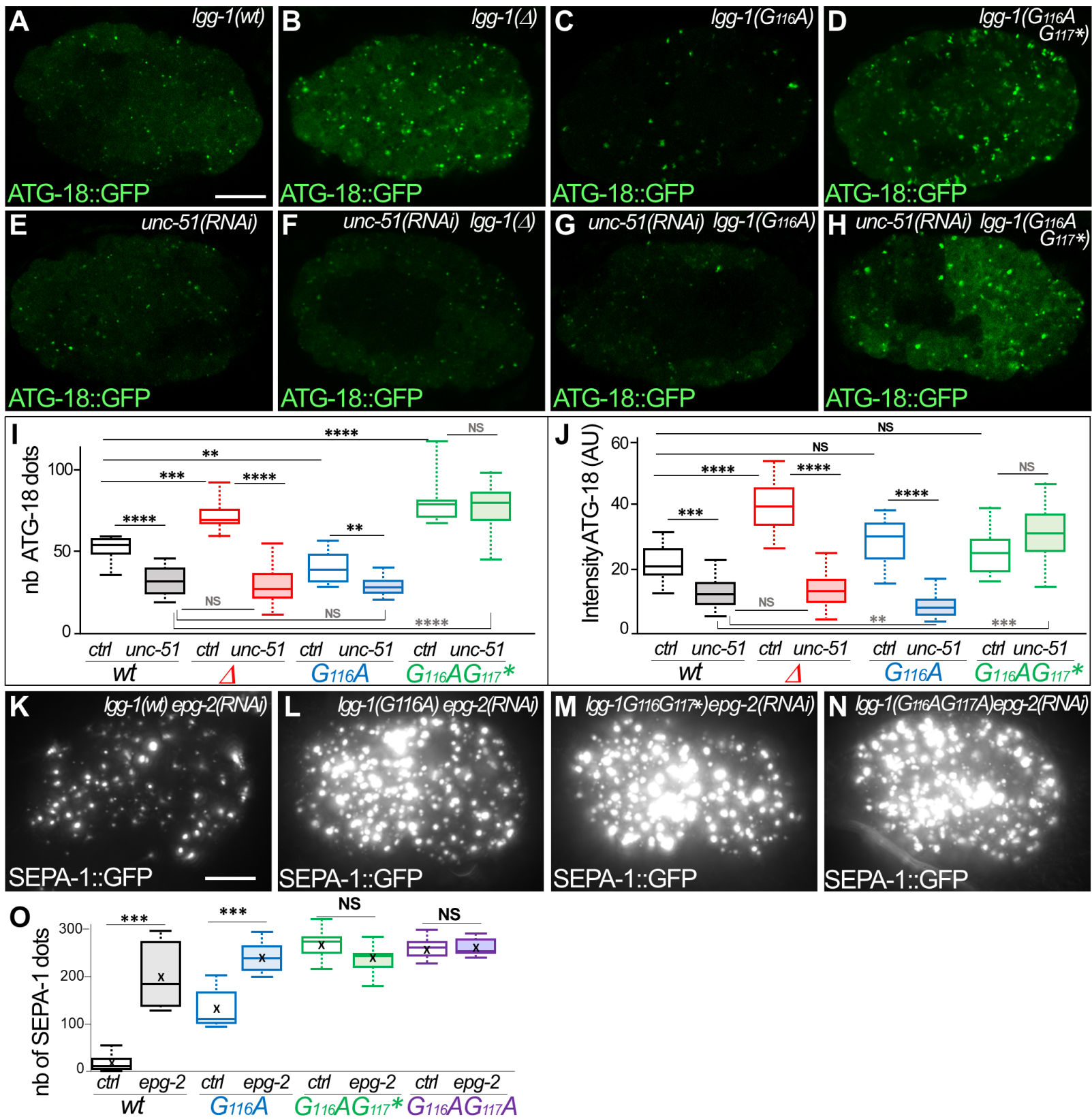


Figure 7-figure supplement 1 Leboutet et al.