

1 **TBK1-mediated phosphorylation of LC3C and GABARAP-L2 controls au-**
2 **tophagosome shedding by ATG4 protease**

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5 **Running title: TBK1 controls ATG8 processing by ATG4**
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8 Lina Herhaus¹, Ramachandra M. Bhaskara², Alf Håkon Lystad³, Anne Simonsen³,
9 Gerhard Hummer^{2,4}, Ivan Dikic^{1,5*}

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11 Affiliations:

12 ¹ Institute of Biochemistry II, Goethe University School of Medicine, Theodor-Stern-
13 Kai 7, 60590 Frankfurt am Main, Germany

14 ² Department of Theoretical Biophysics, Max Planck Institute of Biophysics, Max-
15 von-Laue Straße 3, 60438, Frankfurt am Main, Germany

16 ³ Department of Molecular Medicine, Institute of Basic Medical Sciences and Cen-
17 tre for Cancer Cell Reprogramming, Institute of Clinical Medicine, Faculty of Med-
18 icine, University of Oslo, 1112 Blindern, 0317 Oslo, Norway

19 ⁴ Institute for Biophysics, Goethe University, 60438, Frankfurt am Main, Germany

20 ⁵ Buchmann Institute for Molecular Life Sciences, Goethe University Frankfurt,
21 Riedberg Campus, Max-von-Laue Straße 15, 60438 Frankfurt am Main, Germany
22

23 * Correspondence should be addressed to:

24 Email: dikic@biochem2.uni-frankfurt.de

25 Phone: +496963015964

26 Fax: +496963015577
27

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31

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33 Abbreviations:

- 34 LC3 interacting motif (LIR)
35 Phosphatidylethanolamine (PE)
36 Ubiquitin-binding domain (UBD)
37 MAP1LC3C (LC3C)
38 GABA Type A Receptor Associated Protein Like 2 (GABARAP-L2)
39 Golgi-Associated ATPase Enhancer Of 16 KDa (GATE-16)
40 Autophagy Related 3 (ATG3)
41 Autophagy Related 5 (ATG5)
42 Autophagy Related 7 (ATG7)
43 Autophagy Related 12 (ATG12)
44 Autophagy Related 16 Like 1 (ATG16L1)
45 TANK binding kinase 1 (TBK1)
46 Optineurin (OPTN)
47 Sequestosome 1 (p62)
48 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
49 Immunoprecipitation (IP)
50 Dulbecco's modified Eagle's medium (DMEM)
51 Phosphate-buffered saline (PBS)
52 Bovine serum albumin (BSA)
53 4',6-diamidino-2-phenylindole (DAPI)
54 Stable isotope labeling with amino acids in cell culture (SILAC)
55 Molecular dynamics (MD)
56 phosphorylated serine (S-PO₄)
57

58 **Abstract**

59 Autophagy is a highly conserved catabolic process through which defective or oth-
60 erwise harmful cellular components are targeted for degradation via the lysosomal
61 route. Regulatory pathways, involving post-translational modifications such as
62 phosphorylation, play a critical role in controlling this tightly orchestrated process.
63 Here, we demonstrate that TBK1 regulates autophagy by phosphorylating autoph-
64 agy modifiers LC3C and GABARAP-L2 on surface-exposed serine residues (LC3C
65 S93 and S96; GABARAP-L2 S87 and S88). This phosphorylation event impedes
66 their binding to the processing enzyme ATG4 by destabilizing the complex. Phos-
67 phorylated LC3C/GABARAP-L2 cannot be removed from liposomes by ATG4 and
68 are thus protected from ATG4-mediated premature removal from nascent autoph-
69 agosomes. This ensures a steady coat of lipidated LC3C/GABARAP-L2 through-
70 out the early steps in autophagosome formation and aids in maintaining a unidi-
71 rectional flow of the autophagosome to the lysosome. Taken together, we present
72 a new regulatory mechanism of autophagy, which influences the conjugation and
73 de-conjugation of LC3C and GABARAP-L2 to autophagosomes by TBK1-medi-
74 ated phosphorylation.

75 **Introduction**

76 The recycling of redundant cytosolic components and damaged organelles is
77 termed autophagy. It is a highly conserved process, which increases during star-
78 vation conditions or during other cellular stresses (Dikic, 2017, Xie & Zhou, 2018).
79 Autophagy involves the formation of the phagophore, a double-membrane cup-
80 shaped structure, which expands to enwrap and enclose the designated cellular
81 cargo to form the autophagosome, which then fuses with lysosomes to enable en-
82 zymatic degradation of its cargo along with its inner membrane (Yang & Klionsky,
83 2010). Upon induction of autophagy, small ubiquitin-like LC3 proteins (autophagy-
84 modifiers) are conjugated to phosphatidylethanolamine (PE) anchoring them to the
85 growing phagophore membrane. This conjugation is carried out by the lipidation
86 cascade enzymes (ATG3, ATG5, ATG7, ATG12, ATG16L1) and allows cargo se-
87 lection and autophagosome formation (Nakatogawa, 2013, Stolz et al., 2014). In
88 humans, there are six autophagy-modifier proteins, grouped into two sub-families:
89 (1) LC3A, LC3B, LC3C, and (2) GABARAP, GABARAP-L1, and GABARAP-
90 L2/GATE-16 (Cemna et al., 2016).
91 LC3 proteins undergo two processing steps, (1) an initial proteolytic cleavage of
92 the peptide bond responsible for the conversion of pro-LC3 to active LC3 and (2)
93 the subsequent cleavage of the amide bond for de-lipidation of LC3-PE from au-
94 topagosomes to regenerate a free cytosolic LC3-pool. Both processing steps are
95 catalyzed by ATG4 (Zhang et al., 2016). Among the four mammalian paralogs of
96 ATG4, ATG4B is the most active protease followed by ATG4A and ATG4C/D,
97 which exhibit minimal protease activity (Li et al., 2011). LC3s are bound by the
98 ATG4B enzyme body and through LC3 interacting motifs (LIRs) located at the N-
99 and C-terminal flexible tails of ATG4B (Maruyama & Noda, 2017). ATG4s are cys-
100 teine proteases that cleave peptide bonds of pro-LC3 to expose the C-terminal
101 glycine and allow conjugation with PE. ATG4 also de-conjugates LC3-PE from the
102 outer membrane of autophagosomes preceding, or just after autophagosome-ly-
103 sosome fusion by cleaving the amide bond between PE and the C-terminal glycine
104 residue of LC3s (Yu et al., 2012). Both processing steps are known to be regulated
105 by direct phosphorylation of ATG4B itself. Phosphorylation of ATG4B at S383/392
106 increases its protease activity (Yang et al., 2015), especially during the LC3 de-
107 lipidation phase, whereas ULK1-mediated phosphorylation of ATG4B at S316 (in
108 humans) (Pengo et al., 2017) or at S307 (in yeast) (Sanchez-Wandelmer et al.,
109 2017) reduces pro-LC3 binding and C-terminal tail cleavage. Likewise, oxidation
110 of ATG4 by H₂O₂ also attenuates its activity and blocks LC3 de-lipidation (Scherz-
111 Shouval et al., 2007).
112 The serine-threonine kinase TBK1 has been implicated in the selective degrada-
113 tion of depolarized mitochondria (mitophagy) and intracellular pathogens (xenoph-
114 agy) (Randow & Youle, 2014, Richter et al., 2016b, Wild et al., 2011). Specific

115 autophagic cargo marked with an ubiquitin signal is recognized by autophagy re-
116 ceptor proteins such as Optineurin (OPTN) and p62 (Herhaus & Dikic, 2015,
117 Herhaus & Dikic, 2018). They physically bridge the cargo to the nascent phago-
118 phore by binding to ubiquitin via their UBD and to LC3 family proteins via their
119 conserved LIR motifs, respectively. These autophagy receptors also recruit TBK1
120 to the site of autophagosome formation. Local accumulation of active TBK1 phos-
121 phosphorylates p62 and OPTN, which increases their binding affinity for polyubiquitin
122 chains and the LC3 family proteins, thereby driving autophagy (Heo et al., 2015,
123 Lazarou et al., 2015, Matsumoto et al., 2011, Ordureau et al., 2015, Pilli et al.,
124 2012, Richter et al., 2016a, Thurston et al., 2009, Wild et al., 2011).
125 In this study we expand the role of TBK1 in the autophagic process by demonstrat-
126 ing its ability to directly phosphorylate LC3C on S93/96 and GABARAP-L2 on
127 S87/88. We study the consequences of LC3 phosphorylation during autophagy
128 and show that this phosphorylation primarily impedes ATG4-mediated processing
129 of LC3 on the liposomes, adding a new layer of regulation.

130 **Results**

131

132 **TBK1 phosphorylates LC3C and GABARAP-L2 *in vitro***

133 The serine-threonine kinase TBK1 has previously been shown to phosphorylate
134 autophagy receptors such as OPTN and p62 (Pilli et al., 2012, Richter et al., 2016a,
135 Wild et al., 2011). To test if recombinant TBK1 can also directly phosphorylate
136 autophagy modifiers, we performed an *in vitro* kinase assay. Four out of the six
137 autophagy modifier proteins: LC3A, LC3C, GABARAP-L1 and GABARAP-L2 are
138 directly phosphorylated by TBK1 *in vitro* (**Figure 1A**). The S/T phosphorylation
139 sites of LC3-family proteins were identified by mass spectrometry with significant
140 PEP scores (**Figure 1B**) and we decided to further investigate the TBK1-mediated
141 phosphorylation sites of LC3C at S93 and S96 and GABARAP-L2 at S87 and S88
142 in detail. The TBK1-mediated phosphorylation sites of LC3C (**Figure 1C**) and
143 GABARAP-L2 (**Figure 1D**) are topologically equivalent and are present in surface
144 exposed loops (depicted in red). This loop is on the opposite face of the LIR binding
145 pocket indicating that LIR-mediated interactions of LC3C might not be affected di-
146 rectly upon phosphorylation.

147

148 **TBK1 phosphorylates and binds LC3C and GABARAP-L2 *in cells***

149 To test if TBK1 also phosphorylates LC3C in cells, HEK293T cells were SILAC
150 labeled and either WT TBK1 (heavy label) or TBK1 kinase dead (K38A; light label)
151 were overexpressed along with GFP-LC3C or with GFP-GABARAP-L2. GFP-pro-
152 teins were immunoprecipitated and analyzed by mass spectrometry. Phosphory-
153 lation at positions S96 and S93 of LC3C was enhanced in the presence of WT
154 TBK1 (factors 6 and 10 respectively; **Figure 2A**), as compared to TBK1 K38A.
155 Similarly, the presence of WT TBK1 resulted in enhanced phosphorylation of
156 GABARAP-L2 at S87 and S88 (by factors 13 and 2, respectively; **Figure 2A**). Un-
157 fortunately, our efforts to generate phospho-specific antibodies against GABA-
158 RAP-L2 S87-PO₄ and GABARAP-L2 S87/88-PO₄ failed (**Figure S1**). To confirm
159 the phosphorylation event directly, we visualized it by using phos-tagTM polyacryla-
160 mide gels, where phosphorylated proteins are retained by the phos-tag reagent
161 and appear at a higher molecular weight. Overexpression of WT TBK1, but not
162 TBK1 kinase dead, induced an upward shift and retention of phosphorylated LC3C
163 (**Figure 2B**). The ratio of phosphorylated to unphosphorylated LC3C is also higher
164 upon the induction of mitophagy, by the addition of Parkin, an E3 ligase and CCCP,
165 the mitochondrial depolarization agent (compare lanes two and five of phosphory-
166 lated upper HA-band from phos-tag gel, Figure 2B). Moreover, the endogenous
167 TBK1 from HeLa or HEK293T cell lysate binds to GST-LC3C and GST-GABARAP-
168 L2 (**Figure 2C**) indicating direct physical interaction. The binding of TBK1 to LC3C
169 and GABARAP-L2 is independent of its catalytic activity (**Figure 2D**) and could be

170 mediated through its C-terminal coiled-coil region (**Figure 2D**), which is known to
171 bind OPTN (Freischmidt et al., 2015).

172

173 **Phospho-mimetic LC3C impedes ATG4 cleavage and binding**

174 To understand the consequences of phosphorylated-LC3C, we looked at its phos-
175 pho-sites in more detail. The LC3C phosphorylation sites S93 and S96 are situated
176 on the face opposite to the hydrophobic pocket enabling LIR binding (**Figure 1C**)
177 and are therefore less likely to influence the direct binding of LC3C to autophagy
178 receptors or adaptors. However, they are in close proximity to the C-terminal tail
179 of LC3C which is proteolytically processed. ATG4 mediated processing of the
180 LC3C C-terminal tail allows lipid-conjugation and adherence to autophagosomes.
181 To test if phosphorylation of these residues could impair the proteolytic cleavage
182 of the LC3C C-terminal tail by ATG4, an *in vitro* cleavage assay was performed.
183 Double-tagged LC3C WT, S93/96A or phospho-mimetic LC3C S93/96D were in-
184 cubated with ATG4B for indicated times and the C-terminal cleavage of LC3C was
185 monitored by detecting the appearance of truncated His-LC3C protein (**Figure 3A**).
186 ATG4B cleaves the entire pool of LC3C WT or S93/96A within 10 minutes,
187 whereas only half of the phospho-mimetic LC3C S93/96D pool is cleaved (**Figure**
188 **3A**). When LC3 proteins are overexpressed in HEK293T cells, they are rapidly
189 processed by endogenous ATG4 proteins. The C-terminal tail of LC3C that is
190 cleaved by ATG4s is considerably larger (21 residues) than that of other LC3 family
191 proteins. Hence, a pro-form of LC3C S93/96D could be visualized by separating
192 cell lysate on a 15% polyacrylamide gel (**Figure 3B**).

193 The inability of ATG4 to process phosphorylated LC3C might be distinct during
194 stress conditions. To test this, we induced mitophagy in HEK293T cells by adding
195 CCCP. Upon induction of mitophagy LC3C S93/96D could not be completely pro-
196 cessed by ATG4s (**Figure 3C**). Similarly, GABARAP-L2 phospho-mimetic (S88D)
197 could not be cleaved by endogenous ATG4s, impairing subsequent lipidation (**Fig-**
198 **ure 3D**). We reasoned that this inability of ATG4 to process LC3C S93/96D and
199 GABARAP-L2 S87/88D could be due to an impediment in direct protein binding,
200 and therefore tested this by co-expressing GFP-LC3 proteins with either Flag-
201 ATG4A or Flag-ATG4B in HEK293T cells and subjected them to GFP-immunopre-
202 cipitation (**Figure 3E**). Phospho-mimetic mutants LC3C S93/96D and GABARAP-
203 L2 S87/88D displayed reduced binding to ATG4A and B. To understand this re-
204 duced binding, we modelled the full-length LC3C-ATG4B complex based on the
205 core crystal structure (Satoo et al., 2009) (see Methods). We tested the effect of
206 phosphorylation at both these sites (S93 and S96) by modeling phosphate groups
207 onto serine residues in the LC3C-ATG4B complex and performed molecular dy-
208 namics (MD) simulations (up to 1.5 μ s). We found that the WT LC3C-ATG4B com-

209 plex with and without additional LIR interactions between ATG4B and LC3C re-
210 mained stable. The C-terminal tail of LC3C remained bound and strongly anchored
211 to the active site of ATG4B throughout the simulation. In the complex, the phospho-
212 sites S93 and S96 of LC3C (red cartoon in **Figure 3F**) are in close-proximity to the
213 ATG4B interacting surface (grey surface). LC3C S96 forms a hydrogen-bond in-
214 teraction with ATG4B E350, and LC3C S93 is close to a network of hydrogen
215 bonds and salt bridges stabilizing. In MD simulations, double phosphorylation of
216 S93 and S96 interfered with these interactions and disrupted the binding interface
217 between ATG4B and LC3C (**Movies SM1, SM2**). The phosphorylated serine resi-
218 dues detached from the ATG4B surface and partially dislodged the LC3C, resulting
219 in partial retraction of the LC3C C-terminal tail from the ATG4B active site. The
220 negative charge introduced by phosphorylation severely weakens complex stabil-
221 ity based on calculated binding energies (**Table S2**), with electrostatic interactions
222 as the dominant factor. Figure S2 shows residue-wise contributions to the binding
223 energy mapped onto the LC3C structure. According to these calculations, phos-
224 phosphorylated S93 and S96 are strongly destabilizing (**Figure S2**; red thick cartoon),
225 whereas unphosphorylated S93 and S96 are favorable (**Figure S2**; blue thin car-
226 toon). The MD simulations and binding energy calculations indicate that phosphor-
227 ylation disrupts the LC3C-ATG4B interface and destabilizes the complex.

228

229 **Phosphorylation at S93 and S96 affects LC3C C-terminal tail structure and** 230 **thereby impedes ATG4-mediated cleavage**

231 Based on the simulation results for the LC3C-ATG4B complex, we hypothesized
232 that phosphorylation of unbound LC3C could affect its C-terminal tail structure and
233 prevent binding to the ATG4B active site. In MD simulations (see Methods) of free
234 LC3C, we found that the C-terminal tail of LC3C (126-147) was disordered and
235 highly dynamic (**Movie SM3**). By contrast, in the phosphorylated variants (S93-
236 PO₄ LC3C and S96-PO₄ LC3), the C-terminal tail adopted more ordered confor-
237 mations (**Movies SM4, SM5; Figure 4A-B**). The phosphoserines formed intramo-
238 lecular salt bridges with R134 (**Figures 4A and 4B**) that pulled the C-terminal tail
239 of LC3C towards the protein, structuring it locally. In repeated simulations ($n = 6$
240 each) of unphosphorylated and phosphorylated variants of LC3C (**Figures S3A-**
241 **C**), we observed a total of six salt-bridge formation events, indicating that the in-
242 tramolecular salt-bridge formation between the phosphoserines and R134 is ro-
243 bust. We observed the salt bridge formation on a sub-microsecond time scale (**Fig-**
244 **ure 4D and 4E**). To confirm this finding and the role of R134, we performed ATG4-
245 mediated *in vitro* cleavage experiments of double tagged LC3C WT, S93/96D,
246 S93/96D R134A, and S93/96D R142A (a control mutation in the C-terminal tail).
247 The LC3C C-terminal cleavage was monitored by the disappearance of its C-ter-
248 minal Strep-tag. The mutation of S93/96D delayed the cleavage of the C-terminal

249 tail of LC3C by ATG4B (**Figure 4F**). The R134A mutation could partially rescue
250 this phenotype of S93/96D, whereas the other C-terminal tail mutation, R142A,
251 could not (**Figure 4F**). The results of the ATG4-mediated cleavage assay are thus
252 consistent with R134-phosphoserine interactions sequestering the LC3C C-termi-
253 nal tail and preventing access to ATG4B and subsequent cleavage.

254

255 **Phospho-mimetic LC3C and GABARAP-L2 cannot form autophagosomes in** 256 **cells**

257 GABARAP-L2 lacks the C-terminal tail, and the ATG4B-mediated processing re-
258 moves only a single C-terminal residue (F117), which exposes G116 for lipidation.
259 Therefore, we hypothesized that phosphorylating S87 and S88 in GABARAP-L2
260 weakens binding to ATG4B and in turn slows down proteolytic processing. Accord-
261 ingly, we tested if phospho-mimetic GABARAP-L2 S88D and LC3C S93/96D can
262 form autophagosomes, despite not being processed by ATG4B. U2OS cells were
263 co-transfected with HA-Parkin and GFP-GABARAP-L2 WT, S88D, S88A (**Figure**
264 **5A and Figure S4A**) or GFP-LC3C WT, S93/96D, S93/96A (**Figure 5B and Fig-**
265 **ure S4B**) and mitophagy was induced by the addition of CCCP for 3 hours. Upon
266 induction of mitophagy GABARAP-L2 WT and S88A formed autophagosomes. By
267 contrast, the phospho-mimetic GABARAP-L2 S88D remained dispersed through-
268 out the cell and no autophagosome formation was observed (**Figure 5A and Fig-**
269 **ure S4A**). Likewise, phospho-mimetic LC3C S93/96D did not form autophago-
270 somes upon induction of mitophagy unlike WT and S93/96A LC3C (**Figure 5B and**
271 **Figure S4B**).

272

273 **Phospho-mimetic Δ C-terminal LC3C and GABARAP-L2 are not lipidated and** 274 **do not form autophagosomes**

275 Since LC3 family proteins can only be integrated into autophagosomes after C-
276 terminal cleavage by ATG4, we tested whether artificially truncated LC3C or
277 GABARAP-L2 (Δ C-term: LC3C (1-126) and GABARAP-L2 (1-116)) could circum-
278 vent ATG4-mediated processing, undergo lipidation, and form autophagosomes.
279 U2OS cells were co-transfected with HA-Parkin and GFP-GABARAP-L2 Δ C-term
280 WT, S88A or S88D (**Figure 6A and Figure S5A**) or GFP-LC3C Δ C-term WT,
281 S93/96A or S93/96D (**Figure 6B and Figure S5B**) and mitophagy was induced by
282 the addition of CCCP for 3 hours. Upon induction of mitophagy, GABARAP-L2 or
283 LC3C Δ C-term WT and alanine mutants formed autophagosomes, while phospho-
284 mimetic mutants with truncated C-terminus (GABARAP-L2 Δ C-term S88D or
285 LC3C Δ C-term S93/96D) remained dispersed throughout the cell and no autoph-
286 agosome formation could be observed (**Figure 6A and Figure 6B**). Upon induction
287 of mitophagy, GABARAP-L2 lipidation can be observed by the appearance of a

288 lower band on Western Blots (**Figure 3D**), which can also be observed during mi-
289 tophagy induction of GABARAP-L2 Δ C-term WT and S88A, but not with phospho-
290 mimetic GABARAP-L2 Δ C-term S88D (**Figure S5C**). Hence, the phosphorylation
291 of LC3C or GABARAP-L2 not only impedes their C-terminal cleavage by ATG4,
292 but also their lipidation by the lipidation cascade enzymes ATG12-5-16L1. ATG7
293 function is similar to ubiquitin-activating (E1) enzymes; it recruits ATG3 (an E2-like
294 enzyme), which then catalyzes the conjugation to the lipid moiety (PE) to the C-
295 terminal exposed glycine of the truncated LC3 Δ C-term. Binding of the ATG12-5-
296 16L1 complex (E3-like enzyme) to ATG3 enhances the lipidation of LC3, since the
297 ATG5-ATG12 complex ensures that nascently lipidated LC3 is incorporated into
298 the phagophore membrane (Nakatogawa, 2013). In order to test if phosphorylation
299 of LC3C/GABARAP-L2 Δ C-term impedes their processing by the lipidation cas-
300 cade enzymes, we also performed an *in vitro* lipidation assay (**Figure 6C**). LC3C
301 Δ C-term WT and LC3C Δ C-term S93/96D or GABARAP-L2 Δ C-term WT and
302 GABARAP-L2 Δ C-term S87/88D were incubated with ATP, liposomes, hATG3,
303 hATG7, and hATG12-5-16L1 (reaction mix). WT LC3C and GABARAP-L2 could
304 be successfully lipidated, while S93/96D LC3C and S87/88D GABARAP-L2 could
305 not be conjugated to membrane *in vitro* (**Figure 6C,D**) indicating that phosphory-
306 lation of LC3s also affects lipid conjugation.

307

308 **TBK1-mediated GABARAP-L2 phosphorylation impedes its premature cleav-** 309 **age from autophagosomes by ATG4**

310 TBK1 is recruited to the site of autophagosome formation by autophagy receptor
311 proteins, where TBK1 phosphorylates OPTN and p62 to promote autophagy flux
312 (Heo et al., 2015, Lazarou et al., 2015, Matsumoto et al., 2011, Ordureau et al.,
313 2015, Pilli et al., 2012, Richter et al., 2016a, Thurston et al., 2009, Wild et al.,
314 2011). Hence, it is most likely that LC3C and GABARAP-L2 are phosphorylated by
315 TBK1 during autophagosome formation and not during the initial processing step
316 of pro-LC3 cleavage post ribosomal release. In order to test whether the TBK1-
317 mediated phosphorylation of LC3C and GABARAP-L2 has an impact on ATG4-
318 mediated de-lipidation of LC3s from the mature autophagosome, an *in vitro* de-
319 lipidation assay of LC3C and GABARAP-L2 proteins was performed. The fractions
320 of PE-conjugated LC3C Δ C-term WT, phospho-mimetic S93/96D and PE-conju-
321 gated GABARAP-L2 Δ C-term WT and phospho-mimetic S87/88D (see Methods)
322 were enriched and used as substrates for the de-lipidating enzymes ATG4A or
323 ATG4B (**Figure 7A,B**). We found that neither LC3C Δ C-term WT, nor LC3C Δ C-
324 term S93/96D could be de-lipidated and released from the liposome by ATG4A
325 (**Figure 7A,B**). In contrast, ATG4B is able to de-lipidate and cleave small amounts
326 of LC3C Δ C-term WT (**Figure S6A**) from liposomes, but has no activity towards
327 LC3C Δ C-term S93/96D. This indicates that lipidated LC3C could be targeted

328 specifically by other isoforms of ATG4 enzymes (ATG4C or ATG4D) but not by
329 ATG4A and ATG4B. WT GABARAP-L2 could be de-lipidated from liposomes by
330 ATG4B and ATG4A (at a slower rate) in a dose dependent manner (**Figure S6B**).
331 On the contrary, we found that GABARAP-L2 S87/88D is not a target of ATG4A or
332 B (**Figure 7A,B**).

333 In addition, we also tested whether the phosphorylation of GABARAP-L2 has an
334 impact on its de-lipidation from the phagophore by other proteases such as RavZ
335 (**Figure 7C**). RavZ is a bacterial effector protein from the intracellular pathogen
336 *Legionella pneumophila* that interferes with autophagy by directly and irreversibly
337 uncoupling GARARAP-L2 attached to PE on autophagosome membranes (Choy
338 et al., 2012, Kwon et al., 2017). We found that small amounts of RavZ could re-
339 move GARABAP-L2 WT and S87/88D mutant from autophagosomes (**Figure 7C**),
340 indicating its effectiveness in circumventing *Legionella* growth restriction via xe-
341 nophagy (when TBK1 is also activated). Likewise, RavZ is also able to cleave
342 LC3C WT and S93/96D mutant from liposomes *in vitro* (**Figure S6A**).

343 Finally, we tested if phosphorylated LC3C or GABARAP-L2 adhered to autophago-
344 somes are still functional to perform downstream reactions. LC3 family proteins
345 interact with autophagosome receptors such as p62, which link the growing au-
346 topagosome to cargo (Pankiv et al., 2007, Zheng et al., 2009). Both LC3C Δ C-
347 term WT and phospho-mimetic S93/96D can bind to p62 (**Figure 7D**). Similarly,
348 p62 and OPTN can be recruited to autophagosomes by WT as well as S87/88D
349 GABARAP-L2 (**Figure 7E**) (Wild et al., 2011, Wong & Holzbaaur, 2014). Once all
350 of the cargo has been engulfed by the autophagosome, degradation can take place
351 through the fusion with lysosomes (Nakamura & Yoshimori, 2017). GABARAP
352 family proteins mediate autophagosomal-lysosomal fusion by binding to the au-
353 topagy adaptor protein PLEKHM1 (McEwan et al., 2015, Wang et al., 2015).
354 Phosphorylation of GABARAP-L2 by TBK1 does not interfere with its ability to bind
355 to PLEKHM1 (**Figure 7E**).

356 Hence, TBK1 mediated phosphorylation of GABARAP-L2 and LC3C protects them
357 from premature autophagosome removal by ATG4, but does not interfere with
358 downstream reactions like cargo binding and lysosomal fusion.

359

360 Discussion

361 The autophagy pathway is tightly regulated to ensure proper recycling and disposal
362 of cellular material during nutrient shortage. Here, we present a new regulatory
363 mechanism of autophagy, which influences the conjugation and de-conjugation of
364 LC3C and GABARAP-L2 to autophagosomes. The kinase TBK1 fulfils several
365 roles during selective autophagy. Upon autophagy induction, TBK1 is recruited to
366 the site of autophagosome formation and gets activated by trans-autophosphory-
367 lation after accumulation (Ma et al., 2012, Shu et al., 2013). We show that, at this
368 stage, TBK1 can phosphorylate LC3C and GABARAP-L2 at specific serine resi-
369 dues to protect them from ATG4-mediated premature removal from autophago-
370 somes.

371 ATG4 mediates regular processing of pro-LC3s post ribosomal release, de-lipida-
372 tion of incorrectly lipidated LC3-PE on other endomembranes, and favors incorpo-
373 ration of LC3s into autophagosomes by ATG12-5-16L1. Spatial and temporal reg-
374 ulation of recruitment and dissociation of LC3 family proteins to and from autoph-
375 agosomes is achieved through regulation of ATG4 activity (Pengo et al., 2017,
376 Sanchez-Wandelmer et al., 2017, Scherz-Shouval et al., 2007, Yang et al., 2015,
377 Yu et al., 2012). ATG4 constitutively de-conjugates LC3 family proteins from all
378 endomembranes except from autophagosomes, to maintain a pool of unlipidated
379 LC3 (Nakatogawa et al., 2012). This suggests that LC3-PE conjugated to autoph-
380 agosomes is protected from premature de-lipidation by a timely regulatory mech-
381 anism. This regulation is achieved through the phosphorylation and dephosphory-
382 lation of ATG4 itself (Pengo et al., 2017, Sanchez-Wandelmer et al., 2017) and the
383 phosphorylation of LC3s by TBK1.

384 The kinase activity of TBK1 is tightly regulated (Xu 2017) and during xenophagy
385 and mitophagy, TBK1 phosphorylates autophagy receptor proteins (Heo et al.,
386 2015, Lazarou et al., 2015, Ordureau et al., 2015, Pilli et al., 2012, Richter et al.,
387 2016a, Thurston et al., 2009, Wild et al., 2011).

388 The active recruitment of TBK1 to the sites of autophagosome formation (Lazarou
389 et al., 2015, Richter et al., 2016a) makes it likely that TBK1-mediated phosphory-
390 lation occurs on nascent phagophores, resulting in phosphorylated forms of mem-
391 brane-embedded LC3s. Phosphorylation prevents premature removal of lipidated
392 LC3C/GABARAP-L2 from growing autophagosomes by ATG4. Molecular model-
393 ing and atomistic simulations of the ATG4B-LC3C complex revealed that LC3C
394 phosphorylation impedes binding to ATG4. The weakened binding slows down de-
395 lipidation, which ensures that a steady coat of lipidated LC3C/GABARAP-L2 is
396 maintained throughout the early steps in autophagosome formation (**Figure 8**).
397 The phosphorylation of LC3C/GABARAP-L2 does not impede their binding to au-
398 topagy receptors such as p62 or PLEKHM1, which promotes unhindered down-
399 stream steps for, e.g., autophagosome-lysosome fusion (McEwan et al., 2015).

400 Thus, phosphorylation of LC3s aids in maintaining an unperturbed and unidirectional flow of the autophagosome to the lysosome.
401
402 At later stages of autophagosome formation, this process could be slowed-down
403 or reversed by either TBK1 dissociation from autophagosomes or diminished catalytic activity. Alternatively, action of phosphatases could allow de-lipidation prior
404 to autophagosomal-lysosomal fusion, thereby recycling LC3s.
405
406

407 **Materials and Methods**

408 Expression constructs

409 Expression constructs of indicated proteins were cloned into indicated vectors using PCR or the gateway system. Site-directed mutagenesis was performed by PCR
410 using PCR or the gateway system. Site-directed mutagenesis was performed by PCR
411 to introduce desired amino acid substitutions. All expression constructs were sequenced by SeqLab.
412

413

414 Protein expression and purification

415 GST or His-tagged fusion proteins were expressed in *E. coli* strain BL21 (DE3).
416 Bacteria were cultured in LB medium supplemented with 100 µg/mL ampicillin at
417 37°C in a shaking incubator (150 rpm) until OD₆₀₀ ~0.5-0.6. Protein expression
418 was induced by the addition of 0.5 mM IPTG and cells were incubated at 16°C for
419 16 hours. Bacteria were harvested by centrifugation (4000 rpm) and lysed by sonication in GST lysis buffer (20 mM Tris HCl, pH 7.5, 10 mM EDTA, pH 8.0, 5 mM EGTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 1 mM PMSF) or His lysis buffer (25 mM Tris HCl, pH 7.5, 200 mM NaCl, 0.1% β-mercaptoethanol, 1 mM PMSF, 1mg/ml lysozyme). For the purification of ATG4 the use of PMSF was omitted.
424 Lysates were cleared by centrifugation (10000 rpm), 0.05% of Triton X-100 was added and the lysates were incubated with glutathione Sepharose 4B beads (GE Life Sciences) or Ni-NTA agarose beads (Thermo Fisher) on a rotating platform at 4°C for 1 hour. The beads were washed five times either in GST wash buffer (20 mM Tris HCl, pH 7.5, 10 mM EDTA, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 0.1% β-mercaptoethanol, 1 mM PMSF) or His wash buffer (25 mM Tris HCl, pH 7.5, 200 mM NaCl, 0.05% Triton X-100, 10 mM Imidazole). The immobilized proteins were reconstituted in GST storage buffer (20 mM Tris HCl, pH 7.5, 0.1% Na₃N, 0.1% β-mercaptoethanol) or eluted with His elution buffer (25 mM Tris HCl, pH 7.5, 200 mM NaCl, 300 mM Imidazole) and dialysed in (25 mM Tris HCl, pH 7.5, 200 mM NaCl) at 4°C over night.
434

435 Recombinant GST-TBK1 was obtained from the MRC PPU DSTT in Dundee, UK (#DU12469).
436

437 Purification of proteins used for *in vitro* lipidation/de-lipidation: Full-length hATG7, hATG3 and hATG5-12-ATG16L1 complex was expressed and purified from HEK suspension cells (HEK-F, Invitrogen) as previously described (Lystad et al., 2019).
440 To purify mATG3, LC3C, LC3C S93/96D, GABARAP-L2 WT, GABARAP-L2 S87/88D and RavZ, pGEX-6P-1 plasmid containing the corresponding cDNA was transformed into BL21-Gold (DE3) *E. coli*. Cells were grown at 37°C to an OD of 0.6-0.8 before induction with 0.5 mM IPTG. Cells were then grown for 3 additional hours before they were collected by centrifugation. Cells were resuspended in NT350 (20 mM Tris-HCl pH 7.4, 350 mM NaCl) supplemented with a Roche Com-
445

446 plete Protease inhibitor, lysed by sonication and cleared by centrifugation. The su-
447 pernatant was incubated at 4°C with Glutathione Beads (Sigma) for 4 hours. Beads
448 were collected and washed twice with NT350 buffer before HRV 3C protease was
449 added and allowed to cut at 4°C overnight. The next morning, protein fractions
450 were collected and stored at -80°C in 20% glycerol. (mATG3 and RavZ plasmids
451 were a gift from Thomas Melia (Yale University)).

452

453 Cell culture

454 HEK293T and U2OS cells were cultured in Dulbecco's modified Eagle's medium
455 (DMEM; Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine,
456 and 1% penicillin/streptomycin and maintained at 37°C in a humidified atmosphere
457 with 5% CO₂. CCCP was resuspended in DMSO and cells were treated with 40
458 µM for 3 hours. Plasmid transfections were performed with 3 µl GeneJuice (Merck
459 Millipore), 0.5 µg plasmid DNA in 200 µl Opti-MEM (Life Technologies). After incu-
460 bation for 15 min, the solution was added to the cells, which were lysed in lysis
461 buffer or fixed with 4% paraformaldehyde 48 hours later.

462

463 Immunofluorescence microscopy

464 Transfected U2OS cells were seeded onto glass coverslips in 12-well culture
465 dishes and treated accordingly. Cells were washed in phosphate-buffered saline
466 (PBS) before fixation with 4% paraformaldehyde for 10 minutes at room tempera-
467 ture. The coverslips were washed a further three times before permeabilization of
468 the cells with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. Cells
469 were rinsed with PBS before being incubated for 1 hour in 1% bovine serum albu-
470 min (BSA) in PBS for 1 hour. Primary antibody incubation was done for 1 hour in
471 a humidified chamber with 1% BSA in PBS. After thorough washes in PBS, cells
472 were incubated with Alexa Fluor secondary antibodies 1% BSA in PBS for 1 hour
473 in the dark. Cells were washed three more times in PBS and once with deionized
474 water before being mounted onto glass slides using ProLong Gold mounting rea-
475 gent (Life Technologies), which contained the nuclear stain 4',6-diamidino-2-phe-
476 nylindole (DAPI). Slides were imaged using a Leica microscope Confocal SP 80
477 fitted with a 60x oil-immersion lens.

478

479 Cell lysis

480 For lysis, cells were scraped on ice in lysis buffer (50 mM Hepes, pH 7.5, 150 mM
481 NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM NaF, 5% glycerol, 10µM
482 ZnCl₂) supplemented with complete protease inhibitors (cOmplete, EDTA-free;
483 Roche Diagnostics) and phosphatase inhibitors (P5726, P0044; Sigma-Aldrich).
484 Extracts were cleared by centrifugation at 15000 rpm for 15 minutes at 4°C.

485

486 Immunoprecipitation and protein binding assays

487 Cleared cell extracts were mixed with glutathione-Sepharose beads (GE
488 Healthcare) conjugated to LC3 family proteins or GST, FLAG-agarose beads
489 (Sigma-Aldrich) or GFP-Trap_A beads (ChromoTek) for 2 hours at 4°C on a rotat-
490 ing platform. The beads were washed four times in lysis buffer. Immunoprecipi-
491 tated and input samples were reduced in SDS sample buffer (50 mM Tris HCl, pH
492 6.8, 10% glycerol, 2% SDS, 0.02% bromophenol blue, 5% β-mercaptoethanol) and
493 heated at 95°C for 5 minutes (Herhaus et al., 2013, Herhaus et al., 2014).

494

495 Western blotting

496 For immunoblotting, proteins were resolved by SDS-PAGE and transferred to
497 PVDF membranes. Blocking and primary antibody incubations were carried out in
498 5% BSA in TBS-T (150 mM NaCl, 20 mM Tris, pH 8.0, 0.1% Tween-20), secondary
499 antibody incubations were carried out in 5% low-fat milk in TBS-T and washings in
500 TBS-T. Blots were developed using Western Blotting Luminol Reagent (sc-2048;
501 Santa Cruz). All Western blots shown are representative.

502

503 Antibodies

504 The following antibodies were used in this study: anti-HA-tag (11867423001;
505 Roche), anti-FlagM2-tag (F3165; Sigma-Aldrich), anti-GFP-tag (Living Colors
506 632592; Clontech), anti-Strep-tag (34850; Qiagen), anti-His-tag (11922416001;
507 Roche), anti-vinculin (V4505; Sigma), anti-tubulin (T9026; Sigma), anti-TBK1
508 (#3013; Cell Signaling Technology), anti-pTBK1 (pS172; #5483; Cell Signaling
509 Technology). Secondary HRP conjugated antibodies goat anti-mouse (sc-2031;
510 Santa Cruz), goat anti-rabbit (sc-2030; Santa Cruz) and goat anti-rat (sc-2006;
511 Santa Cruz), IgGs were used for immunoblotting. Donkey anti-rat Alexa Fluor 647
512 (A-21247; Life Technologies) was used for immunofluorescence studies.

513

514 Kinase assays

515 LC3 family proteins were incubated in 20 μL phosphorylation buffer (50 mM Tris
516 HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM EGTA, 20 mM β-glycerophosphate, 1 mM
517 DTT, 0.1 mM Na₃VO₄, 0.1 mM ATP or γ^{32P} ATP (500 cpm/pmol)) with 50 ng of
518 recombinant GST-TBK1 for 15 minutes at 30°C. The kinase assay was stopped
519 by adding SDS sample buffer containing 1% β-mercaptoethanol and heating at
520 95°C for 5 minutes. The samples were resolved by SDS-PAGE, and the gels were
521 stained with InstantBlue (Expedeon) and dried. The radioactivity was analysed by
522 autoradiography (Herhaus et al., 2015).

523

524 SILAC-IP and phosphopeptide identification

525 Cells were maintained in custom-made SILAC DMEM (heavy: R10, K8/light R0,
526 K0) for 14 days, treated accordingly and lysed (as stated above). Incorporation of
527 labeled amino acids to more than 95% was verified by Mass spectrometry. Lysates
528 of SILAC-labeled cells expressing GFP-tagged LC3C or GFP-tagged GABARAP-
529 L2, TBK1 WT (heavy labeled) and TBK1 K38A (light labeled) were combined at
530 equal amounts and incubated with GFP-Trap beads for 1 hour, followed by washes
531 under denaturing conditions (8 M Urea, 1% SDS in PBS). Bound proteins were
532 eluted in NuPAGE LDS Sample Buffer (Life Technologies) supplemented with 1
533 mM DTT, boiled at 70°C for 10 minutes, alkylated and loaded onto 4-20% gradient
534 SDS-PAGE gels. Alternatively, *in vitro* phosphorylated LC3 family proteins (as
535 stated above) were used to determine TBK1-dependent phosphorylation sites.
536 Proteins were stained using InstantBlue and digested in-gel with trypsin. Peptides
537 were extracted from the gel, desalted on reversed phase C18 StageTips and ana-
538 lyzed on an Orbitrap Elite™ mass spectrometer (ThermoFisher) (Richter et al.,
539 2016a).

540

541 Phos-tag™ SDS-PAGE

542 Phos-tag™ acrylamide (Wako) gels were used as indicated by the supplier. Gels
543 were prepared with 10% acrylamide, 50 μM phos-tag™ and 100 μM MnCl₂. Cells
544 were lysed in SDS sample buffer supplemented with 10 μM MnCl₂.

545

546 ATG4 cleavage assay

547 Proteins were purified as described above and incubated in buffer (50 mM Tris
548 HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1.5 mM DTT) at 37°C for indicated time
549 points. The assay was stopped by adding SDS sample buffer containing 1% β-
550 mercaptoethanol and heating at 95°C for 5 minutes. The samples were resolved
551 by SDS-PAGE and imaged by Western blotting.

552

553 Modeling and MD simulations with analysis

554 Starting from the human LC3C (8-125) structure (PDB Id: 3WAM; (Suzuki et al.,
555 2014)), we added N- and C-terminal overhangs using Modeller (Sali & Blundell,
556 1993) to construct full-length LC3C (1-147). The LC3C-ATG4B complex was mod-
557 eled using the core complex structure of LC3B(1-124)-ATG4B(1-357) (PDB id:
558 2Z0E; (Satoo et al., 2009)) as template using Modeller (Sali & Blundell, 1993). The
559 C-terminal tails of both LC3C and ATG4B were modeled in extended confor-
560 mations without steric clashes across their interface. Additional unresolved loops
561 in ATG4B were modeled using the loop modeling protocol of Modeller (Sali &
562 Blundell, 1993). ATG4B contains an N- and a C-terminal LIR motif, both of which
563 can, in principle, interact with the WXXL-binding sites on either non-substrate or

564 substrate LC3. Therefore, we modeled an alternative WT complex structure includ-
565 ing the interaction between the C-terminal LIR of ATG4B and the WXXL-binding
566 site on substrate LC3C. Phosphoserines at S93 and S96 were modeled using
567 CHARMM:GUI (Jo et al., 2008). All structures were solvated in TIP3P water and
568 150 mM NaCl. After energy minimization, MD simulations of different phosphory-
569 lation states were performed using gromacs v5.1 (Pronk et al., 2013), with position-
570 restrained NVT equilibration and NPT equilibration runs for 1000 ps each. Produc-
571 tion runs at 310 K and 1 atm were simulated for different times (see Table S1). We
572 used the CHARMM36m force field (Huang et al., 2017), the Nosé-Hoover thermo-
573 stat (Nosé, 1984), the Parinello-Rahman barostat (Parrinello & Rahman, 1981),
574 and a time step of 2 fs. For each of the LC3C-systems (Table S1), six replicates
575 were simulated with different initial velocities. We also used the molecular mechan-
576 ics Poisson-Boltzmann surface area (MM-PBSA) to compute the binding energies
577 of the phosphorylated and unphosphorylated LC3C-ATG4B complexes as imple-
578 mented in g_mmpbsa (Kumari et al., 2014). These binding energies contain mo-
579 lecular mechanical (MM), polar, and non-polar solvation energies. MM energies
580 depend on bonded and non-bonded terms including electrostatic (E_{elec}) and van
581 der Waals (E_{vdW}) contributions. The polar solvation energies were computed at an
582 ionic strength of 150 mM, a solvent dielectric constant of 80, and a protein dielectric
583 constant of 2 by solving the linearized Poisson-Boltzmann equation with a fine-grid
584 width of 0.5 Å and a coarse grid width of 1.5 times the long axis of the complex, as
585 implemented in Assisted Poisson-Boltzmann Solver (APBS) (Konecny et al.,
586 2012). The non-polar solvation contributions were estimated with the SASA model
587 using a probe radius of 1.4 Å, a surface tension of $\gamma = 0.0226$ kJ/mol/Å² and an
588 offset of 3.84 kJ/mol (Lee et al., 2000). Binding free energies were estimated as
589 the difference energies between bound and free states,

590 $\Delta G_{Binding} = G_{LC3C-ATG4B} - G_{LC3C} - G_{ATG4B}$, where the free energy contributions of the
591 protein-complex and free proteins are decomposed into a sum of molecular-mechan-
592 ics, solvent, and configurational entropy contributions,

$$593 G = \Delta E_{MM} + \Delta G_{Solv} - T\Delta S$$

$$594 G = \Delta E_{bonded} + \Delta E_{vdW} + \Delta E_{ele} + \Delta G_{polar} + \Delta G_{non-polar} - T\Delta S$$

595 The binding energies were evaluated at intervals of 10 ns from the 1000-ns MD
596 trajectories and averaged (see Table S2). Double differences between unphos-
597 phosphorylated and phosphorylated complexes minimize systematic errors caused by
598 possible energy-function inaccuracy. For the dynamic LC3C-ATG4B protein com-
599 plexes studied here, these calculated free energy differences point to trends, but
600 should not be interpreted in terms of, say, dissociation constants.

601

602 Liposome and proteoliposome preparation

603 All lipids were purchased and dissolved in chloroform from Avanti Polar Lipids (Al-
604 abaster, AL). Liposomes were prepared by combining 55 mol % 1,2-dioleoyl-sn-
605 glycerol-3-phosphoethanolamine (DOPE), 35 mol % 1-palmitoyl-2-oleoyl-sn-glyc-
606 ero-3-phosphocholine (POPC), and 10 mol % bovine liver phosphoinositol (PI).
607 The lipids were dried under nitrogen gas and the lipid film was further dried under
608 vacuum for 1 hour. The lipids were reconstituted in NT350 buffer (350 mM NaCl,
609 20 mM Tris-HCl pH 7.4) and subjected to 7 cycles of flash-freezing in liquid nitro-
610 gen and thawing in a 37°C bath. Liposomes were further sonicated immediately
611 prior to the lipidation reaction.

612

613 Lipidation reaction of LC3C and GABARAP-L2

614 For a full lipidation reaction LC3C, LC3C S93/96D, GABARAP-L2 WT or GABA-
615 RAP-L2 S87/88D (10 µM) were mixed with hATG7 (0.5 µM), hATG3 (1 µM), Atg12-
616 5-16L1 (0.25 µM), sonicated liposomes (3 mM) and 1 mM DTT. Lipidation was
617 initiated by adding 1 mM ATP and reactions were incubated at 30°C for 90 minutes.
618 Samples were mixed with LDS loading buffer and immediately boiled to stop fur-
619 ther lipidation. The reactions were run on a 4-20% SDS-PAGE gel and visualized
620 by Coomassie blue stain and analyzed with Image Lab 6.0 (Biorad).

621

622 De-lipidation reaction of LC3C and GABARAP-L2

623 For a full lipidation reaction LC3C, LC3C S93/96D, GABARAP-L2 WT or GABA-
624 RAP-L2 S87/88D (10 µM) were mixed with hATG7 (0.5 µM), mAtg3 (containing an
625 extended N-terminal amphipathic helix that permits lipidation in absence of
626 ATG12-5-16L1) (1 µM), sonicated liposomes (3 mM) and 1 mM DTT. Lipidation
627 was initiated by adding 1 mM ATP and reactions were incubated at 30°C for 90
628 minutes. After the reaction was complete, the lipidation reaction was run on a
629 Nycodenz density gradient. The bottom layer of the gradient consisted of 150 µL
630 of 80% Nycodenz and 150 µL of the lipidation reaction. The second layer consisted
631 of 250 µL of 30% Nycodenz while the top layer was 50 µL of NT350 buffer. Gradi-
632 ents were centrifuged at 27000 rpm at 4°C for 4 hours in a Beckman SW55 rotor.
633 Liposomes with the conjugated LC3C or GABARAP-L2 protein were collected from
634 the top of the tube before use in subsequent de-lipidation experiments. To meas-
635 ure the activity of proteases, 10 µM of proteoliposomes (concentration estimated
636 by Coomassie blue stain) were mixed with NT350 buffer and kept on ice until ac-
637 tivity assays were initiated by the addition of 2 µM (or indicated amounts) of either
638 ATG4A, ATG4B or RavZ. Reactions were incubated at 37°C for 1 hour. Samples
639 were mixed with LDS loading buffer and immediately boiled to stop proteolysis.
640 The reactions were run on a 4-20% SDS-PAGE gel and visualized by Coomassie
641 blue stain and analyzed with Image Lab 6.0 (Biorad).

642

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655

656 **Author contributions**

657 LH and ID conceived the study. LH designed and performed most of the experi-
658 ments. RMB developed structural models and performed MD simulations and anal-
659 ysis of the data with help and supervision from GH. AHL performed *in vitro* (de-
660 lipidation assays in the lab of AS. LH wrote the manuscript with contribution from
661 all authors. All authors approved the final version of the manuscript.

662

663 **Conflict of Interest**

664 The authors declare no conflict of interest.

665

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821

822

823

824 **Figure legends**

825 **Figure 1:**

826 **TBK1 phosphorylates LC3C and GABARAP-L2 *in vitro*.** (A) Coomassie stain
827 and autoradiography of SDS-PAGE after an *in vitro* kinase assay with His-TBK1
828 and His-LC3 family proteins as substrates. TBK1 phosphorylates LC3A, LC3C,
829 GABARAP-L1 and GABARAP-L2 *in vitro*. (B) Identification of phosphosites by
830 mass spectrometry following an *in vitro* TBK1 kinase assay. (C) Structure of
831 LC3C₈₋₁₂₅ (PDB: 3WAM) with modeled phosphate groups (red sticks) at S93 and
832 S96. (D) Topologically equivalent positions in GABARAP-L2 (PDB: 4CO7), S87
833 and S88 are also phosphorylated (red sticks) by TBK1. Phosphorylation sites are
834 on the opposite face of the LIR binding pocket of LC3-proteins.

835

836 **Figure 2:**

837 **TBK1 phosphorylates and binds LC3C and GABARAP-L2 *in cells*.** (A) Identifi-
838 cation of phosphosites by mass spectrometry following GFP-LC3C or GFP-
839 GABARAP-L2 immunoprecipitation (IP) in SILAC labeled cells. TBK1 WT was
840 overexpressed in heavy and TBK1 kinase dead K38A was overexpressed in light
841 labeled cells. (B) SDS-PAGE and Western blot of phos-tag™ gel with HEK293T
842 cell lysates. Cells were transfected with HA-LC3C, TBK1 WT or K38A and GFP-
843 Parkin and left untreated or treated with CCCP (3 hours, 40 μM) to induce mitoph-
844 agy. (C) SDS-PAGE and Western blot of HEK293T and HeLa cell lysates and
845 GST-LC3C or GST-GABARAP-L2 immunoprecipitations. (D) SDS-PAGE and
846 Western blot of HEK293T cell lysates transfected with full-length TBK1, a C-termi-
847 nal truncation mutant (TBK1 ΔC), a kinase dead version (TBK1 K38A) and GST-
848 LC3C or GST-GABARAP-L2 immunoprecipitations.

849

850 **Figure 3:**

851 **Phospho-mimetic LC3C and GABARAP-L2 impede ATG4 cleavage and bind-**
852 **ing.** (A) SDS-PAGE and Western blotting of *in vitro* ATG4 cleavage assay. Purified
853 double tagged His-LC3C-Strep WT and mutants were incubated with ATG4B for
854 indicated time points. LC3C S93/96D mutation slows down C-terminal cleavage of
855 LC3C by ATG4B. (B) SDS-PAGE and Western blot of HEK293T cell lysates trans-
856 fected with LC3C WT or mutants. S93/96D mutation of LC3C impedes cleavage
857 of pro-LC3 by endogenous ATG4s. (C,D) SDS-PAGE and Western blot of
858 HEK293T cell lysates transfected with LC3C WT or mutants (C) or GABARAP-L2
859 WT or mutants (D). Cells were left untreated or treated with 40 μM CCCP for 3
860 hours to induce mitophagy. (E) SDS-PAGE and Western blot of HEK293T cell ly-
861 sates and GFP immunoprecipitations. Cells were transfected with Flag-tagged
862 ATG4A or ATG4B and GFP-tagged LC3C or GABARAP-L2 WT or mutants and
863 lysates used for GFP IPs. S93/96D mutation of LC3C and S87/88D mutation of

864 GABARAP-L2 impede binding to ATG4A or B. (F) Full-length LC3C (red cartoon)
865 binds to ATG4B (grey surface) with its C-terminal tail accessible to the active site
866 of ATG4B. Phosphorylation of LC3C at S93 and S96 (sticks) affect binding to
867 ATG4B. Zoom-up showing the side and top view of LC3C-ATG4B interface. S96
868 of LC3C and E350 of ATG4B form direct hydrogen-bonds across the interface.
869 S93 position is central to a network of polar interactions (blue dashed lines; side
870 chains shown as sticks) across the interface.

871

872 **Figure 4:**

873 **Phosphorylation at S93 and S96 affects LC3C C-terminal tail structure in pro-**
874 **LC3C, thereby impeding ATG4-mediated processing.** (A-C) Representative
875 snapshots from all-atom MD simulations (see Movies SM3-5) of unphosphorylated
876 (red), S93-PO₄ LC3C (blue) and S96-PO₄ LC3C (green). (D, E) Salt-bridge for-
877 mation dynamics in MD simulations represented by the time-dependent minimum
878 distance between side chain heavy atoms of R134 to (D) S93 and (E) S96 in phos-
879 phorylated (blue) and unphosphorylated (red) LC3C simulations. Black line repre-
880 sents cut-off distance (0.6 nm) for favorable salt-bridge formation. (F) SDS-PAGE
881 and Western blotting of *in vitro* ATG4 cleavage assay. Purified double tagged His-
882 LC3C-Strep WT and mutants were incubated with ATG4B in buffer for indicated
883 time points. LC3C S93/96D R134A mutation enables C-terminal cleavage of LC3C
884 by ATG4B.

885

886 **Figure 5:**

887 **Phospho-mimetic LC3C and GABARAP-L2 cannot form autophagosomes.**
888 (A,B) U2OS cells were transfected with GFP-GABARAP-L2 (A) or GFP-LC3C (B)
889 WT or mutants and HA-Parkin. Mitophagy was induced by the addition of 40 μM
890 CCCP for 3 hours. GFP-expressing cells were counted and segregated into clas-
891 ses with greater and less than 10 autophagosomes per cell. Bars represent
892 mean±SD from three separate experiments. * P<0.05, ** P<0.01, ***P<0.001, as
893 analyzed by unpaired Student's t-test.

894

895 **Figure 6:**

896 **Phospho-mimetic Δ C-terminal LC3C or GABARAP-L2 cannot form autophago-**
897 **somes because they cannot be lipidated.** (A,B) U2OS cells were transfected
898 with GFP-GABARAP-L2 Δ C-terminal (A) or GFP-LC3C Δ C-terminal (B) WT or
899 mutants and HA-Parkin. Mitophagy was induced by the addition of 40 μM CCCP
900 for 3 hours. GFP-expressing cells were counted and segregated into classes with
901 greater and less than 10 autophagosomes per cell. Data is presented as mean±SD
902 from three separate experiments. *P<0.05, **P<0.01, ***P<0.001 as analyzed by
903 Students T-test. (C) *In vitro* lipidation reactions containing 10 μM LC3C WT, LC3C

904 S93/96D, GABARAP-L2 (GL2) WT or GABARAP-L2 S87/88D incubated with or
905 without 0.5 μ M hATG7, 1 μ M hATG3, 0.25 μ M hATG12-5-16L1 β , 3 mM lipid (soni-
906 cated liposomes composed of 10 mol% bi-PI, 55 mol% DOPE, and 35 mol%
907 POPC), 1 mM DTT and 1 mM ATP were incubated at 30°C for 90 minutes. The
908 reactions were analyzed by SDS-PAGE and visualized by Coomassie blue stain.
909 (D) The extent of lipidation in (C) was quantified and plotted as percentage of total
910 protein (conjugated and unconjugated). Data is presented as mean \pm SEM from
911 three separate experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, as an-
912alyzed by One-way Anova followed by Bonferonis multiple comparison test.

913

914 **Figure 7:**

915 **TBK1-mediated GABARAP-L2 phosphorylation impedes its premature cleav-**
916 **age from autophagosomes by ATG4.** (A) LC3C WT-, LC3C S93/96D, GABA-
917 RAP-L2 (G.-L2) WT- or GABARAP-L2 S87/88D-conjugated liposomes were
918 treated or not with 2 μ M ATG4A or ATG4B for 1 hour at 37°C. Samples were then
919 subjected to SDS-PAGE together with unconjugated LC3C and GABARAP-L2 pro-
920 teins. (B) The extent of de-lipidation in (A) was quantified and plotted as percent-
921 age of total protein (conjugated and unconjugated). Data is presented as
922 mean \pm SEM from three separate experiments. *P<0.05, **P<0.01, ***P<0.001,
923 ****P<0.0001, as analyzed by Two-way Anova followed by Bonferonis multiple
924 comparison test. (C) GABARAP-L2 WT- or GABARAP-L2 S87/88D-conjugated lip-
925 osomes (G.-L2-II) were treated or not with different amounts (2-0.125 μ M) of RavZ
926 for 1 hour at 37°C. Samples were then subjected to SDS-PAGE together with un-
927 conjugated GABARAP-L2 (G.-L2-I). (D) SDS-PAGE and Western blot of HEK293T
928 cell lysates and GFP IPs. Cells were transfected with GFP, GFP-LC3C Δ C-termi-
929 nal WT or S93/96D and lysates used for GFP IPs. WT and S93/96D LC3C bind
930 endogenous p62. (E) SDS-PAGE and Western blot of HEK293T cell lysates and
931 GFP IPs. Cells were transfected with GFP, GFP-GABARAP-L2 Δ C-terminal WT
932 or S87/88D and lysates used for GFP IPs. WT and S87/88D GABARAP-L2 bind
933 endogenous p62, Optineurin (OPTN) and PLEKHM1.

934

935 **Figure 8:**

936 **Model of TBK1-mediated LC3C and GABARAP-L2 phosphorylation.**

937 TBK1 recruitment to autophagosomes promotes phosphorylation of membrane-
938 embedded LC3C and GABARAP-L2. Phosphorylation prevents premature re-
939 moval of LC3C and GABARAP-L2 from autophagosomes, ensuring an unper-
940 turbed and unidirectional flow of the autophagosome to the lysosome.

941

942 **Supplementary Figure legends**

943 **Supplementary Figure 1:**

944 **phospho-GABARAP-L2 antibody validation.** SDS-PAGE and Western blot of *in*
945 *vitro* TBK1 kinase assay with His-GABARAP-L2 WT or mutants as substrates to
946 test the respective phospho-GABARAP-L2 antibodies for their specificity.

947

948 **Supplementary Figure S2:**

949 **Phosphorylation of S93 and S96 of LC3C affects ATGB binding energy.** (A)
950 WT LC3C-ATG4B complex (B) with additional LIR interactions and (C) with phos-
951 phosphorylated LC3C residues (S93 and S96) subjected to MD simulations and binding
952 free energy computations using MM-PBSA (see Methods) approach. A residue-
953 wise decomposition of the total binding free energy mapped onto the LC3C struc-
954 ture displays locally favorable (blue), neutral (white) and unfavorable (red) residue
955 interaction with ATG4B (grey surface). Note that S93 and S96 positions in WT
956 complexes contribute favorably (blue color), whereas in the phosphorylated com-
957 plex contribute unfavorably (red) towards complex formation. The thickness of the
958 backbone scales linearly with the binding energy of LC3C-ATG4B complexes.

959

960 **Supplementary Figure 3:**

961 **Phosphorylation at S93 and S96 affects LC3C C-terminal tail structure,**
962 **thereby impeding ATG4-mediated cleavage.** Plots of the minimum distance be-
963 tween R134 and serines S93 and S96 report on salt-bridge formation in MD simu-
964 lations of the LC3C-ATG4B complex with (A) unphosphorylated S93 and S96, (B)
965 S93-PO₄, and (C) S96-PO₄. N=6.

966

967 **Supplementary Figure 4:**

968 **Phospho-mimetic LC3C and GABARAP-L2 cannot form autophagosomes.**
969 (A,B) U2OS cells were transfected with GFP-GABARAP-L2 (A) or GFP-LC3C (B)
970 WT or mutants and HA-Parkin. Mitophagy was induced by the addition of 40 μM
971 CCCP for 3 hours. WT and S87/88A GABARAP-L2 form autophagosomes,
972 whereas S87/88D GABARAP-L2 remains dispersed in the cytosol (A). WT and
973 S93/96A LC3C form autophagosomes that localize with HA-Parkin, whereas
974 S93/96D LC3C remains dispersed in the cytosol (B).

975

976 **Supplementary Figure 5:**

977 **Phospho-mimetic Δ C-terminal LC3C or GABARAP-L2 cannot form autopa-**
978 **gosomes.** (A,B) U2OS cells were transfected with GFP-GABARAP-L2 Δ C-termi-
979 nal (A) or GFP-LC3C Δ C-terminal (B) WT or mutants and HA-Parkin. Mitophagy
980 was induced by the addition of 40 μM CCCP for 3 hours. WT and S87/88A Δ C-
981 terminal GABARAP-L2 form autophagosomes, whereas S87/88D Δ C-terminal

982 GABARAP-L2 remains dispersed in the cytosol (A). WT and S93/96A Δ C-terminal
983 LC3C form autophagosomes that localize with HA-Parkin, whereas S93/96D Δ C-
984 terminal LC3C remains dispersed in the cytosol (B). (C) SDS-PAGE and Western
985 blot of HEK293T cell lysates transfected with HA-GABARAP-L2 Δ C-terminal WT
986 or mutants. Cells were left untreated or treated with 40 μ M CCCP for 3 hours to
987 induce mitophagy.

988

989 **Supplementary Figure 6:**

990 **De-lipidation of GABARAP-L2 and LC3C by ATG4A, B and RavZ is dose de-**
991 **pendent.**

992 (A) LC3C WT-, LC3C S93/96D-, GABARAP-L2 (G.-L2) WT- or GABARAP-L2
993 S87/88D-conjugated liposomes were treated or not with 2 μ M ATG4A, ATG4B or
994 RavZ for 1 hour at 37°C. Samples were then subjected to SDS-PAGE. (B) GABA-
995 RAP-L2 WT- or GABARAP-L2 S87/88D-conjugated liposomes (G.-L2-II) were
996 treated or not with different amounts (2-0.125 μ M) of ATG4A or ATG4B for 1 hour
997 at 37°C. Samples were then subjected to SDS-PAGE together with unconjugated
998 GABARAP-L2 (G.-L2-I).

999

1000 **Supporting Movie legends**

1001 **SM1:**

1002 **MD simulation of phosphorylated LC3C=ATG4B complex.** First 200 ns of a
1003 1158 ns trajectory showing the effect of phosphorylation of LC3C (red cartoon) on
1004 ATG4B interaction (grey surface). Phosphorylation at S93 and S96 (sticks) induces
1005 strong electrostatic effects that partially dislodge LC3C from the surface of ATG4B
1006 (first ~10-50 ns). The phosphorylated S93-PO₄ and S96-PO₄ detach from the
1007 ATG4B surface, opening a gap in the binding interface. The LC3C structure is dis-
1008 torted upon phosphorylation of S93 and S96. Its C-terminal tail retracts partially
1009 from the ATG4B active site. However, the ATG4B C-terminal LIR motif maintains
1010 stable interactions with the LIR binding pocket of LC3C (on the opposite face, Mod-
1011 eled).

1012

1013 **SM2:**

1014 **MD simulation of unphosphorylated LC3C=ATG4B complex.** First 200 ns of a
1015 1536 ns trajectory showing the dynamics of unphosphorylated LC3C (red cartoon)
1016 on ATG4B interaction (grey surface). S93 and S96 (sticks) are strongly bound to
1017 the ATG4B and buried in the interface. S93 and S96 of LC3C (red cartoon) mediate
1018 a series of polar contacts across the interface that provide specificity to ATG4B
1019 binding. S96 forms hydrogen-bonds with E350 of ATG4B. S93 is part of a network

1020 of polar contacts involving Y239, R229, N172 of ATG4B and Y86 of LC3C. Inter-
1021 actions across the extended interface allow stable sequestering of the C-terminal
1022 tail of LC3C close to the ATG4B active site throughout the simulation.

1023

1024 **SM3:**

1025 **MD simulation of full-length unphosphorylated LC3C₁₋₁₄₇.** First 500 ns from a
1026 representative trajectory (run 1 in Figure S3A) of full-length LC3C₁₋₁₄₇ (red) show-
1027 ing large fluctuations of the unprocessed C-terminal tail. The large fluctuations of
1028 the C-terminal tail prevent R134 (sticks) to approach the phosphorylation sites S93
1029 and S96 (sticks). The ATG4B binding site (grey surface) displays minimal fluctua-
1030 tions due to C-terminal tail dynamics.

1031

1032 **SM4:**

1033 **MD simulation of S93-PO₄-LC3C₁₋₁₄₇.** First 500 ns from an example trajectory
1034 (run 3 in Figure S3B) of S93-PO₄-LC3C₁₋₁₄₇ (blue) showing the formation of a sta-
1035 ble salt bridge between S93-PO₄ and R134 (sticks). The movie shows the initial
1036 fluctuations of C-terminal tail enabling contact between S93-PO₄ and R134 fol-
1037 lowed by rearrangement of the loop to form a stable salt bridge between. The
1038 ATG4B binding site (grey surface) is perturbed by the formation of salt bridge.

1039

1040 **SM5:**

1041 **MD simulation of S96-PO₄-LC3C₁₋₁₄₇.** First 500 ns from an example trajectory (run
1042 5 in Figure S3C) of S96-PO₄-LC3C₁₋₁₄₇ (green) showing the quick formation and
1043 disassociation of a salt bridge between S96-PO₄ and R134 (sticks; within 80-100
1044 ns). Subsequently, due to loop-rearrangement in the C-terminal tail, the R134 ap-
1045 proach to S96-PO₄ is restricted, leading to weak interactions in close-proximity
1046 (~0.5-0.6 nm). The ATG4B binding site (grey surface) is perturbed upon phosphor-
1047 ylation.

1048

1049 **Table S1: Molecular dynamics simulations of LC3C and LC3C-ATG4B com-**
 1050 **plexes.** The table lists the different simulations of LC3C₁₋₁₄₇ and of LC3C-ATG4B
 1051 complexes, including the phosphorylation state, the number of runs, the total sim-
 1052 ulation time, the number of ions and water molecules, the total number of atoms,
 1053 and the number of salt-bridge formation events between phosphorylated S93 or
 1054 S96 and R134. In the column “interface interactions,” we give a qualitative assess-
 1055 ment on the preservation of the interface structure and interactions in the LC3C-
 1056 ATG4B complex simulations.

LC3C	No of replicates	Total Simulation time (μs)	Ions (Na+/Cl-)	Tip3p Water	Total number of atoms	Salt-bridge formation events
WT	6	7.380	47/51	17078	53723	0
S93-PO ₄	6	7.155	48/50	17057	53663	4
S96-PO ₄	6	7.420	48/50	17101	53795	2
S93/S93D	6	7.181	60/62	21127	65896	-
LC3C-ATG4B Complex	No of replicates	Simulation time (μs)	Ions (Na+/Cl-)	Tip3p Water	Total number of atoms	Interface interactions
WT	1	1.475	106/91	30583	100457	++
WT + LIR	1	1.535	179/164	56164	177357	+++
S93/S96-PO ₄ LC3C + LIR	1	1.158	110/89	31711	103863	---

1057

1058 **Table S2: Binding free energy computations for LC3C-ATG4B complexes.**

1059 The table lists different energetic contributions (mean \pm s.d.) to the binding of LC3C
 1060 and ATG4B in different phosphorylation states and with modeled LIR-WXXL inter-
 1061 actions. The different binding energy contributions were computed using the MM-
 1062 PBSA approach implemented in *g_mmpbsa* (see Methods) from MD simulations
 1063 of LC3C-ATG4B complexes. The non-bonded energy terms (van der Waals and
 1064 electrostatic) contribute significantly to the molecular-mechanics interaction en-
 1065 ergy of the complex, whereas changes in the bonded terms (bond-length, angle,
 1066 and dihedral terms) do not contribute significantly to the interaction energy during
 1067 complex formation.

System/ Energy terms [kJ/mol]	LC3C-ATG4B (unphosphorylated)	LC3C-ATG4B (unphosphorylated + LIR)	LC3C-ATG4B (S93/S96-PO₄ + LIR)
van der Waals	-1036.2 \pm 110.2	-985.9 \pm 91.6	-970.1 \pm 128.4
Electrostatic	-4652.7 \pm 536.4	-4495.8 \pm 315.7	-1610.2 \pm 293.4
Polar solvation	2940.0 \pm 406.0	2883.0 \pm 339.2	2672.4 \pm 311.0
SASA	-136.2 \pm 14.5	-134.8 \pm 10.2	-125.2 \pm 14.0
Total binding energy	-2885.1 \pm 305.2	-2733.5 \pm 204.0	-33.1 \pm 202.4
$\Delta\Delta G$	-	151.6 \pm 367.1	2851.9 \pm 366.2

1068

Figure 1: TBK1 phosphorylates LC3C and GABARAP-L2 *in vitro*

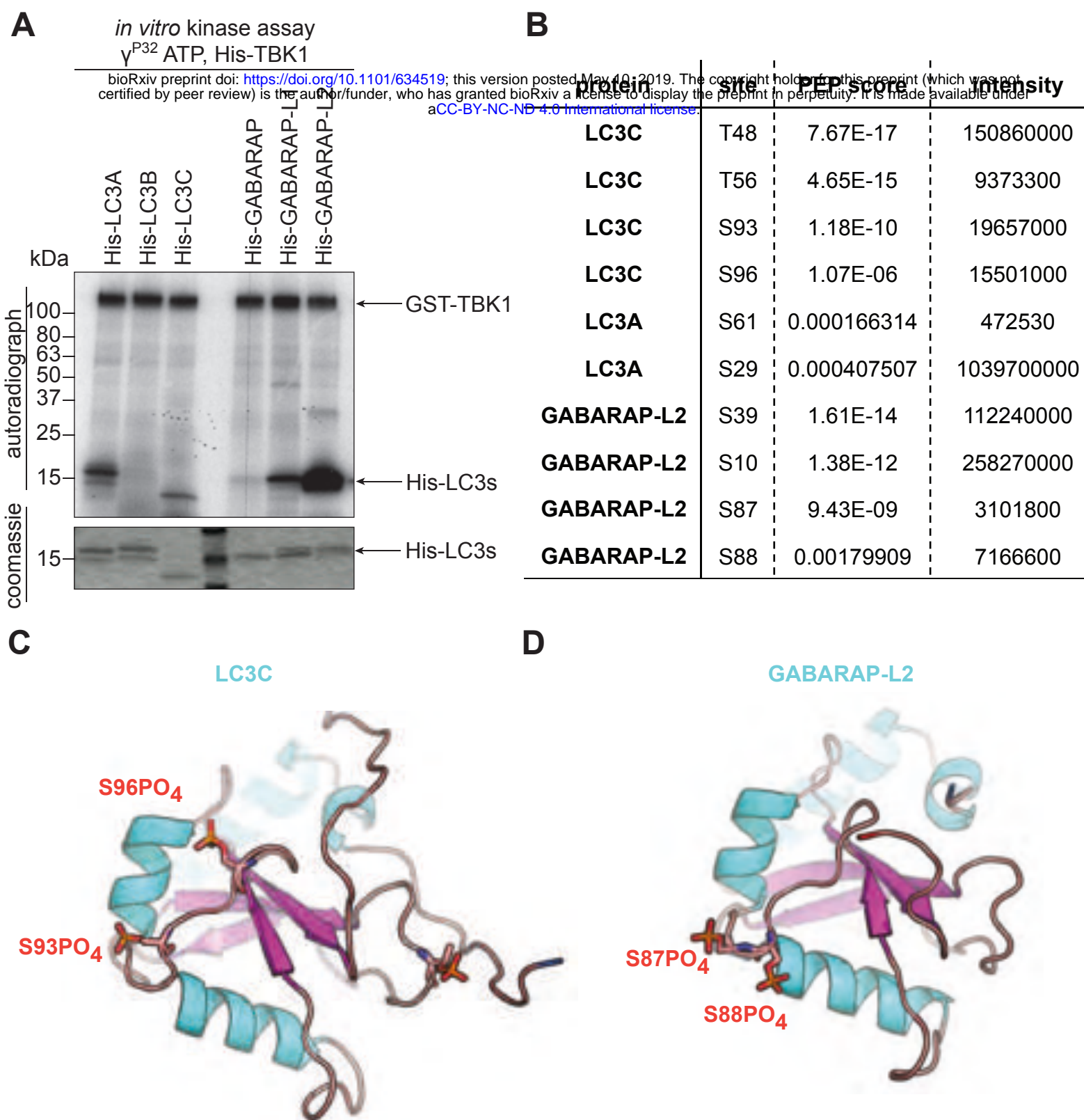


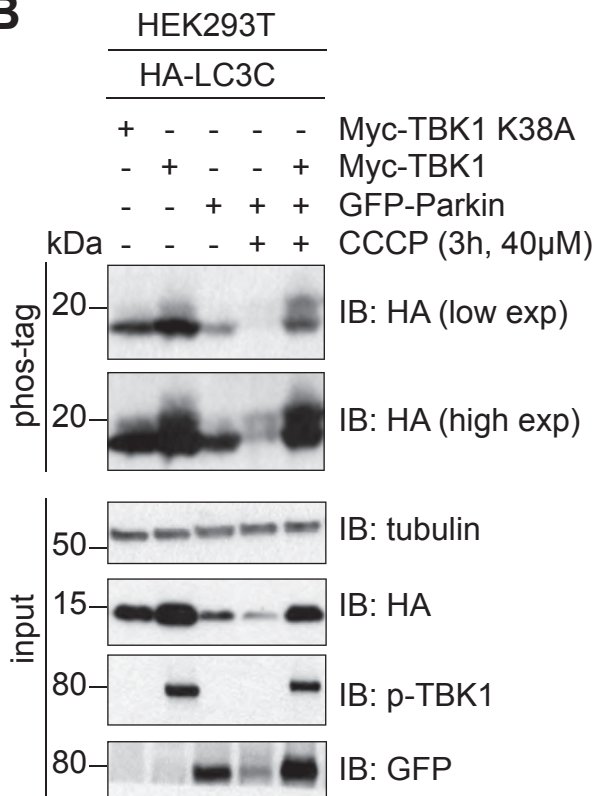
Figure 2: TBK1 phosphorylates and binds LC3C and GABARAP-L2 in cells

A

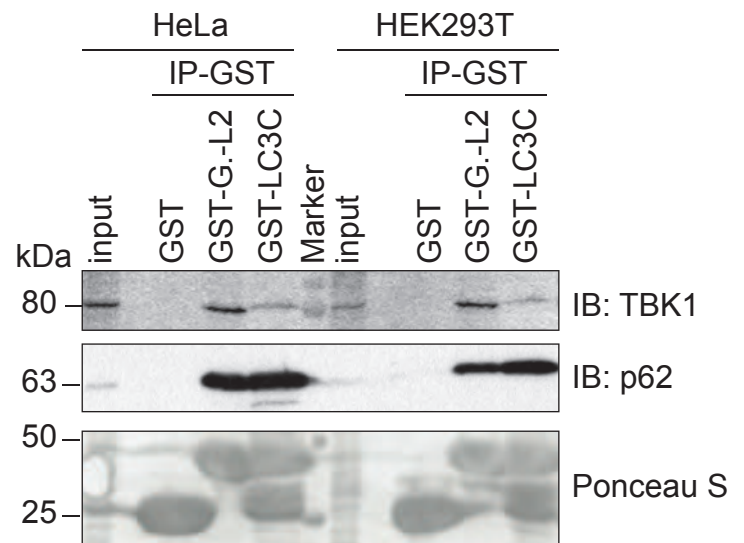
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protein	site	PEP score	H/L normalized	H/L normalized total protein
LC3C	S96	1.41E-12	6.0957	0.9665
LC3C	S93	1.67E-06	9.8225	0.9665
GABARAP-L2	S87	6.87E-09	13.65	1.0037
GABARAP-L2	S88	5.06E-05	2.7345	1.0037

B



C



D

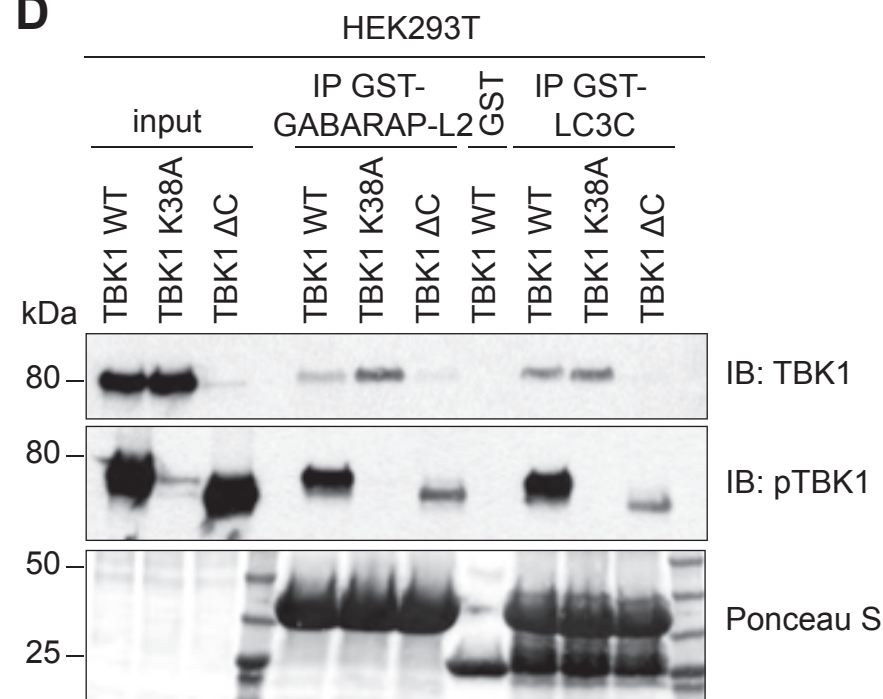
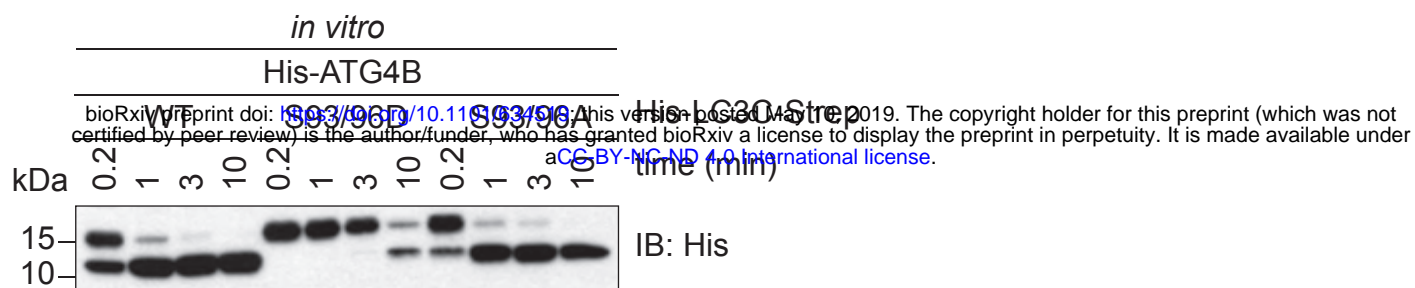
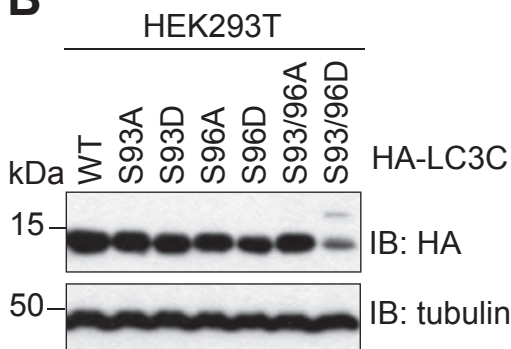


Figure 3: Phospho-mimetic LC3C and GABARAP-L2 impede ATG4 cleavage and binding

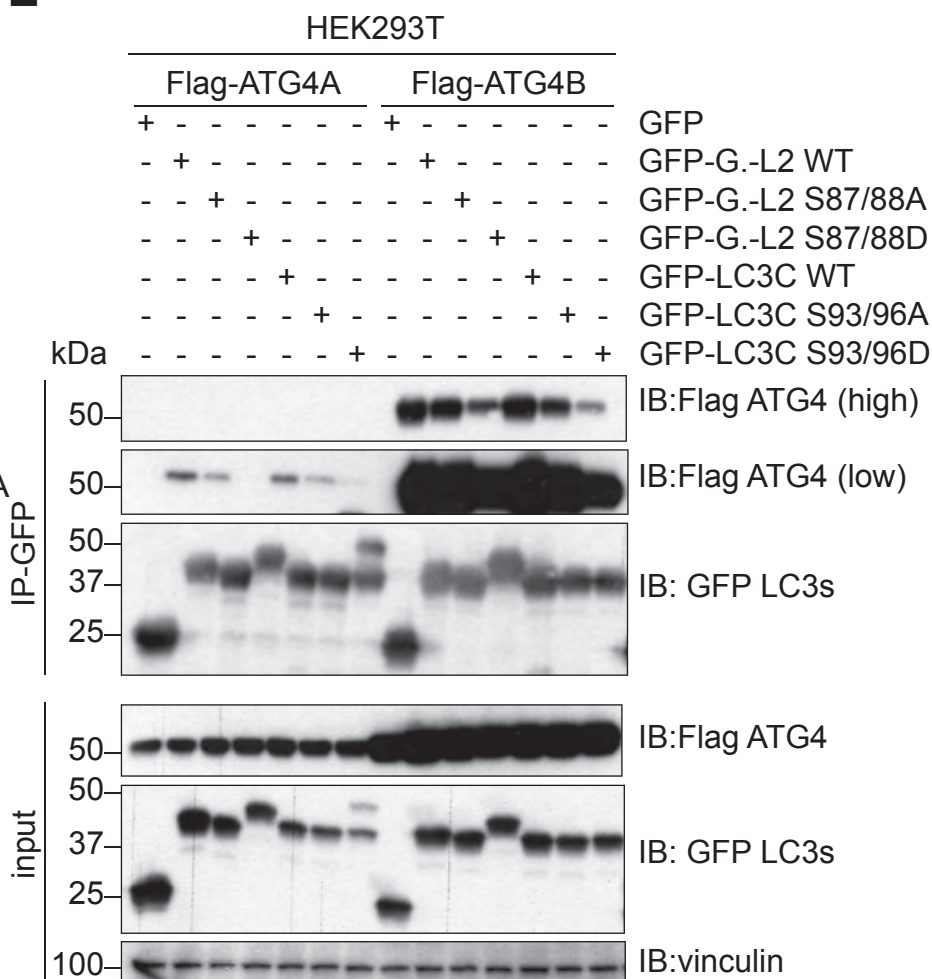
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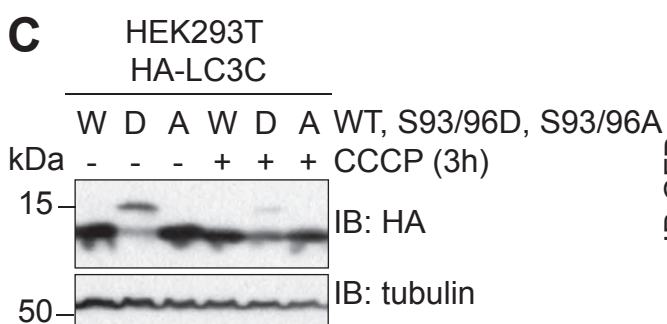
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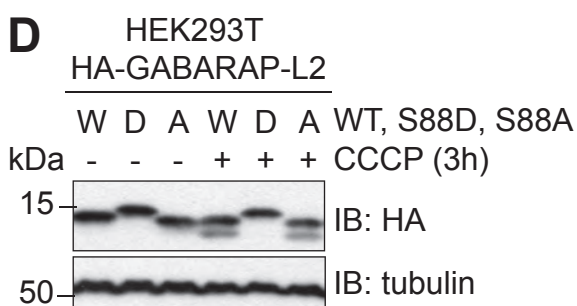
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F

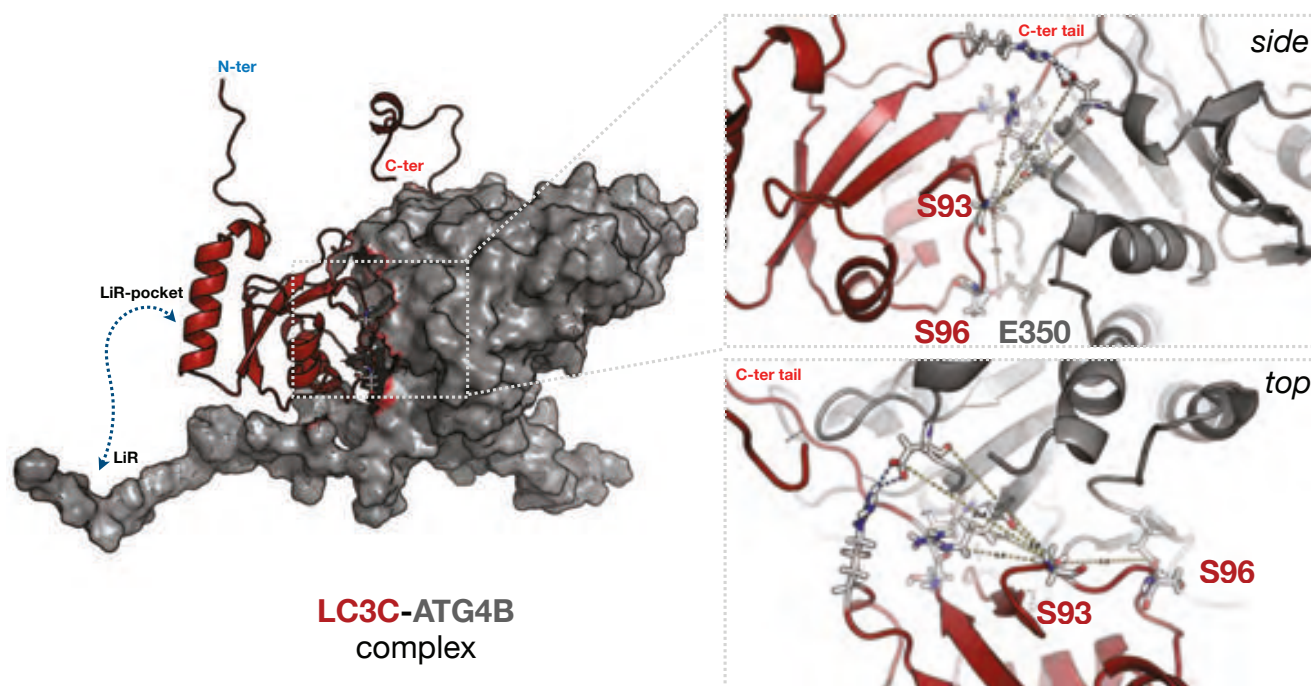


Figure 4: The C-terminus of LC3C phospho-S93 folds back onto itself and thereby impedes ATG4-mediated cleavage

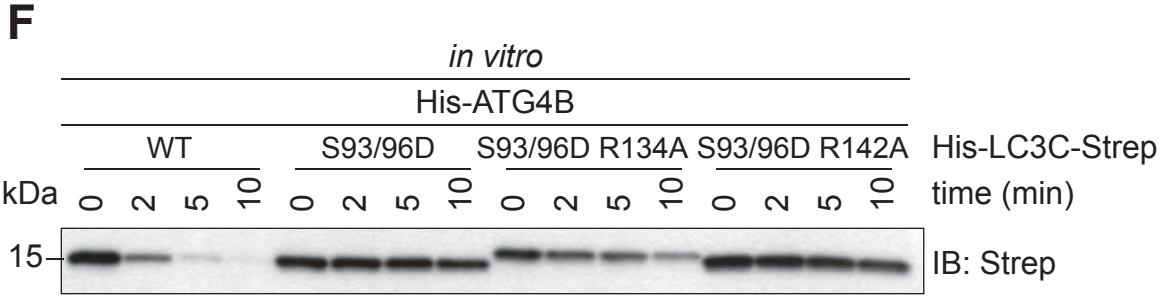
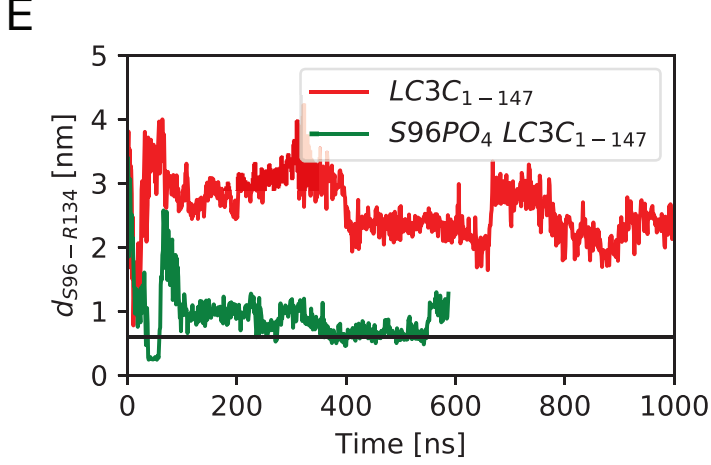
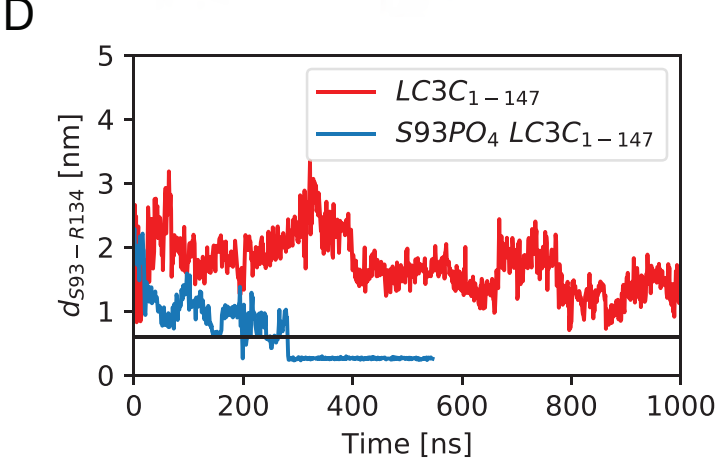
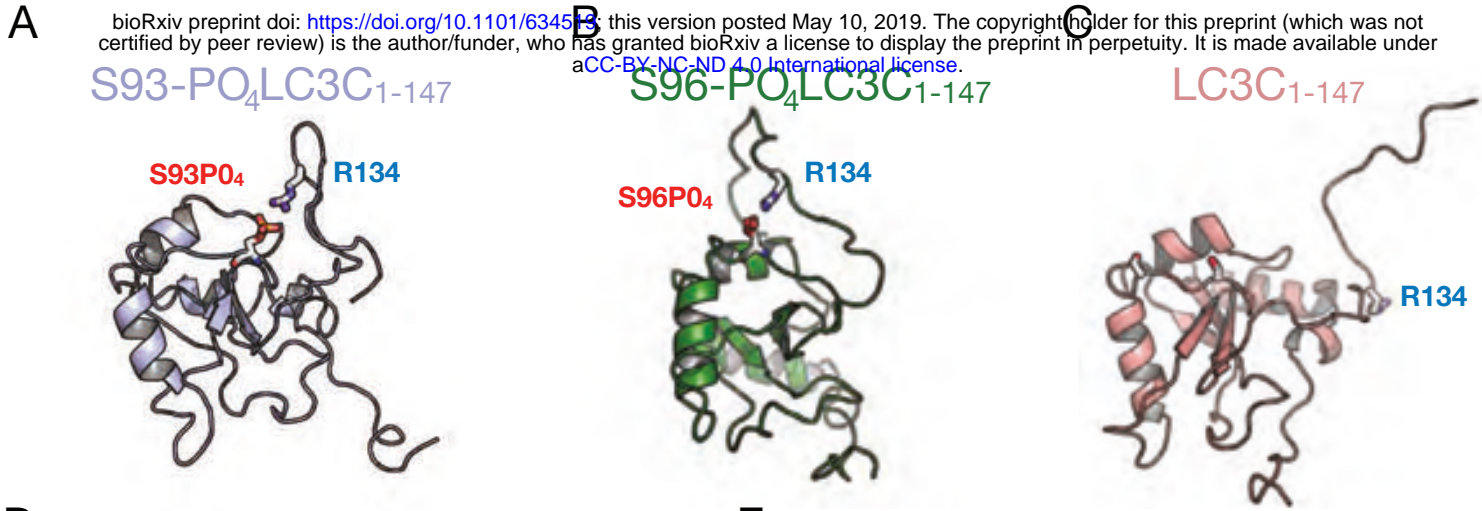
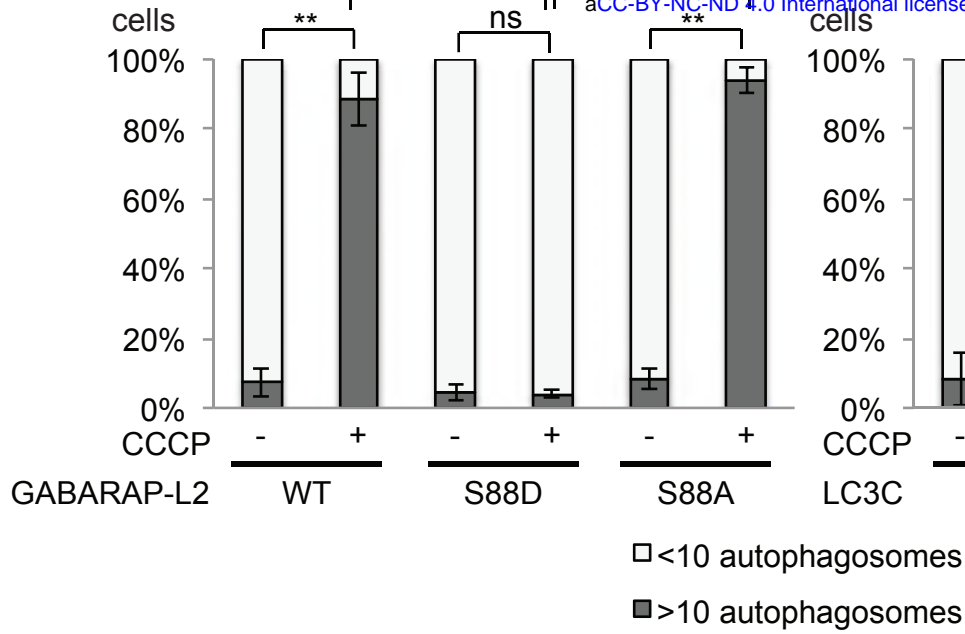


Figure 5: Phospho-mimetic LC3C and GABARAP-L2 cannot form autophagosomes

A

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B

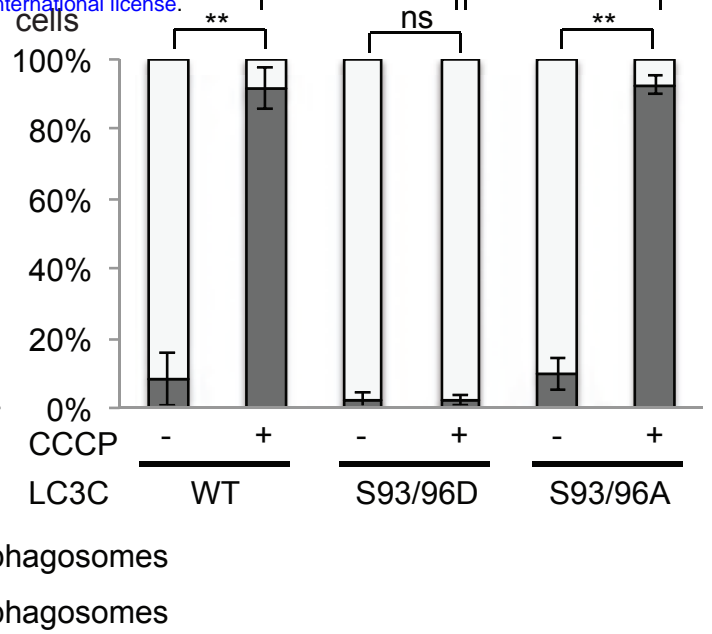
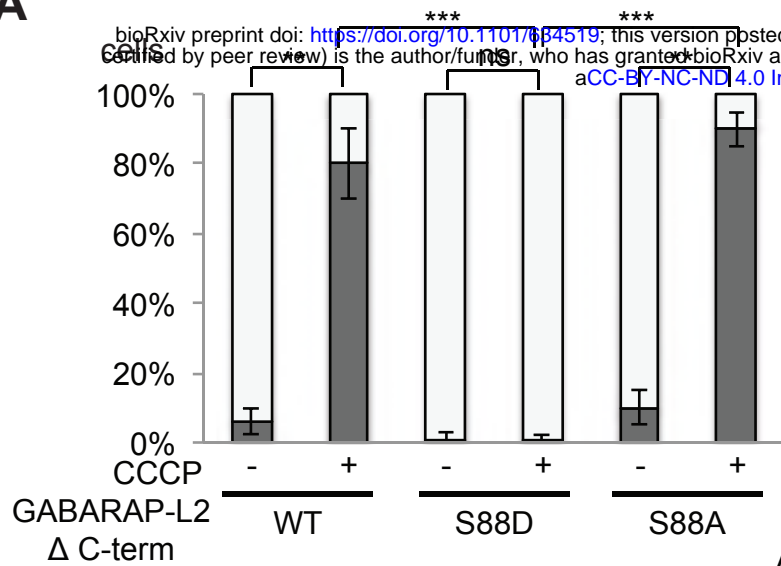
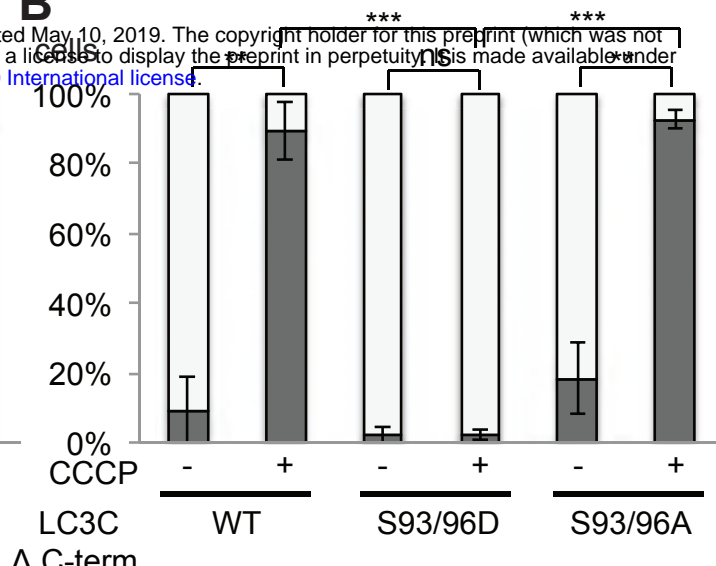


Figure 6: Phospho-mimetic delta C-terminal LC3C or GABARAP-L2 cannot form autophagosomes as they cannot be lipidated

A



B

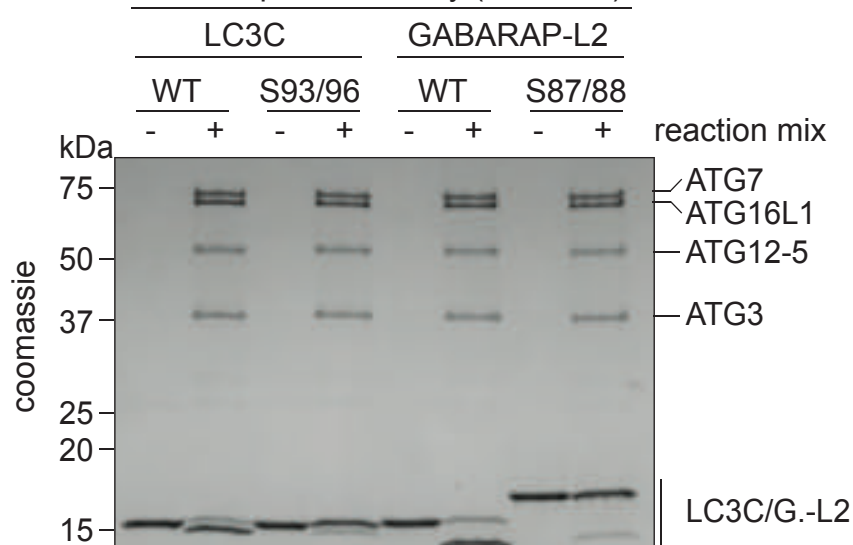


□ <10 autophagosomes

■ >10 autophagosomes

C

in vitro lipidation assay (Δ C-term)



D

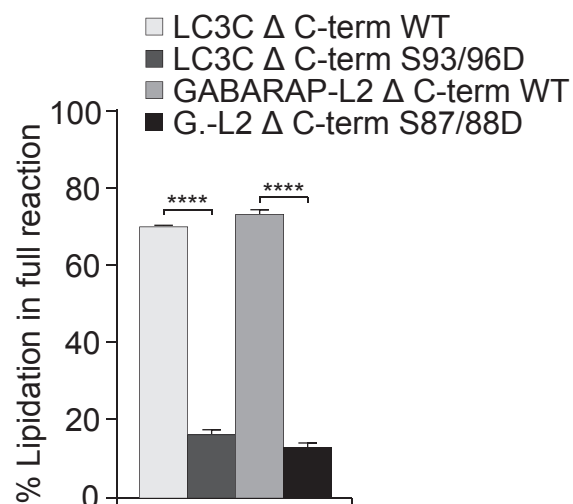
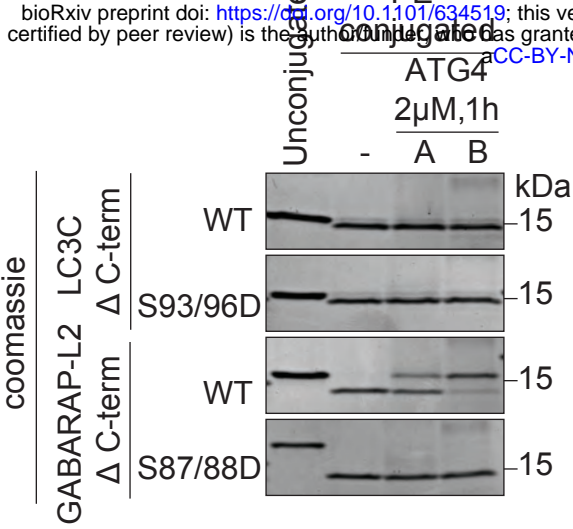
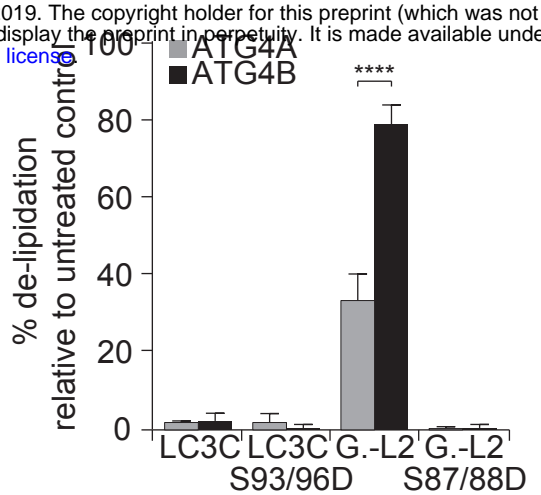


Figure 7: TBK1-mediated GABARAP-L2 phosphorylation impedes its premature cleavage from autophagosomes by ATG4

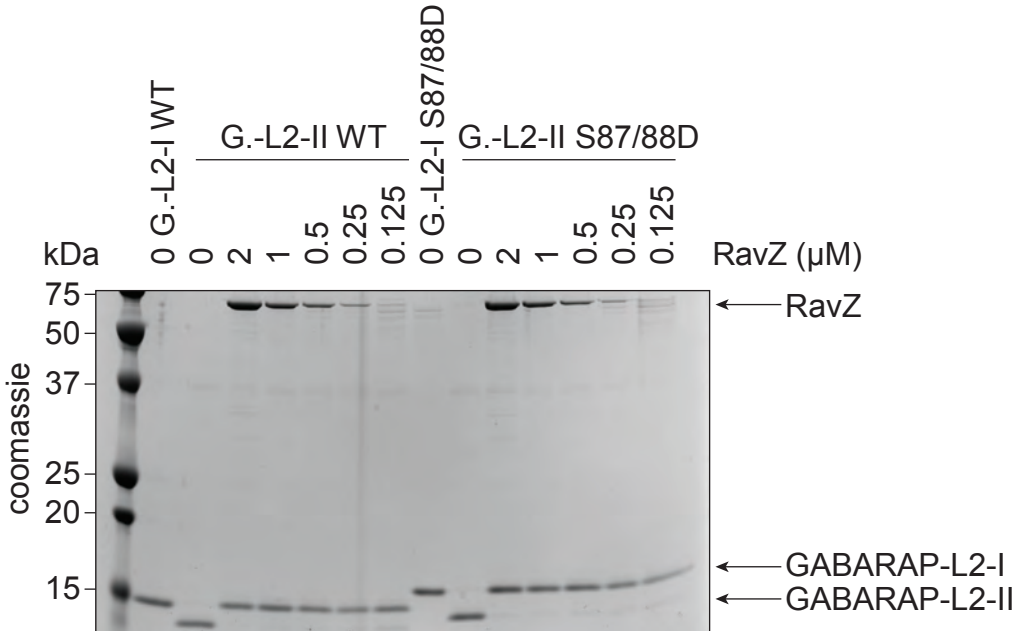
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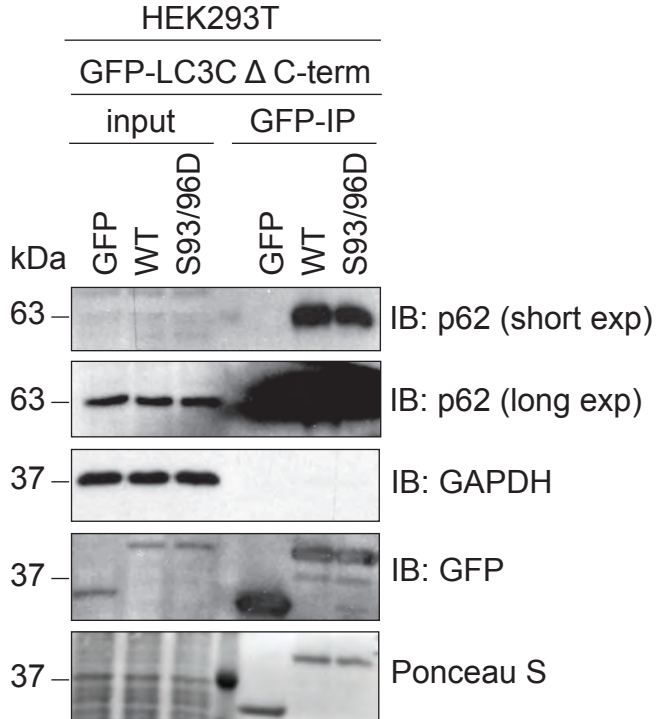
B



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E

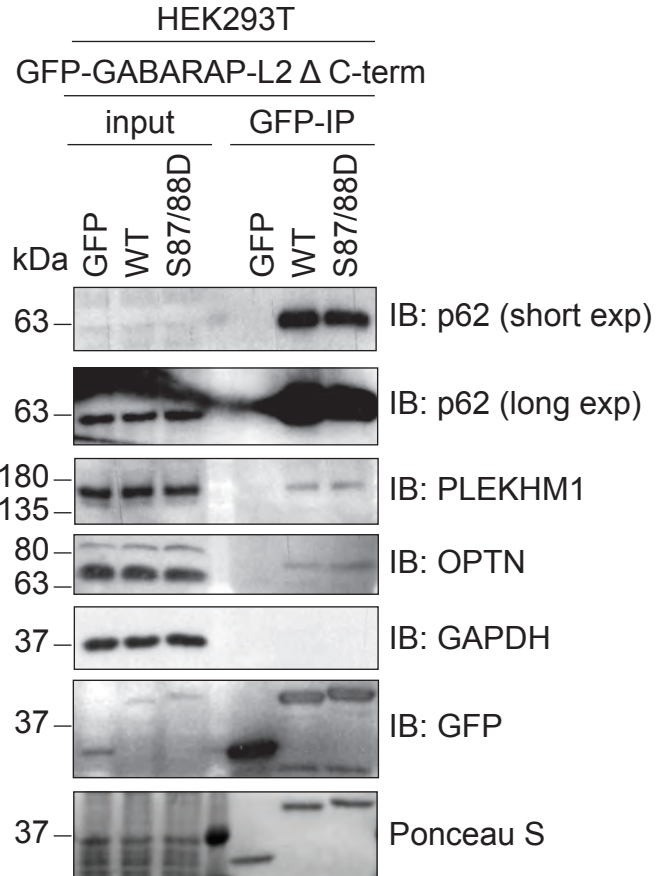
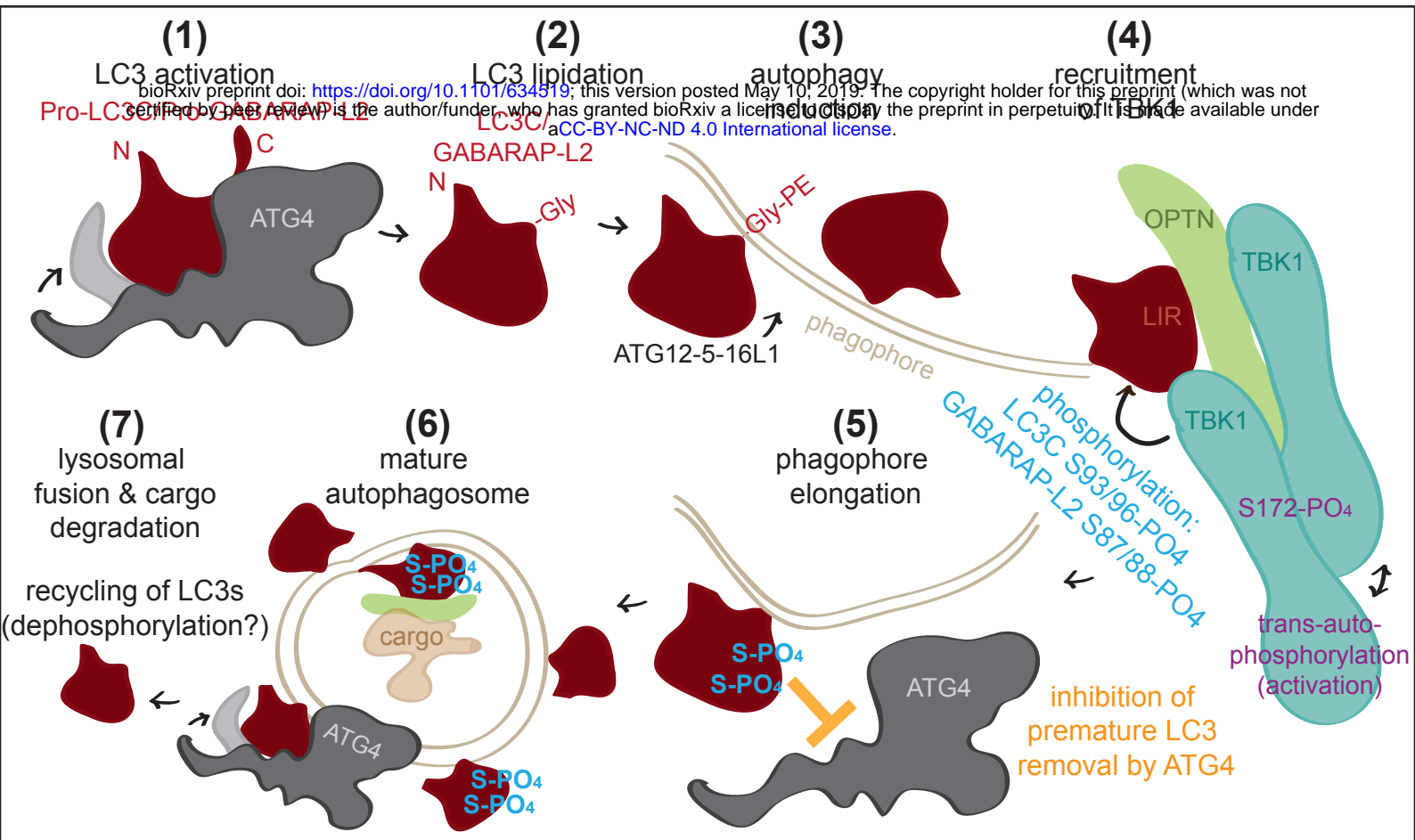
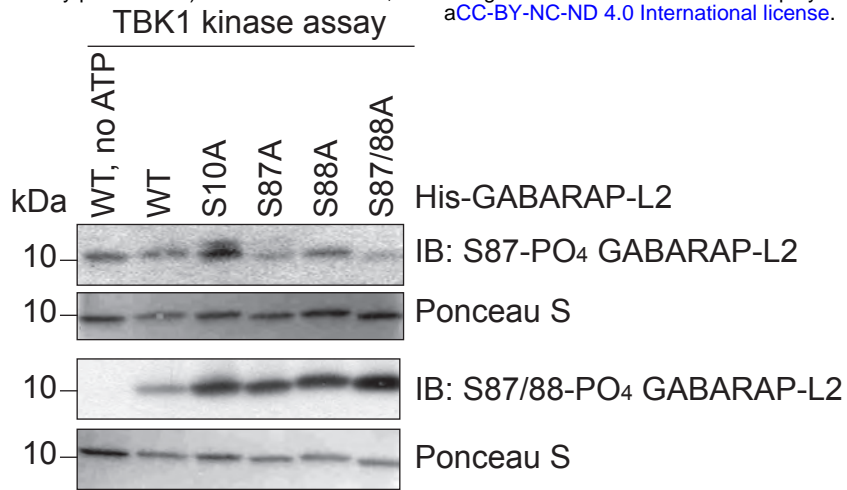


Figure 8: Model of TBK1-mediated LC3C and GABARAP-L2 phosphorylation

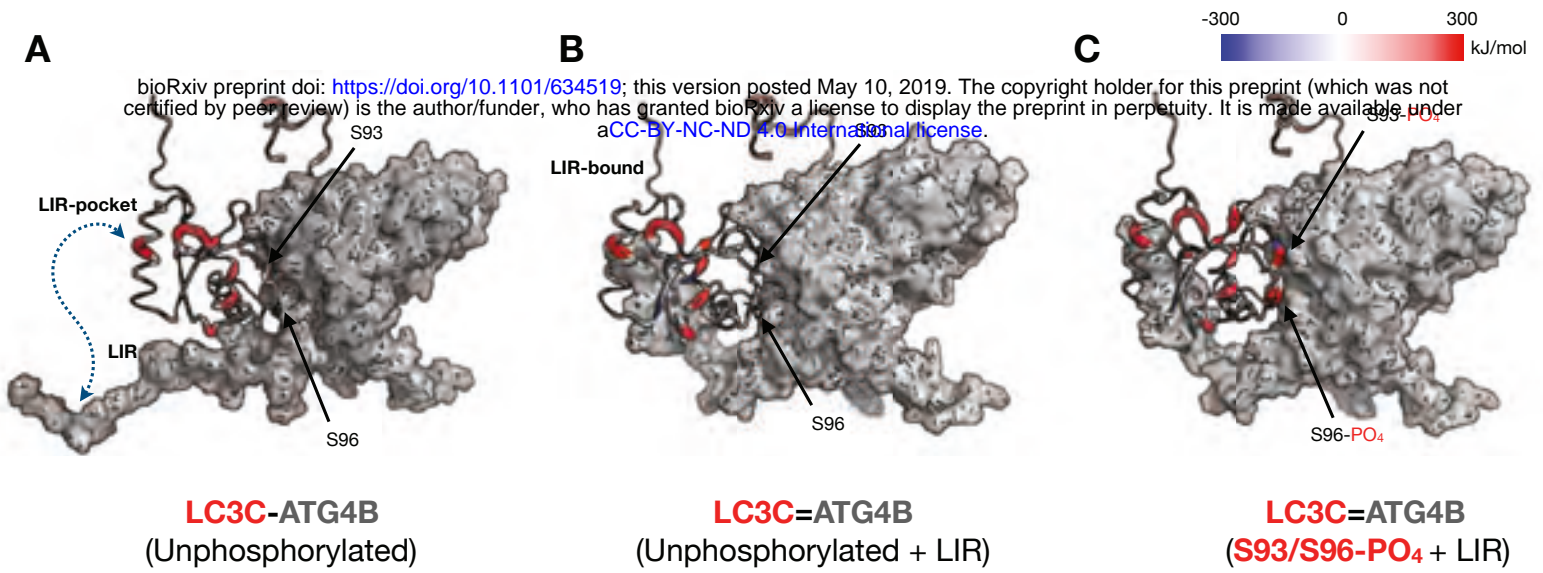


Supplementary Figure 1:

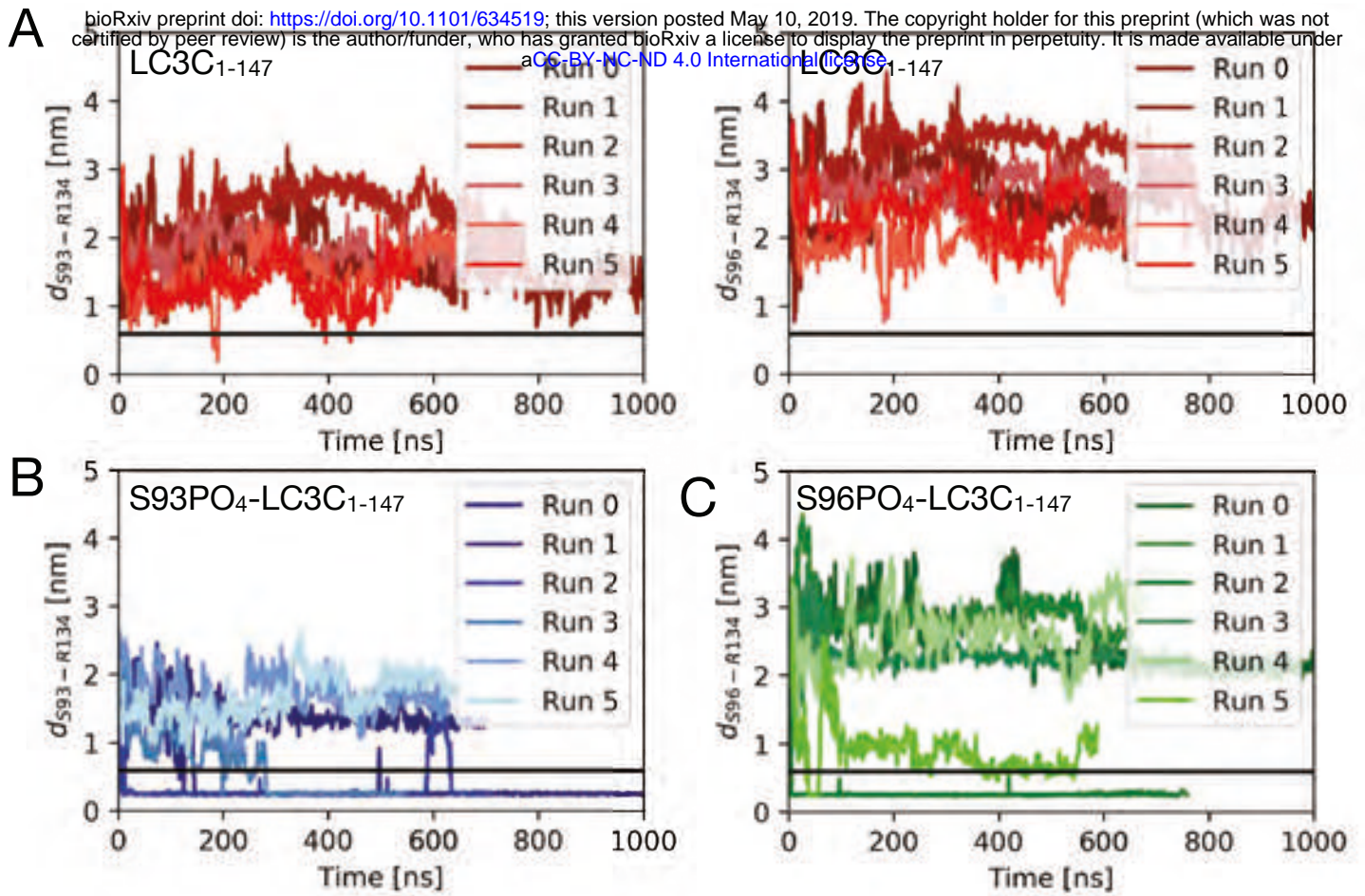
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Supplementary Figure 2:

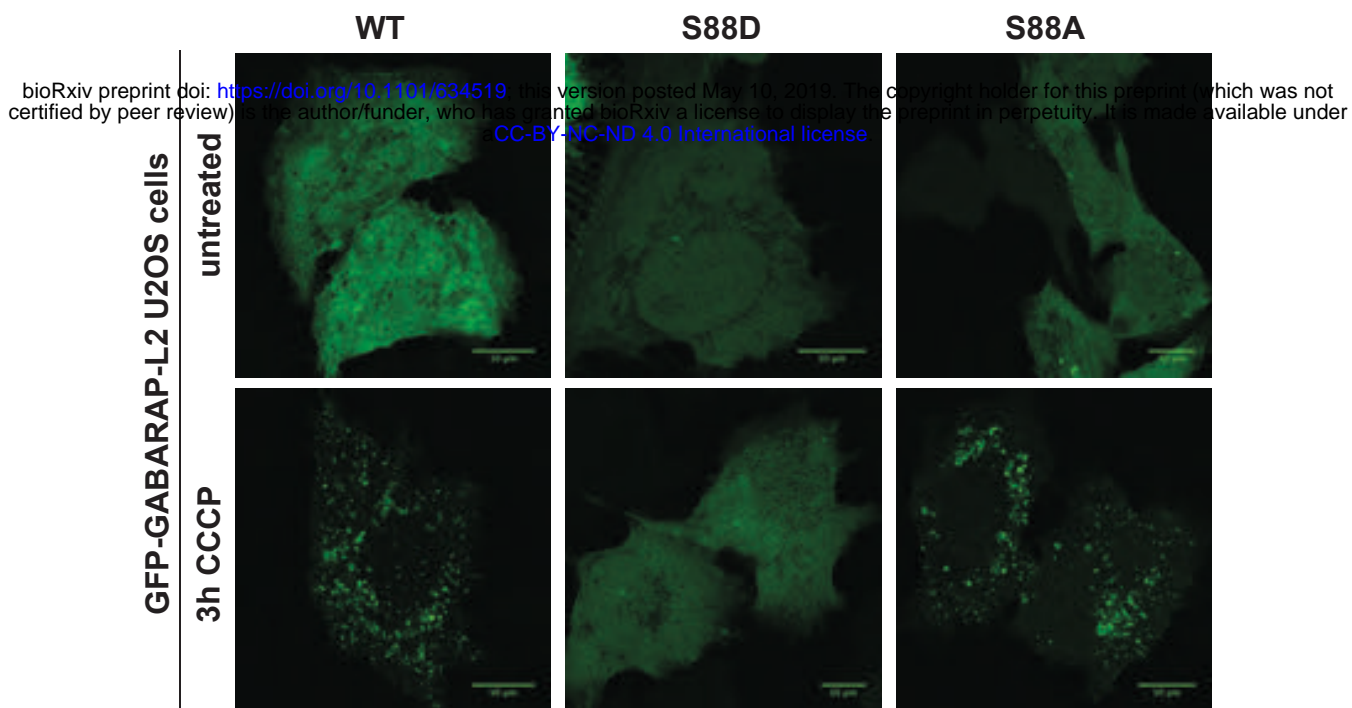


Supplementary Figure 3:

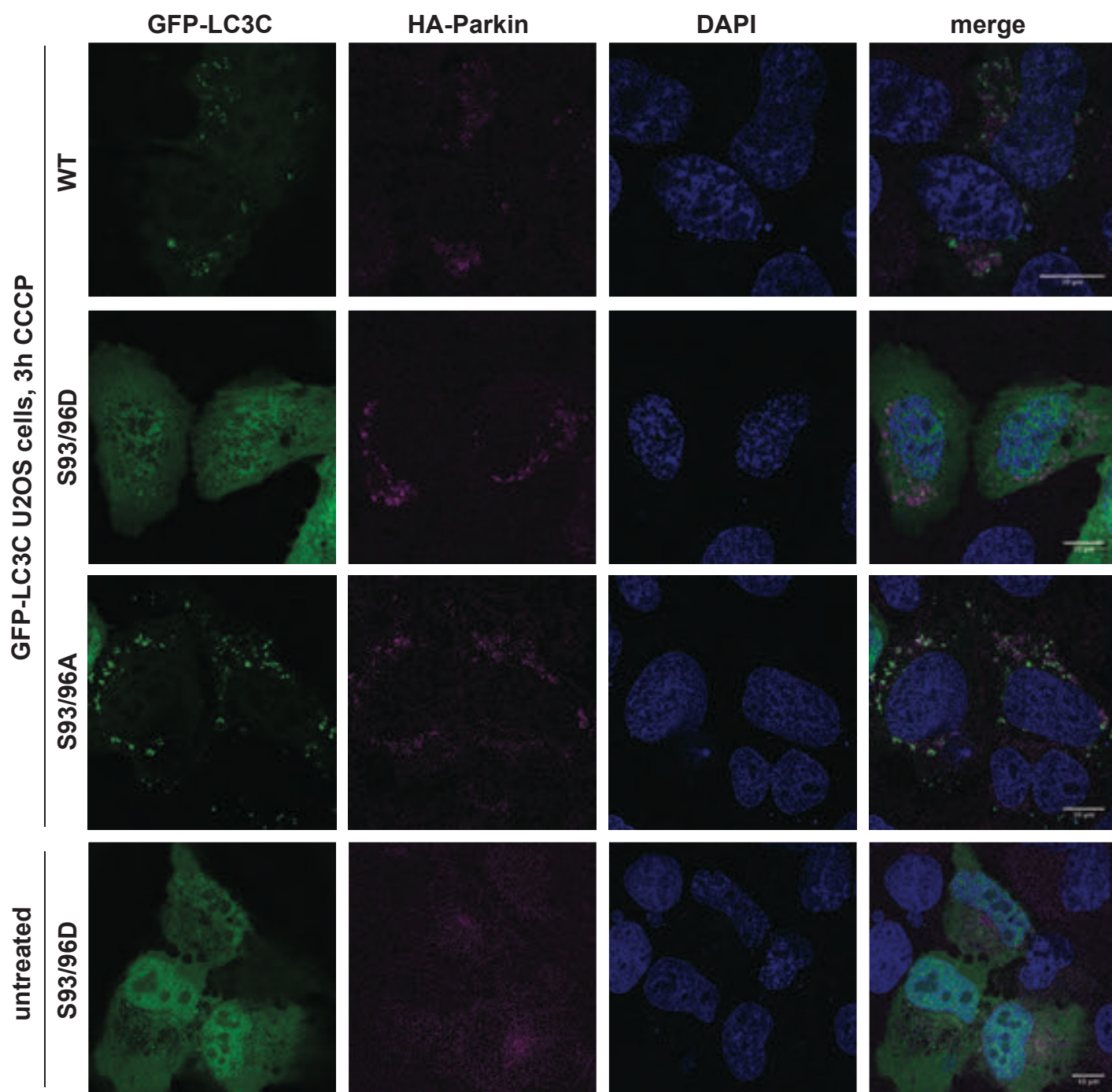


Supplementary Figure 4:

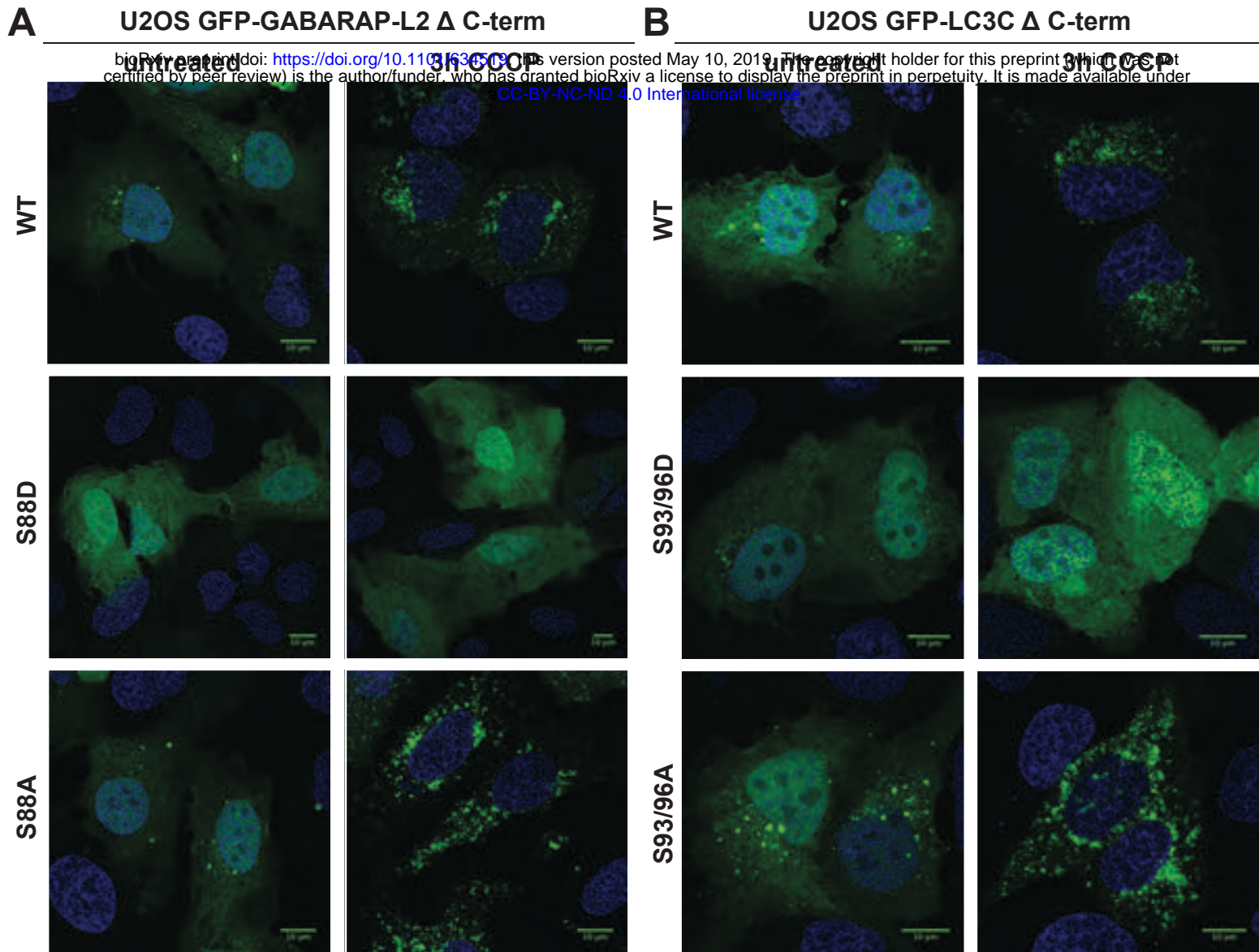
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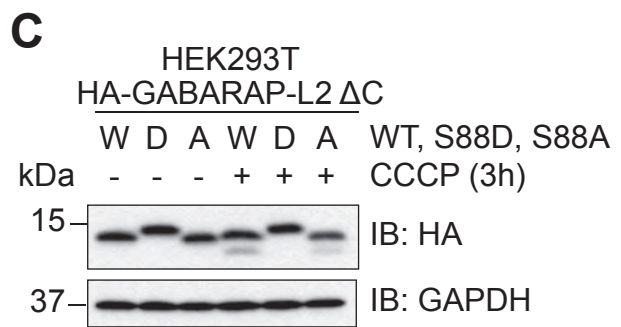
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Supplementary Figure 5:



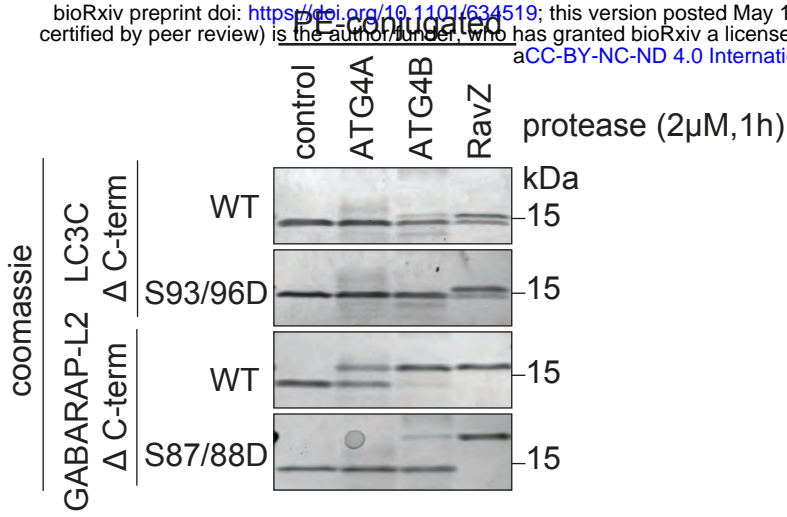
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Supplementary Figure 6:

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B

