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Title: The *Chlamydia* effector CT622/TaiP

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targets a non-autophagy related function of ATG16L1

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19 **Keywords:** host-pathogen interactions, autophagy, ATG16L1, intracellular traffic, *Chlamydia*

20 *trachomatis*

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23 **Abstract**

24 The obligate intracellular bacteria *Chlamydia trachomatis*, the causative agent of trachoma and
25 sexually transmitted diseases, multiply in a vacuolar compartment, the inclusion. From this niche, they
26 secrete “effector” proteins, that modify cellular activities to enable bacterial survival and proliferation.
27 Here, we show that the host protein ATG16L1 restricts inclusion growth, and that this effect is
28 counteracted by the secretion of the bacterial effector CT622/TaiP (Translocated ATG16-L1
29 interacting Protein). ATG16L1 is mostly known for its role in the lipidation of the human homologs of
30 ATG8 (*i.e.* LC3 and homologs) on double membranes during autophagy, as well as on single
31 membranes during LC3-associated phagocytosis and other LC3-lipidation events. Unexpectedly, the
32 LC3 lipidation-related functions of ATG16L1 are not required for restricting inclusion development. We
33 show that the carboxy-terminal domain of TaiP exposes a mimic of an eukaryotic ATG16L1-binding
34 motif, that binds to ATG16L1’s WD40 domain. By doing so, TaiP prevents ATG16L1 interaction with
35 the integral membrane protein TMEM59, and allows the rerouting of Rab6-positive compartments
36 towards the inclusion. The discovery that one bacterial effector evolved to target ATG16L1’s
37 engagement in intracellular traffic rather than in LC3 lipidation brings this “secondary” activity of
38 ATG16L1 in full light, and emphasizes its importance for maintaining host cell homeostasis.

39

40 **Significance statement:**

41 Some intracellular bacteria develop inside a vacuole, which expands during the infection process.
42 We show here that the protein ATG16L1 restricts the expansion of the *Chlamydia trachomatis*
43 vacuole. ATG16L1 is well known for its role in autophagy, a process that contributes to the elimination
44 of intracellular microbes. However, the restriction exerted by ATG16L1 on vacuole expansion relies on
45 a different ATG16L1 function. We demonstrate that the bacteria secrete an effector protein that
46 prevents ATG16L1 binding to TMEM59, and allows rerouting of vesicular traffic to the vacuole. The
47 discovery that one bacterial effector evolved to target ATG16L1’s engagement in intracellular traffic
48 emphasizes the importance this “secondary” activity of ATG16L1 for maintaining host cell
49 homeostasis.

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52 **Introduction:**

53 *Chlamydia trachomatis* is an obligate intracellular pathogen responsible for the most common
54 sexually transmitted bacterial infection (1). The bacteria reside within a vacuolar compartment, called
55 the inclusion, which expands throughout the developmental cycle. The host and the bacteria
56 contribute collectively to the making of this compartment. In particular, host lipids are diverted to the
57 inclusion membrane both through vesicular and non-vesicular traffic (2). The nature of the intercepted
58 vesicles is not fully understood, and the presence of many different Rab GTPases at the inclusion
59 membrane suggests that several trafficking pathways are involved (3). Key players in this rerouting of
60 host-derived vesicles are the bacterial Inc proteins, that are inserted into the inclusion membrane, and
61 that interact with regulators of intracellular traffic (4). However, Inc proteins are confined to the
62 inclusion membrane, which limits their range of action. We recently observed that the loss of
63 expression of the soluble effector CT622 in a *Ctr*^{ΔCT622} strain resulted in several deficiencies, including
64 a defect in inclusion growth, supporting the hypothesis that this soluble effector might contribute to the
65 diversion of host-derived material towards the inclusion (5). In the present study, we identify the host
66 protein ATG16L1 as a target of CT622. ATG16L1 is best known for its role as part of the ATG12-
67 ATG5-ATG16L1 complex, which catalyzes the lipidation of the human homologs of ATG8 (*i.e.* LC3
68 and homologs) on double membranes during autophagy, as well as on single membranes during LC3-
69 associated phagocytosis and other LC3-lipidation events (6-9). ATG16L1 also plays an important role
70 in the control of inflammation through its ability to bind NOD1 and NOD2 (10). Very unexpectedly, we
71 show here that the ATG16L1-driven function that is targeted by CT622 is not related to its LC3
72 lipidation capacity, nor to its ability to bind NODs, but to its involvement in regulating intracellular traffic
73 by interacting with the transmembrane protein TMEM59. We show that CT622 inhibits the formation of
74 the ATG16L1/TMEM59 complex, allowing the rerouting of vesicular traffic to the inclusion, thereby
75 rescuing inclusion growth in the *Ctr*^{ΔCT622} strain.

76

77 **Results**

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79 **CT622 binds to ATG16L1 through its carboxy-terminal domain**

80 The observation that loss of CT622 impaired inclusion growth suggested that this soluble effector
81 might contribute to the diversion of host-derived material towards the inclusion. To identify the targets

82 of CT622 in the host cytoplasm we performed two independent pull-down experiments. We identified
83 33 proteins that were significantly enriched in the GST-CT622 pulled-down fraction compared to GST,
84 three of which being recovered in the two independent experiments (Table S1). The autophagy related
85 (ATG) proteins ATG16L1 and ATG5 were recovered in the two experiments, with the highest total
86 peptide counts. To test their ability to interact with CT622 we first performed co-immunoprecipitation
87 experiments in cells transfected with plasmids expressing Flag-CT622, GFP-ATG5 and/or GFP-
88 ATG16L1. After cell lysis, we immunoprecipitated Flag-CT622, separated the proteins by SDS-PAGE
89 and probed this fraction with antibodies against GFP by western blot. GFP-ATG16L1 co-
90 immunoprecipitated with Flag-CT622 while GFP-ATG5 did not (Fig1A). This result suggests that
91 CT622 interacts with ATG16L1. The recovery of ATG5 in the pull-down but not in the co-
92 immunoprecipitation suggests that, when all protein were expressed at the endogenous level, ATG5
93 co-fractionated with CT622 via its ability to bind ATG16L1. By immunofluorescence, we observed that
94 co-expression of GFP-ATG16L1 with Flag-CT622 led to the relocation of Flag-CT622 to GFP-
95 ATG16L1 puncta, further supporting the hypothesis that the two proteins interact (SI Appendix Fig.
96 1A).

97 CT622 exhibits a highly conserved carboxy-terminal domain (CT622^{Cterm}) and a somewhat less
98 conserved amino-terminal domain (CT622^{Nterm}) (5). Co-immunoprecipitation experiments with each of
99 these domains expressed individually revealed that the interaction with ATG16L1 occurred via
100 CT622^{Cterm} (Fig1B). To confirm this interaction in the context of an infection and in the absence of
101 protein overexpression we used the *Ctr*^{Δct622} strain complemented with *ct622* with a carboxy-terminal
102 Flag tag (*Ctr*^{Δct622+CT622-Flag}), which we had characterized previously (5). Cells were infected for 35 h,
103 lysed and anti-Flag antibodies were used to immunoprecipitate CT622-Flag. We detected endogenous
104 ATG16L1 in the immunoprecipitated fraction, supporting the hypothesis that CT622 interacted with
105 ATG16L1 in infection (Fig. 1C). Finally, to determine whether the interaction was direct, we incubated
106 purified ATG16L1 with either recombinant GST-CT622 or recombinant GST-CT622^{Nterm} as negative
107 control. We pulled-down GST using glutathione-bound resin and measured the levels of ATG16L1 that
108 co-purified in these fractions by quantifying band intensities in western blot. ATG16L1 co-purified with
109 GST-CT622, demonstrating that the interaction between the two proteins was direct (Fig. 1D). Based
110 on these data we propose to rename CT622 Translocated ATG16L1 Interacting Protein (TaiP), which
111 is consistent with the nomenclature for other soluble chlamydial effectors (e.g. TarP, TepP, TmeA).

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ATG16L1 restricts *C. trachomatis* development and the restriction is exacerbated in the absence of TaiP.

To study the role of ATG16L1 in *C. trachomatis* infection, we generated *atg16l1* KO HeLa cells (Fig. 1E), in which we verified that LC3B lipidation was fully impaired (SI Appendix Fig. 1B). We compared the ability of wild type bacteria (Ctr^{WT}), $Ctr^{\Delta taiP}$ and $Ctr^{\Delta taiP+TaiP-Flag}$ to establish an infection in this cellular background compared to the parental HeLa cells. The loss of ATG16L1 resulted in a 20% increase in the size of the inclusions of Ctr^{WT} and $Ctr^{\Delta taiP+TaiP-Flag}$ (Fig. 1E), indicating that ATG16L1 restricts the development of *C. trachomatis*. Strikingly, the increase in inclusion diameter was much more pronounced (~ 70% increase) for the $Ctr^{\Delta taiP}$ strain. As a result, the $Ctr^{\Delta taiP}$ inclusions in the *atg16l1* background reached the average size for Ctr^{WT} in control cells (Fig. 1 E). The recovery in inclusion size was observed in four independent *atg16l1* KO clones, and is therefore not an clonal effect (SI Appendix Fig. 1C). The same observations were made when using siRNA targeting ATG16L1 in HeLa cells, as well as in the endometrial epithelial cell line HEC-1-B (SI Appendix Fig. 1D- E). These data indicate that at least part of the decrease in inclusion growth observed in the $Ctr^{\Delta taiP}$ strain is due to its inability to counteract an ATG16L1-driven restriction on inclusion development. In support of this, we observed that the transfection of Flag-CT622 prior to infection resulted in a 50% increase in inclusion size for the $Ctr^{\Delta taiP}$ strain, and a 40% increase for the Ctr^{WT} strain (Fig. 1E). A construct expressing an irrelevant Flag-tagged protein (CymR) expressed at similar levels as Flag-TaiP, was used as a negative control. Altogether, we concluded from these series of experiments that ATG16L1 hinders inclusion development, and that one of the roles of the effector TaiP is to counteract this brake by binding to ATG16L1. Importantly, the absence of ATG16L1 resulted in a ~ two-fold increase in progeny for the $Ctr^{\Delta taiP}$ strain (SI Appendix Fig. 1F). This is modest compared to the 25-fold difference of infectivity that exists between the $Ctr^{\Delta taiP}$ and Ctr^{WT} strains (5), implicating that while the inclusions recovered a normal size, the other phenotype associated to TaiP loss, *i.e.* defect of infectivity of EBs, remained. This is not surprising, considering that the loss of progeny in the $Ctr^{\Delta taiP}$ strain is largely due to the formation of non-functional EBs (*e.g.* defects in TarP secretion for instance), which is likely disconnected from the defect on the inclusion size. The absence of ATG16L1 did not significantly affect the progeny of the Ctr^{WT} or $Ctr^{\Delta taiP+TaiP-Flag}$ strains (SI Appendix Fig. 1F).

142 **ATG16L1 restricts inclusion growth through its WD40 domain**

143 ATG16L1 amino-terminal part is structurally and functionally conserved in yeast and is responsible
144 for its role in LC3 lipidation. Its carboxy-terminal part is made of an unstructured region followed with
145 seven WD40 repeats (amino acids 320 to 607) and is absent in the yeast ortholog (Fig. 2A). The
146 seven WD40 repeats form part of a beta-propeller domain which recruits several ATG16L1 effector
147 proteins including NOD1, NOD2 and TLR2 (11-13). Notably, while the WD40 domain is dispensable
148 for LC3 lipidation on double membrane autophagosomes during classical autophagy, it was shown to
149 be required for ATG12-ATG5-ATG16 complex-mediated LC3 lipidation at single membranes (7, 9). To
150 investigate the mechanism by which ATG16L1 restricts inclusion development we expressed in wild-
151 type or *atg16l1* KO HeLa either GFP, full length GFP-ATG16L1 (GFP-ATG16L1^{FL}), a truncated form of
152 ATG16L1 lacking the WD40 domain (GFP-ATG16L1¹⁻³¹⁹), or a truncated form of ATG16L1 lacking the
153 ATG5-binding and coiled-coil domains (GFP-ATG16L1²⁶⁶⁻⁶⁰⁷). As expected, expression of GFP-
154 ATG16L1^{FL} and of GFP-ATG16L1¹⁻³¹⁹ in *atg16l1* KO cells rescued LC3B lipidation, and GFP-
155 ATG16L1²⁶⁶⁻⁶⁰⁷ did not (SI Appendix Fig. 2A). These cells were infected with *Ctr*^{AtaiP}, and the median
156 size of inclusions in the GFP-positive cells was measured (SI Appendix Fig. 2B). We observed that the
157 expression of GFP-ATG16L1^{FL} and GFP-ATG16L1²⁶⁶⁻⁶⁰⁷ decreased the size of the *Ctr*^{AtaiP} inclusions
158 compared to GFP expressing cells, whereas the expression of GFP-ATG16L1¹⁻³¹⁹ did not (Fig. 2B).
159 These experiments demonstrate that the E3-ligase activity of ATG16L1 is dispensable for the
160 restriction this protein exerts on *C. trachomatis* development. Interestingly, the presence of LC3B at
161 the inclusion periphery had been reported in a previous study, and the authors had concluded that this
162 observation did not depend on a functional autophagy machinery (14). In agreement with that report,
163 we observed an enrichment of LC3B around the inclusion, labeled with an antibody against the
164 inclusion protein Cap1 (Fig. 2C). Strikingly, the presence of LC3B around the inclusion was
165 independent of the LC3B lipidation by the ATG12-ATG5-ATG16 complex since it was also observed in
166 *atg16l1* KO HeLa cells (Fig. 2C), or in *atg16l1* KO or *atg3* KO HEK293 cells (Fig S2B). It was also
167 independent of the expression of TaiP, as *Ctr*^{AtaiP} inclusions were also decorated with LC3B (Fig. 2C).
168 Thus, while the presence of LC3B at the inclusion membrane is an intriguing observation, we
169 concluded from these experiments that it is not related to the TaiP/ATG16L1 interaction.

170 Since the expression of the WD40 domain was sufficient to restrict the growth of *Ctr*^{AtaiP} inclusions
171 we tested whether this domain was implicated in the interaction between ATG16L1 and TaiP. Purified

172 GST-CT622 was incubated with lysates from *atg16l1* KO cells expressing either GFP-ATG16L1¹⁻³¹⁹ or
173 GFP-ATG16L1^{FL}, before purifying GST-TaiP together with associated proteins (Fig. 2D). We worked in
174 an *atg16l1* KO background to avoid possible dimerization of the expressed constructs with
175 endogenous ATG16L1. ATG16L1^{FL} co-purified with GST-TaiP while ATG16L1¹⁻³¹⁹ did not, indicating
176 that the WD40 is necessary for the formation of the TaiP/ATG16L1 complex (Fig. 2D). Consistent with
177 this result, we observed that Flag-TaiP no longer relocalized to GFP-ATG16L1 puncta in the absence
178 of the WD40 (Fig. 2E). Altogether, we concluded from these experiments that TaiP targets the WD40
179 domain of ATG16L1. This interaction results in a gain in inclusion growth through a pathway that does
180 not require the LC3-lipidation capacity of ATG16L1.

181

182 **TaiP promotes inclusion growth by disrupting ATG16L1/TMEM59 interaction.**

183 To identify the ATG16L1-related complex or pathway that is targeted by TaiP we reasoned that
184 silencing the expression of proteins involved in this process should result in the same phenotype as
185 ATG16L1 silencing, *i.e.* a rescue of the growth of the *Ctr*^{Δ*taiP*} strain. We thus transfected cells with
186 siRNA against the five best characterized binding partners of the WD40 domain of ATG16L1, TLR2,
187 NOD1, TMEM59, T3JAM and DEDD2 (12). NOD2 was not tested as it is not expressed in HeLa cells.
188 siRNA against ATG16L1 was included in the screen as a positive control, and the efficiency of the
189 silencing was verified by qRT-PCR (SI Appendix Fig. 3A). Thirty hours later, the cells were infected
190 with *Ctr*^{Δ*taiP*}. The cells were fixed 20 hrs after infection and processed for measuring the size of the
191 inclusions by immunofluorescence. We observed that silencing the expression of the protein TMEM59
192 phenocopied the phenotype observed with ATG16L1 silencing, *i.e.* an increase in the size of *Ctr*^{Δ*taiP*}
193 inclusions (Fig. 3A), a result we confirmed in the HEC-1-B epithelial cell line (SI Appendix Fig. 3B). To
194 avoid the possible sampling bias inherent to microscopy quantification we measured the percentage of
195 infected cells detected by flow cytometry. When working at low MOI, this parameter is directly linked to
196 bacterial load, because only cells bearing sufficient bacteria are recorded as infected (15). We
197 observed that silencing the expression of the protein TMEM59, but not of the other proteins tested,
198 significantly increased the percentage of infected cells, confirming the results obtained on inclusion
199 size measurement (SI Appendix Fig. 3C). These results indicate that the restriction ATG16L1 exerts
200 on the growth of *C. trachomatis* inclusions depends on its ability to interact with TMEM59. To
201 strengthen this hypothesis, we tested whether TaiP interfered with the formation of ATG16L1/TMEM59

202 complexes. We first confirmed, using HeLa cells expressing GFP-ATG16L1 and HA-TMEM59, that the
203 two proteins co-immunoprecipitate (Fig. 3B). When the immunoprecipitation was performed on cell
204 infected with *Ctr*^{WT} or *Ctr*^{ΔtaiP+TaiP-Flag} strains, we observed a decrease in the amount of ATG16L1 that
205 co-immunoprecipitated with TMEM59. In contrast, infection with *Ctr*^{ΔtaiP} did not prevent the interaction
206 of ATG16L1 with TMEM59 (Fig. 3B). This result strongly supports an inhibitory role of TaiP on the
207 formation of an ATG16L1/TMEM59 complex. To confirm these data, we expressed separately HA-
208 TMEM59 and GFP-ATG16L1 in HeLa cells by transfection, then mixed cell lysates in the presence of
209 purified GST-TaiP. GST-TaiP^{Nterm} was used as negative control in this assay since it does not bind to
210 ATG16L1 (Fig. 1B). After incubation, HA-TMEM59 was immunoprecipitated, and we analyzed by
211 western blot the levels of GFP-ATG16L1 in this fraction. In the presence of GST-TaiP^{Nterm} GFP-
212 ATG16L1 co-immunoprecipitated together with HA-TMEM59. However, in the presence of GST-TaiP,
213 the quantity of GFP-ATG16L1 that co-precipitated with HA-TMEM59 amounted to the signal observed
214 in the absence of expression of HA-TMEM59, and thus corresponded to non-specific GFP-ATG16L1
215 binding to the beads (Fig. 3C). Interestingly, addition of GST-TaiP did not compromise the ability for
216 ATG16L1 to interact with NOD1, NOD2 nor TLR2 (SI Appendix Fig. 3D). Thus, TaiP blocks specifically
217 TMEM59/ATG16L1 interaction in epithelial cells.

218

219 **TaiP mimics a eukaryotic domain for binding to ATG16L1 WD40 domain and D480 is a** 220 **critical residue for TaiP/ATG16L1 interaction**

221 Our results indicate that TaiP targets the ATG16L1/TMEM59 complex. TMEM59 is type I
222 transmembrane protein whose role is poorly understood. Initially described as residing in the Golgi
223 apparatus, it was later described as a player in endocytic trafficking from late endosomes to
224 lysosomes, with a clear colocalization with lysosomal markers (12, 16). Overexpression of TMEM59
225 induced LC3 lipidation of the compartment in which the protein resides, through its ability to attract
226 ATG16L1 (12). The Pimentel-Muñoz' laboratory identified in its short cytoplasmic tail a WD40 binding
227 motif defined as [YW]-X3-[ED]-X4-[YWF]-X2-L (12). This motif was found in several other proteins that
228 bind to ATG16L1, such as TLR2 and the CARD domain of NOD2. We analysed TaiP sequence, and
229 found a single matching motif **Y⁴⁷⁴AAALSD⁴⁸⁰GYSAY⁴⁸⁵KTL⁴⁸⁸**, that corresponds to the 6th helix of
230 TaiP^{Cter}, which is well exposed at the surface of the protein (5). To test whether this motif was
231 implicated in ATG16L1/TaiP interaction we mutated aspartate 480 into an alanine (D480A). Co-

232 immunoprecipitation experiments showed that the introduction of this point mutation reduced the
233 ability for TaiP to interact with ATG16L1 by about 50% (Fig. 3D). To confirm the engagement of this
234 motif in ATG16L1/TaiP interaction we next measured the gain in inclusion size in HeLa cells
235 transfected with either TaiP or TaiP^{D480A}, and infected with *Ctr*^{ΔtaiP}. Transfection of an irrelevant Flag-
236 tagged construct, Flag-CymR, was used as a negative control. As previously observed, expression of
237 TaiP resulted in increased *Ctr*^{ΔtaiP} inclusion size. However, the mutated form of TaiP was unable to
238 rescue inclusion growth (Fig. 3E). We concluded from these experiments that TaiP^{Cterm} 6th helix mimics
239 the ATG16L1 binding motif [YW]-X3-[ED]-X4-[YWF]-X2-L, thereby allowing the bacterial effector to
240 disrupt TMEM59/ATG16L1 interaction and favor inclusion growth.

241

242 **TaiP diverts Rab6 and ATG16L1-dependent vesicular traffic towards the inclusion**

243 We next analysed TMEM59 localisation in *Ctr*^{WT} and *Ctr*^{ΔtaiP} infected HeLa cells. We observed
244 TMEM59 in punctate structures, with no enrichment at the inclusion membrane, neither in cells
245 infected with wild-type nor with *Ctr*^{ΔtaiP} bacteria (SI Appendix Fig. 4). Our data show that silencing
246 ATG16L1 or TMEM59 converge to a similar phenotype, *i.e.* a rescue of the growth of *Ctr*^{ΔtaiP}
247 inclusions. One possible explanation for this phenomenon is that in the absence of those players (or in
248 the presence of TaiP, that disrupts their interaction), a pool of vesicles becomes available for inclusion
249 growth. To strengthen this scenario, we looked for a molecular marker associated with such a pool of
250 vesicles. Among the proteins that were significantly enriched in the GST-TaiP pull-down compared to
251 GST alone (Table S1) we had identified several small Rab GTPases: Rab5, Rab7 and small GTPases
252 that could not be identified because the peptide recovered was common to several Rab proteins
253 (Rab6, 27, 34, 39, 41 and 44). Out of these potential candidates, Rab 6 and Rab39 are recruited to the
254 chlamydial inclusion, (17, 18). Furthermore, silencing Rab6 reduces the delivery of the lipid ceramide
255 to the inclusion (19). To test if TaiP was able to interact with Rab proteins as indicated by the mass
256 spectrometry data we performed co-immunoprecipitation experiments in cells co-transfected with flag-
257 tagged TaiP and GFP-tagged Rab proteins. We tested several of the Rab proteins that the pull-down
258 assay had hinted to as potential interactors (Rab 5, 6, 7, 39), as well as Rab14 because it is one of the
259 Rab proteins recruited to the inclusion membrane (20). TaiP co-immunoprecipitated with Rab6a and
260 Rab39a, and with none of the other Rab proteins tested (Fig. 4A). Deletion of the carboxy-terminal
261 domain of TaiP abolished these interactions, indicating that this domain is involved (Fig. 4B). To

262 determine if the growth defect of the *Ctr*^{Δ*taiP*} strain was due to an inability to mobilize Rab6 or Rab39-
263 regulated vesicular traffic, we tested the effect of silencing Rab6 or Rab39 on bacterial development.
264 Silencing Rab39 had no effect on the ability for the bacteria to infect and grow in HeLa cell, and this
265 small GTPase was not investigated further. Silencing of Rab6 resulted in a 25 % decrease in the
266 inclusion size of wild type bacteria, confirming its role in feeding *Chlamydia*-inclusion growth (19) (Fig.
267 4C). In contrast, depleting Rab6 had no significant impact on the size of *Ctr*^{Δ*taiP*} inclusions. This result
268 suggests that the TaiP/ATG16L1-dependent vesicular traffic that contributes to the growth of the
269 inclusion in the wild-type strain requires Rab6. To further link Rab6 to the TaiP/ATG16L1 dependent
270 growth of *Chlamydia* inclusions we looked at the consequence of silencing Rab6 in the wild-type
271 versus the *atg16l1* KO background. We observed that the benefice, for the *Ctr*^{Δ*taiP*} strain, of knocking-
272 out ATG16L1 expression was lost when Rab6 was silenced (Fig. 4C). This observation indicates that
273 the source of membrane that allows faster growth of the *Ctr*^{WT} strain compared to the *Ctr*^{Δ*taiP*} strain is
274 Rab6 dependent. Rab6 is highly enriched in the Golgi apparatus, which is localized close to the
275 inclusion (SI Appendix Fig. 4). To facilitate the quantification of vesicular Rab6 enrichment at the
276 inclusion periphery we applied a short treatment with nocodazole, an inhibitor of microtubule
277 polymerization, that results in the dispersion of Golgi stacks (21). Infected cells were fixed and stained
278 for endogenous Rab6 and for the inclusion protein CT813 (Fig. 4D). We observed a stronger
279 enrichment in Rab6 at the periphery of *Ctr*^{Δ*taiP*} inclusions compared to *Ctr*^{Δ*taiP*}, an observation fully
280 consistent with the hypothesis that TaiP is required for efficient recruitment of Rab6 positive vesicles
281 to the inclusion. Furthermore, the recruitment of Rab6 to *Ctr*^{Δ*taiP*} inclusions, but not to *Ctr*^{WT} inclusions,
282 increased significantly in the *atg16l1* KO background, indicating that ATG16L1 restricts Rab6 traffic
283 towards the inclusion in the absence of TaiP (Fig. 4D). Altogether, our data converge to establish that
284 one the functions of the chlamydial effector TaiP is to disrupt ATG16L1/TMEM59 interaction, through
285 mimicry of WD40-binding motif. This unleashes access to a Rab6-dependent supply of membrane,
286 that feeds inclusion growth (Fig. 5A). Our model predicts that Rab6-positive vesicles normally feed
287 TMEM59-positive compartments, and that this pathway requires ATG16L1/TMEM59 interaction. The
288 large overlap of Rab6 and TMEM59 positive compartments observed by immunofluorescence is
289 consistent with this prediction (SI Appendix Fig. 4). To test it further we co-expressed GFP-Rab6 and
290 HA-TMEM59 in wild type and in the *atg16l1* KO background. We observed that indeed Rab6 co-
291 immunoprecipitated with TMEM59 in the wild type HeLa but not in the absence of ATG16L1 (Fig. 5B).

292 Thus, our study has uncovered a novel trafficking pathway, controlled by ATG16L1, that feeds
293 TMEM59 positive compartments with Rab6-positive material.

294

295 **Discussion**

296 The observation that the absence of expression of TaiP resulted in several developmental defects
297 had led us to hypothesize that this effector may contribute to multiple key events in infection (5). In this
298 report, we show that the carboxy-terminal part of TaiP is engaged in the regulation of membrane
299 supply to the inclusion, accounting for the defect in inclusion growth of the *Ctr^{ΔtaiP}* strain. At the
300 molecular level, we demonstrated that TaiP interacted with the host protein ATG16L1 through its C-
301 terminal domain. Moreover, we found that TaiP competitively inhibited the interaction of TMEM59 and
302 ATG16L1, *in vitro* and in the infectious context. Remarkably, silencing either TMEM59 or ATG16L1
303 expression allows to revert the inclusion growth defect of the *Ctr^{ΔtaiP}* strain.

304 Most work on ATG16L1 has focused on its role in ATG8 lipidation as part of the ATG12-ATG5-
305 ATG16 complex. We observed that silencing *atg16l1*, or disrupting its expression, enhanced
306 *Chlamydia* growth, even for the wild-type strain. This observation led us to hypothesize that ATG16L1
307 might restrict *Chlamydia* growth through an autophagy-related mechanism. However, we
308 demonstrated that it was not the case since the N-terminal part of ATG16L1, required and sufficient for
309 ATG8 lipidation, was not able to restrict *Chlamydia* growth. Conversely, expression of the C-terminal
310 part of ATG16L1, that contains the WD40, was sufficient for that effect. Therefore, the restriction of
311 bacterial growth exerted by ATG16L1 is not due to its ability to mediate ATG8 lipidation, but is
312 provided by a separate function of the WD40 domain.

313 ATG16L1's WD40 domain is proposed to interact with a variety of proteins (13, 22). A motif
314 common to several ATG16L1 binding proteins had been identified as [YW]-X3-[ED]-X4-[YWF]-X2-L
315 (12). TaiP^{Cterm} contains a single sequence that matches this definition: **Y⁴⁷⁴AAALSDGYSAYKTL⁴⁸⁸**,
316 and we showed that the D480A mutation impaired TaiP binding to ATG16L1. Thus, our data confirm
317 the privileged interaction between the [YW]-X3-[ED]-X4-[YWF]-X2-L motif and ATG16L1 WD40.
318 Interestingly, the interaction between ATG16L1 and TLR2 or NOD2, which also carry the [YW]-X3-
319 [ED]-X4-[YWF]-X2-L motif, appear insensitive to the addition of TaiP. This might be explained by
320 difference in affinities and/or by the limitation of our read-out (co-immunoprecipitation), that might not
321 be sensitive enough to reveal competition between these ATG16L1 binders. Also, other surfaces are

322 likely also implicated in the interactions between these different molecules and ATG16L1, as
323 exemplified by NOD1, which binds ATG16L1 but does not present the motif (12). These additional
324 binding surfaces could compensate for the competition exerted by helix 6 of TaiP.

325 We observed that exogenous expression of TaiP (by transfection) partially reverted the inclusion
326 size defect of the *Ctr*^{Δct622} strain. Importantly, TaiP D480A point mutation abolished both the beneficial
327 effect of TaiP expression on inclusion growth, and the disruptive effect of TaiP on ATG16L1/TMEM59
328 complexes, strongly supporting the hypothesis that TaiP supports *C. trachomatis* growth by disrupting
329 ATG16L1/TMEM59 interaction. In favor of this scenario, we showed that TMEM59 silencing led to a
330 similar phenotype as ATG16L1 silencing, *i.e.* a recovery of *Ctr*^{ΔtaiP} inclusion size. TMEM59 is a
331 transmembrane protein associated to several compartments, including the Golgi apparatus and late
332 endocytic compartments, but its role in membrane traffic remains unclear. Our results converge to the
333 hypothesis that ATG16L1/TMEM59 interaction limits bacterial access to host vesicular traffic (Fig. 5A).
334 Translocation of TaiP disrupts ATG16L1/TMEM59 interaction, unleashing access to a membrane pool,
335 that feeds inclusion growth. TaiP was detected by immunofluorescence in the bacteria throughout the
336 developmental cycle, and in the cytosol late in the cycle, probably due to a detection threshold (5). Our
337 new data are consistent with a role of TaiP throughout the inclusion growth phase, and in particular in
338 the first half of the developmental cycle, when the difference in inclusion sizes compared to wild-type
339 inclusions is the strongest. We showed that the ability of this membrane pool to support inclusion
340 growth was dependent on the expression of the small GTPase Rab6. Rab6 is associated to several
341 exocytic pathways emerging from the Golgi apparatus (23). Our data confirm the role of Rab6 in
342 feeding *Chlamydia*-inclusion growth (19), and revealed the control, by ATG16L1, of a Rab6-positive
343 membrane flow towards TMEM59 compartments.

344 ATG16L1 has attracted a lot of attention since the identification of an amino acid substitution
345 (T300A) that sensitizes the protein to caspase-3 processing, and that is associated with diminished
346 autophagy and increased risk of developing Crohn disease (24, 25). The molecular links between this
347 variant and the susceptibility to Crohn disease are still unclear (26), and could involved impaired
348 trafficking events (8, 27). The finding that ATG16L1T300A protein functions as a dominant negative
349 raises the possibility that the cleavage products have deleterious activity of their own (28). The
350 cleavage site liberates the WD40 domain, that we show here to be implicated in the control of the
351 traffic of at least a subset of Rab6 positive vesicles. The fact that evolution shaped a bacterial effector

352 that targets this WD40 domain in order to redirect intracellular traffic to the bacterial compartment
353 indicates that the role played by WD40 in this process is very central. Future work will thus need to
354 consider the possibility that impaired ATG16L1-controlled trafficking events could play a major role in
355 the susceptibility to Crohn disease, and other pathologies in which ATG16L1 has been implicated.

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Methods:

Cells and bacteria.

361 HeLa (ATCC), HEC-1-B (ATCC) and HEK293 cells (Invitrogen) were grown in Dulbecco's modified
362 Eagle's medium with Glutamax (DMEM, Invitrogen), supplemented with 10 % (v/v) heat-inactivated
363 fetal bovine serum (FBS). HEK293 *atg3* KO and *atg16l1* KO cells used for SFig. 2 were described in
364 (9). *C. trachomatis* serovar LGV L2 strain 434 (*Ctr*^{WT}) and the *taiP* genetic disruption mutant *Ctr*^{Δ*taiP*}
365 mutant were propagated on HeLa cells as described (5).

Generation of *atg16l1* KO HeLa cells.

367 The *atg16l1* KO HeLa cells were generated as described in (29), inserting sgRNA
368 CACCGCTGCAGAGACAGGCGTTCG (forward) and AAACCGAACGCCTGTCTCTGCAGC (reverse)
369 in the pSpCas9(BB)-puro. After transfection, the cells were treated with 0.5 μg/ml puromycin for 24 h
370 before individual clone selection. After monoclonal expansion, the loss of ATG16L1 expression was
371 verified by western blot. All experiments with cells passaged 4 times or less after freezing.

Pull-down of GST-TaiP partners in infected and non-infected cells and mass spectrometry

373 HeLa cells (about 10⁷ cells per point), infected or not for 24 hrs with *C. trachomatis*, were lysed in
374 0.05% NP40 lysis buffer with gentle rocking at 4 °C (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1 mM
375 EDTA, 1 mM EGTA, 10 mM NaF, 5% glycerol supplemented with 0.5% NP40 (v/v)) for the first 20 min
376 of lysis in a 0.1 ml volume, and diluted 1:10 to reach 0.05% NP40 for the last 20 min of lysis, and the
377 rest of the procedure. Lysates were centrifuged at 17000 xg for 15 minutes at 4 °C and precleaned
378 with glutathione-agarose beads with 20 μg of GST for 90 minutes at 4 °C in a rocking platform. Equal
379 amount of precleaned supernatants were incubated with 50 μl of a 50 % slurry of glutathione
380 sepharose 4B beads and 30 μg of purified GST or GST-TaiP for 90 min at 4 °C, on a rocking platform.
381 After a brief centrifugation, beads were washed five times with cold GST lysis buffer. The bound
382 proteins were eluted with urea buffer (8M urea, 1% SDS, 150 mM NaCl, 30 mM Tris-HCl pH 8.0) then
383 identified by mass spectrometry by the Proteopole of the Institut Pasteur as described in (5).

384 **Recombinant protein purification**

385 GST-TaiP (GST-CT622) purification was described in (5). The same protocol was used to produce
386 GST-TaiP^{Nterm}. ATG16L1 cDNA was cloned into pCoofy 29 vector via Sequence and Ligation
387 Independent Cloning. ATG16L1 protein was expressed in H5 insect cells grown in EX-CELL 420
388 serum-free medium (Sigma-Aldrich). Baculovirus-infected insect cells were added in a ratio of 1:1000.
389 Cultures were shaken for 72 hrs at 25°C and 85 rpm. Insect cells were harvested by centrifugation at
390 2000 x g for 15 min, washed with Dulbecco's phosphate buffered saline (Gibco) and resuspended in
391 lysis buffer (0.1M Tris-HCl pH 8.0, 0.5M NaCl, 20mM imidazole, pH 8.0 , 10% glycerol, 5mM β-
392 mercaptoethanol, 0.5% protease inhibitor cocktail (Sigma)). Cells were lysed using a dounce
393 homogenizer. ATG16L1 was purified from cell lysates using a His-trap Ni-NTA agarose column
394 followed by gel filtration in Superdex 200 column. His tag was removed by preScission protease and
395 the released protein was subjected to size exclusion chromatography. Fractions containing ATG16L1
396 were pooled and concentrated using Vivaspin cellulose centrifugation filters (Sartorius Stedim). The
397 protein was flash frozen in liquid nitrogen and stored at -80°C.

398 ***In vitro* assay of ATG16L1/TaiP interaction and immunoblots**

399 GST-TaiP and GST-TaiP^{Nterm} at 100 μM were incubated with ATG16L1 at 33 μM, in the binding
400 buffer (0.4 M NaCl, 25mM Tris-HCl, pH 7.4) and rocked on an Eppendorf tube roller for 2 h at 4°C.
401 Pre-washed 30 μl slurry Glutathione–Sepharose-4B beads were then added to the protein mix and
402 further incubated for 40 min at 4°C. The beads were centrifuged at 1500xg for 5 min and more than
403 90% of the supernatant was removed. The sedimented beads were washed twice in binding buffer
404 supplemented with 0.1% Triton X-100 and once in binding buffer. The samples were eluted by boiling
405 in SDS sample buffer and analyzed by immunoblotting. Immunoblots were analyzed using horse-
406 radish peroxidase secondary antibodies and chemiluminescence was analyzed on a Syngene Pxi4
407 imaging system. For the quantification the background signal was subtracted from all bands before
408 calculating the fold enrichment to the indicated control.

409 **LC3B-PE turnover measurement**

410 1.5*10⁵ WT and *atg16l1* KO HeLa cells were seeded in duplicate 12 well-plates for each
411 condition. The following day they were treated with vehicle (DMSO - D2650 Sigma) or 10 nM
412 bafilomycinA1 (Sigma B1793). Three hours later, the cells were washed with PBS before lysis with
413 Laemmli buffer supplemented with 2% β-mercaptoethanol and boiled for 5min. The lysats were

414 separated by electrophoresis on SDS-PAGE at 15% acrylamide concentration before being
415 transferred to a PVDF membrane. LC3B and LC3B-PE were revealed using the antibody NB100-2220
416 from Novus Biologicals.

417 **Plasmids and transfections**

418 Genomic DNA from *C. trachomatis* D/UW-3/CX, was prepared from bacteria using the RapidPrep
419 Micro Genomic DNA isolation kit (Amersham Pharmacia Biotech). attB-containing primers (Table S2,
420 Gateway®, Life technologies) were used to amplify and clone *ct622* into a destination vector derived
421 from the mammalian expression vector pCiNeo, providing an amino-terminal 3xflag tag, and into
422 pDEST15 (Gateway), for production of GST-tagged proteins. All constructs were verified by
423 sequencing. EGFP-conjugated ATG16L1 β constructs (WT and GFP-ATG16L1²⁶⁶⁻⁶⁰⁷) were from Dr. E.
424 Morel (INEM, Paris), TMEM59 was Dr. S Lichtenthaler (TUM, Germany), Flag NOD1/2 constructs
425 we're from Dr. L Boyer (University of Nice) and TLR2 from Dr. P Cossart (Institut Pasteur, France),
426 GFP-Rab6 was from B. Goud's laboratory (Institut Curie, France), GFP-Rab5 was obtained from A.
427 Echard (Pasteur Institute), and GFP-Rab7, GFP-Rab14, GFP-Rab39 were kindly provided by M.T.
428 Damiani (Mendoza, Argentina). Single point mutagenesis was performed to generate Flag-TaiP^{D480A}
429 and GFP- GFP-ATG16L1¹⁻³¹⁹ using the Quickchange Lightning Site-Directed Mutagenesis Kit from
430 Agilent following the manufacturer's protocol and primers reported in Table S2.

431 DNA transfection were performed using Jet prime and following the manufacturer's protocol. For
432 siRNA transfection we used RNAi Max as recommended by the manufacturer (see Table S2 for
433 sequences).

434 **Immunofluorescence**

435 1.5×10^5 HeLa cells were seeded on glass cover slips in 12 well plates, before transfection (for
436 expression of Flag-tagged proteins or for silencing) for 24h. Transfected cells were then infected for 20
437 hrs at a multiplicity of infection (MOI)=0.2, before fixation with 4% paraformaldehyde (PFA) in PBS for
438 20 min, followed with 10 min quenching with 50 mM NH₄Cl, in PBS. The cells were washed with PBS,
439 permeabilized with 0.05% saponin, 5 mg/ml BSA in PBS (permeabilization buffer) for 20 min, and
440 immunolabelled for 60 min with primary antibodies diluted in permeabilization buffer. Rabbit antibodies
441 against the bacterial inclusion protein Cap1 are described in (30), mouse antibodies against CT813
442 were kindly provided by Dr. G. Zhong (San Antonio, Texas), mouse antibodies against LC3B were
443 from MBL (#M152-3), antibodies against Flag(M2) were from Sigma, rat antibodies against TMEM59

444 (clone 4E5) were generously provided by Dr. S Lichtenthaler (16). Coverslips were then washed 3
445 times with PBS before incubating for 60 min in fluorochrome-coupled secondary antibodies diluted in
446 permeabilization buffer. DNA was stained using $0.5 \mu\text{g}\cdot\text{mL}^{-1}$ of Hoechst 33342 (Thermo Fisher
447 Scientific) added in the secondary antibody solution. Images were acquired on an Axio observer Z1
448 microscope equipped with an ApoTome module (Zeiss, Germany) and a 63x Apochromat lens.
449 Images were taken with an ORCAflash4.OLT camera (Hamamatsu, Japan) using the software Zen.
450 Quantification of LC3B and Rab6 at the inclusion periphery were performed using the brush tool of
451 ImageJ set, at 9 pixels, and following the inclusion membrane marker (Cap1 and CT813 respectively).
452 To measure Rab6 enrichment at the inclusion periphery, the mean green fluorescence (Rab6) along
453 the this line was normalized to the mean value of green fluorescence in two randomly selected ~ 1
454 μm^2 areas in the cytosol. Since LC3B level in the cytosol was hardly above background, this
455 normalization was not applied for LC3B measurements and the data in Fig. 2C display the mean
456 green (LC3B) fluorescence along the inclusion membrane.

457 **Inclusion size measurements:**

458 $1.5 \cdot 10^5$ HeLa cells were seeded in 12 well-plates before transfection with either DNA or siRNA for
459 24 hrs. The transfected cells were infected in triplicates at $\text{MOI}=0.2$ for 20 hrs before being fixed and
460 permeabilized for immunofluorescence. The inclusion membrane was stained using an antibody
461 against Cap1 and 5 to 10 random pictures were taken for each coverslips as described in the
462 immunofluorescence methods. Using the imageJ software the scale was set from pixels to μm and the
463 area of individual inclusions were measured. Each condition was analyzed in a blind fashion from 3
464 individual coverslips with a minimum of 50 inclusions per simplicite. For Fig 1D, 3C, 4D & SFig1C the
465 size of all inclusions was analyzed, whereas in Fig 1E & 3B the inclusions of only cells positive for
466 Flag were analysed. For Fig2B only inclusions in GFP positive cells were taken into account and were
467 analyzed. Note that we consistently observed that the inclusions grew slower in transfected cells
468 compared to non transfected cells (compare for instance the average diameter of inclusions for Ctr^{WT}
469 in non-transfected cells in Fig.1D and in cells transfected with GFP in Fig. 2B).

470 **Progeny assay and flow cytometry**

471 For progeny assays displayed in SFig. 1F wild-type or *atg16l1* KO HeLa cells infected for 40 h with
472 the indicated strains were detached, lysed using glass beads and the supernatant was used to infect
473 fresh HeLa cells plated the day before (100 000 cells/well in a 24-well plate), in serial dilution. The

474 next day, 3 wells per condition with an infection lower than 30 % (checked by microscopy) were
475 detached, fixed in 70% ethanol and stained with a home-made rabbit antibody against GroEL followed
476 with Alexa488-coupled secondary antibodies. Acquisition was performed using a CytoFLEX S
477 (Beckman Coulter) and 10 000 events per sample were acquired and then analyzed using FlowJo
478 (version 10.0.7) to determine the bacterial titer as described in (15). For determining the consequence
479 of infection on bacterial load in SFig 3C, only the primary infection was analyzed by flow cytometry,
480 after staining the bacteria with anti-GroEL antibodies.

481 **Immunoprecipitation**

482 5×10^6 HeLa cells were seeded in 10 cm² dishes. On the following day, cells were transfected with
483 5 µg of plasmid. One day later, cells were lysed in 250 µl of lysis buffer (150 mM NaCl, 50 mM Tris-
484 HCl pH 7.5, 5% glycerol, 0.5% NP-40) supplemented with a protease cocktail inhibitor (Roche
485 Complete, EDTA-free). The lysates were incubated on a rotating wheel at 4 °C for 20 min before
486 adding 1ml of dilution buffer (150 mM NaCl, 50 mM Tris- HCl pH 7.5 supplemented with Protease
487 cocktail inhibitor) thereby reducing the glycerol to 1% and NP-40 to a final concentration of 0,1%. The
488 lysates were then centrifuged at 10,000 xg for 10 min and the insoluble pellet was discarded. For GFP
489 IP the lysates were then incubated with 2 µg of antibody (Invitrogen, # A11122) at 4 °C for 2 hrs before
490 adding 20 µl of slurry protein G beads (Sigma Fast Flow Protein G Sepharose) for 20 min. For HA and
491 Flag IPs, antibody-coupled beads were used (Sigma). The beads were then washed before adding 20
492 µl of Laemmli buffer supplemented with 2% β-mercaptoethanol and boiled for 5 min. For Fig1A, 1B the
493 plasmids were co-transfected, whereas for Fig 3C an SFig3 A to C, the plasmids were transfected
494 individually before mixing 0.75 ml of each diluted lysats in the presence or absence of 100 pmol of
495 GST-TaiP^{Nterm} or GST-TaiP. For the immunoprecipitation in infected cells shown in Fig. 1C cell lysis
496 was performed in RIPA buffer (150 mM sodium chloride, 0.5% NP-40 and 0.5 Triton X-100, 0.5%
497 sodium deoxycholate, 0.1% sodium dodecyl sulfate and 50 mM Tris, pH 8.0). Antibodies used for
498 probing the membranes are described in the immunofluorescence section, in addition antibodies
499 againstGFP (#NB600-308), ATG16L1 (#PM040) and HA (clone 12CA5) were purchased from Novus,
500 MBL and Sigma, respectively.

501 **Pull down assays**

502 2.5×10^6 HeLa cells were seeded in 6 cm² dish the day before transfection with 2.5 µg of plasmid.
503 On the following day, the cells were lysed in 250 µl of lysis buffer (150 mM NaCl, 50 mM Tris- HCl pH

504 7.5, 5% glycerol, 0.5% NP-40) supplemented with a protease cocktail inhibitor (Roche Complete,
505 EDTA-Free). The lysates were incubated on a rotating wheel at 4 °C for 20 min before reducing the
506 glycerol to 1% and NP-40 to a final concentration of 0.1% as above. The lysates were then incubated
507 with 100 pmol of GST-TaiP for 60 min on a spinning wheel at 4 °C before adding 30 µl of slurry
508 Glutathione-sepharose beads for 20 min. The beads were then washed 3 times using lysis buffer
509 before adding 20 µl of Laemmli buffer supplemented with 2% β-mercaptoethanol, and boiled for 5min.

510 **RT-qPCR**

511 Total RNAs were isolated 35 h after transfection with the RNeasy Mini Kit (Qiagen) with DNase
512 treatment (DNase I, Roche). Reverse transcription was performed on 500 ng of total RNA using the
513 high capacity c-DNA Reverse transcription kit (Applied Biosystems) according to manufacturer's
514 instructions. Complementary DNA (cDNA) were diluted 5 times and quantitative PCR (qPCR) was
515 performed on 1 µl of cDNA with the LightCycler 480 system using LightCycler 480 SYBR Green
516 Master I (Roche). Data were analyzed using the $\Delta\Delta C_t$ method with the 36B4 gene as a control gene
517 (31).

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519

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533 **Declaration of interest**

534 The authors declare no competing interests.

535

536 **References**

- 537 1. Brunham RC & Rey-Ladino J (2005) Immunology of *Chlamydia* infection: Implications
538 for a *Chlamydia trachomatis* vaccine. *Nature Reviews Immunology* 5(2):149-161.
- 539 2. Triboulet S & Subtil A (2019) Make It a Sweet Home: Responses of *Chlamydia*
540 *trachomatis* to the Challenges of an Intravacuolar Lifestyle. *Microbiol Spectr* 7(2).
- 541 3. Damiani MT, Tudela JG, & Capmany A (2014) Targeting eukaryotic Rab proteins: a
542 smart strategy for chlamydial survival and replication. *Cellular Microbiology*
543 16(9):1329-1338.
- 544 4. Elwell C, Mirrashidi K, & Engel J (2016) *Chlamydia* cell biology and pathogenesis. *Nat*
545 *Rev Microbiol* 14(6):385-400.
- 546 5. Cossé MM, *et al.* (2018) The Loss of Expression of a Single Type 3 Effector (CT622)
547 Strongly Reduces *Chlamydia trachomatis* Infectivity and Growth. *Front Cell Inf*
548 *Microbiol* 8(145):145.
- 549 6. Ohsumi Y (2014) Historical landmarks of autophagy research. *Cell Res.* 24(1):9-23.
- 550 7. Fletcher K, *et al.* (2018) The WD40 domain of ATG16L1 is required for its non-
551 canonical role in lipidation of LC3 at single membranes. *The EMBO Journal*
552 37(4):e97840-97817.
- 553 8. Cadwell K & Debnath J (2018) Beyond self-eating: The control of nonautophagic
554 functions and signaling pathways by autophagy-related proteins. *J. Cell Biol.*
555 217(3):813-822.
- 556 9. Lystad AH, *et al.* (2019) Distinct functions of ATG16L1 isoforms in membrane binding
557 and LC3B lipidation in autophagy-related processes. *Nat Cell Biol* 21:372-383.
- 558 10. Sorbara MT, *et al.* (2013) The Protein ATG16L1 Suppresses Inflammatory Cytokines
559 Induced by the Intracellular Sensors Nod1 and Nod2 in an Autophagy-Independent
560 Manner. *Immunity* 39(5):858-873.
- 561 11. Bajagic M, Archana A, Büsing P, & Scrima A (2017) Structure of the WD40-domain of
562 human ATG16L1. *Protein Sci.* 26(9):1828-1837.
- 563 12. Boada-Romero E, *et al.* (2013) TMEM59 defines a novel ATG16L1-binding motif that
564 promotes local activation of LC3. *The EMBO Journal* 32(4):566-582.
- 565 13. Slowicka K, *et al.* (2019) Physical and functional interaction between A20 and
566 ATG16L1-WD40 domain in the control of intestinal homeostasis. *Nature*
567 *communications* 10(1):1834.
- 568 14. Al-Younes HM, *et al.* (2011) Autophagy-independent function of MAP-LC3 during
569 intracellular propagation of *Chlamydia trachomatis*. *Autophagy* 7(8):814-828.
- 570 15. Vromman F, Laverriere M, Perrinet S, Dufour A, & Subtil A (2014) Quantitative
571 Monitoring of the *Chlamydia trachomatis* Developmental Cycle Using GFP-Expressing
572 Bacteria, Microscopy and Flow Cytometry. *PLoS One* 9(6):e99197.
- 573 16. Ullrich S, *et al.* (2010) The novel membrane protein TMEM59 modulates complex
574 glycosylation, cell surface expression, and secretion of the amyloid precursor protein.
575 *J. Biol. Chem.* 285(27):20664-20674.
- 576 17. Rzomp KA, Scholtes LD, Briggs BJ, Whittaker GR, & Scidmore MA (2003) Rab GTPases
577 are recruited to Chlamydial inclusions in both a species-dependent and species-
578 independent manner. *Infect. Immun.* 71(10):5855-5870.
- 579 18. Gambarte Tudela J, *et al.* (2015) The late endocytic Rab39a GTPase regulates the
580 interaction between multivesicular bodies and chlamydial inclusions. *J. Cell Sci.*
581 128(16):3068-3081.

- 582 19. Rejman Lipinski A, *et al.* (2009) Rab6 and Rab11 regulate *Chlamydia trachomatis*
583 development and golgin-84-dependent Golgi fragmentation. *PLoS Pathog*
584 5(10):e1000615.
- 585 20. Capmany A & Damiani MaT (2010) *Chlamydia trachomatis* Intercepts Golgi-Derived
586 Sphingolipids through a Rab14-Mediated Transport Required for Bacterial
587 Development and Replication. *PLoS ONE* 5(11):e14084.
- 588 21. Rogalski AA & Singer SJ (1984) Associations of elements of the Golgi apparatus with
589 microtubules. *J. Cell Biol.* 99(3):1092-1100.
- 590 22. Jain BP & Pandey S (2018) WD40 Repeat Proteins: Signalling Scaffold with Diverse
591 Functions. *The protein journal* 37(5):391-406.
- 592 23. Boncompain G & Weigel AV (2018) Transport and sorting in the Golgi complex:
593 multiple mechanisms sort diverse cargo. *Curr. Opin. Cell Biol.* 50:94-101.
- 594 24. Hampe J, *et al.* (2006) A genome-wide association scan of nonsynonymous SNPs
595 identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat. Genet.* 39:207.
- 596 25. Murthy A, *et al.* (2014) A Crohn's disease variant in Atg16l1 enhances its degradation
597 by caspase 3. *Nature* 506(7489):456-462.
- 598 26. Matsuzawa-Ishimoto Y, Hwang S, & Cadwell K (2018) Autophagy and Inflammation.
599 *Annu. Rev. Immunol.* 36:73-101.
- 600 27. Cadwell K, *et al.* (2008) A key role for autophagy and the autophagy gene Atg16l1 in
601 mouse and human intestinal Paneth cells. *Nature* 456(7219):259-263.
- 602 28. Gao P, *et al.* (2017) The Inflammatory Bowel Disease–Associated Autophagy Gene
603 Atg16L1T300A Acts as a Dominant Negative Variant in Mice. *The Journal of*
604 *Immunology* 198:2457-2467.
- 605 29. Ran FA, *et al.* (2013) Genome engineering using the CRISPR-Cas9 system. *Nature*
606 *Protocols* 8(11):2281-2308.
- 607 30. Gehre L, *et al.* (2016) Sequestration of host metabolism by an intracellular pathogen.
608 *Elife* 5:e12552.
- 609 31. Schmittgen TD & Livak KJ (2008) Analyzing real-time PCR data by the comparative CT
610 method. *Nature Protocols* 3(6):1101-1108.
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- 612

613 **Figure Legends**

614

615 **Figure 1: TaiP binds directly to ATG16L1 through its carboxy-terminal domain to promote *C.***
616 ***trachomatis* infection**

617 **A)** HeLa cells were co-transfected with Flag-CT622 and the indicated GFP-
618 tagged construct for 24 hrs, lysed, and immunoprecipitation (IP) was performed with anti-
619 Flag coupled beads. Proteins were separated on SDS-PAGE, transferred on a PVDF
620 membrane and probed with the indicated antibody (IB : immunoblot). An aliquot of each cell
621 lysate was loaded on a separate gel to visualize the expression of Flag-CT622 and of each
622 of the GFP-tagged proteins (input, left panels).

623 **B)** Same as in A, using full-length CT622 or constructs corresponding to the N-
624 terminal (CT622^{Nterm}, amino acids 1-345) or C-terminal (CT622^{Cterm}, amino acids 346-647).

625 **C)** HeLa cells were infected for 35 h with *Ctr*^{Δct622+CT622-Flag} bacteria, lysed, and IP
626 was performed with anti-Flag coupled beads. Proteins were separated on SDS-PAGE,
627 transferred on a PVDF membrane and probed with anti-Flag and anti-ATG16L1 antibody.

628 **D)** Recombinant ATG16L1 (100 nM) was incubated with recombinant GST-
629 CT622 or GST-CT622^{Nterm} (100 nM) for 60 min at 4°C before performing GST-pulldown
630 (PD) using glutathione beads. Pulled-down fractions were analyzed by western blot as in A.
631 GST-CT622^{Nterm}, used here as a negative control, shows the level of non-specific ATG16L1
632 binding to the beads. ATG16L1 was pulled-down together with TaiP, demonstrating that the
633 interaction is direct. The experiment shown is representative of three independent
634 experiments.

635 **E)**Quantification of the effect of knocking-out *atg16l1* on inclusion size. WT or *atg16l1*
636 KO cells seeded on coverslips were infected with either *Ctr*^{WT}, *Ctr*^{ΔtaiP} or *Ctr*^{ΔtaiP+TaiP-Flag} for
637 20 hrs at MOI=0.2. After fixation, infected cells were permeabilized and the inclusion
638 membrane was stained with antibodies against the inclusion protein Cap1. The area of
639 inclusion was measured using imageJ software. The dot-plot shows the median ± SD of 3
640 independent experiments (N>50 in total) and displays the P-values of the Student's t tests.
641 The right panel shows the absence of ATG16L1 in *atg16l1* KO whole cell lysates probed by
642 western blot with anti-ATG16L1 antibodies. ACTIN immunoblot serves as a loading control.

643 **F)** Quantification of the effect of TaiP expression on inclusion size. Cells were
644 transfected with constructs for Flag-CymR or Flag-TaiP for 24 hours before being infected
645 with *Ctr* or *Ct^{ΔtaiP}*. The cells were fixed 20 hrs post infection, permeabilized and the inclusion
646 membrane was stained with antibodies against the inclusion protein Cap1. The area of
647 inclusion was measured using imageJ software. The dot-plot displays the median ± SD of 3
648 independent experiments (N>50 in total) and displays the P-values of the Student's t test.

649

650 **Figure 2: TaiP targets ATG16L1's WD40 domain.**

651 **A)** Schematic representation of ATG16L1 structure including the coiled-coil domain
652 (C.C.), the WD40 domain (WD40). The binding sites to ATG5, WIPI2B and FIP200 are
653 highlighted, along with the serine phosphorylated by ULK1. WD40 binding partners are
654 indicated on the right.

655 **B)** WT or *atg16l1* KO HeLa cells seeded on coverslips were transfected with the
656 indicated constructs for 24 hours. Cells were then infected with *Ct^{ΔtaiP}* for 20 hours at
657 MOI=0.2, before being fixed, permeabilized and the inclusion membrane was stained with
658 antibodies against the inclusion protein Cap1. The graph displays the median ± SD of 3
659 independent experiments (N>50 cells in total) and the P-values of the Student's t test.

660 **C)** WT and HeLa cells were infected with either *Ct^{WT}* or *Ct^{ΔtaiP}* for 20 hours, before
661 fixation, permeabilization and immunostaining with antibodies against LC3B and the bacterial
662 inclusion protein Cap1. LC3B is localized at the inclusion membrane independently of TaiP or
663 ATG16L1. Scale bar: 10 μm. The dot plot displays the median intensity of LC3B staining at
664 the inclusion membrane (N>10 cells from 3 independent experiments). Student's t tests
665 showed no significant difference.

666 **D)** Lysates from *atg16l1* KO cells transfected with either GFP-ATG16L1 or GFP-
667 ATG16L1¹⁻³¹⁹ were incubated with 100 pmol of recombinant GST-TaiP for 1h at 4°C, before
668 performing a GST-pull-down using Glutathione beads. Fractions were analyzed like in Fig 1A.
669 ATG16L1 was no longer pulled-down with TaiP when its WD40 domain was deleted.

670 **E)** *atg16l1* KO HeLa cells were co-transfected with Flag-TaiP and either GFP-ATG16L1 or
671 GFP-ATG16L1¹⁻³¹⁹. The cells were fixed 24 hrs later, permeabilized and stained with anti-

672 Flag antibodies. In the absence of the WD40 domain Flag-TaiP was no longer recruited to
673 ATG16L1 puncta. Scale bar: 10 μ m.

674

675 **Figure 3: TaiP blocks the TMEM59/ATG16L1 complex to promote *C. trachomatis***
676 **inclusion expansion.**

677 **A)** Cells transfected with the indicated siRNA were infected with *Ct* ^{Δ taiP} the following day
678 at MOI=0.2. Cells were fixed 20 hrs later, permeabilized and the inclusion membrane was
679 stained with anti-Cap1 antibodies. Inclusion areas were measured using imageJ software.
680 The Box and whiskers plots represent the median area and 90th percentile of intracellular
681 inclusions (N>50 cells from 3 independent experiments). Statistical analysis was performed
682 using a one -way anova test with a Dunnett's multiple comparison test to siCtrl.

683 **B)** Cells transfected with the indicated plasmids were infected or not 12 h later with *Ct*^{WT},
684 *Ct* ^{Δ taiP} or *Ct* ^{Δ taiP+TaiP-Flag} bacteria at MOI=1. Cells were lysed 30 h later, and IP was performed
685 with anti-HA coupled beads. Proteins were separated on SDS-PAGE, transferred on a PVDF
686 membrane and probed with the indicated antibodies.

687 **C)** Lysates from cells expressing HA-TMEM59, or GFP-ATG16L1, were mixed in the
688 presence of 100 pmol of GST-TaiP or GST-TaiP^{Nterm} (for negative control) for 90 min at 4°C in
689 a finale voume of 1.5 ml. TMEM59 was immunoprecipitated with anti-HA coupled beads and
690 the levels of GFP-ATG16L1 in the IP fraction was analyzed by western blot. Immunoblots of
691 the input fractions show the expression of each individual protein and represents 1.5 % of the
692 total reaction and comassie staining shows purified GST-TaiP and GST-TaiP^{Nterm}. The
693 histogram on the right displays the mean \pm SD of three independent experiments and the P-
694 value of the Student's t test. Addition of GST-TaiP decreased the amount of GFP-ATG16L1
695 that co-immunoprecipitated with HA-TMEM59 by about 50%.

696 **D)** HeLa cells were co-transfected with Flag-TaiP, Flag-TaiP^{D480A} and GFP-ATG16L1
697 constructs for 24 h, lysed, and immunoprecipitation (IP) was performed with anti-Flag
698 antibody. Proteins were separated on SDS-PAGE, transferred on a PVDF membrane and
699 probed with the indicated antibody. An aliquot of each cell lysate was loaded on separate
700 gels to visualize the expression of the tagged proteins (input). Quantification on the right
701 shows the decrease in the amount of ATG16L1 that co-immunoprecipitates with Flag-

702 TaiP^{D480A} compared to Flag-TaiP. Statistical analysis was performed using Student's unpaired
703 t test, n=4.

704 **E)** Quantification of the average inclusion size in cells expressing Flag-CymR, Flag-TaiP
705 and Flag-TaiP^{D480A}. Cells were transfected for 24 hrs before they were infected for 20 hrs with
706 *Ctr*^{Δ*taiP*} at MOI=0.2. After fixation and permeabilization inclusions were stained using
707 antibodies against Cap1. Inclusion areas were measured using imageJ software. The dot-plot
708 shows the median± SD of 3 independent experiments (N>50 cells in each experiment) and
709 displays the P-values of the Student's t test.

710
711

Figure 4: TaiP redirects Rab6-positive vesicular traffic to the inclusion .

712 **A)** HeLa cells were co-transfected with Flag-TaiP and the indicated GFP-tagged Rab
713 constructs for 24 hours. Cells were lysed, and immunoprecipitation (IP) was performed with
714 anti-GFP antibody. Proteins were separated on SDS-PAGE, transferred to a PVDF membrane
715 and probed with the indicated antibody (IB : immunoblot). An aliquot of each cell lysate was
716 loaded on a separate gel to visualize the expression of Flag-TaiP and of each of the GFP-
717 tagged proteins (input panels).

718 **B)** HeLa cells were co-transfected with the indicated constructs for 24 hrs and analyzed
719 as in B.

720 **C)** Wild type and *atg16l1* KO cells were transfected as indicated and infected with *Ctr*^{WT}
721 or *Ctr*^{Δ*taiP*} the following day at MOI=0.2. Cells were fixed 20 hrs post infection, permeabilized,
722 and the inclusion membrane was stained with anti-Cap1 antibodies. Inclusion areas were
723 measured using imageJ software. The Box and whiskers plots display the median area of
724 intracellular inclusions (N>50 cells from 3 independent experiments). Statistical analysis was
725 performed using a one-way anova with a Dunnett's multiple comparison test to siCtrl in WT
726 HeLa. The inset shows the efficacy of the siRNA against Rab6 at silencing Rab6 expression
727 probed by western blot.

728 **D)** Wild type and *atg16l1* KO cells were infected with *Ctr*^{WT} or *Ctr*^{Δ*taiP*} the following day at
729 MOI=0.2. 20 h after infection the cells were treated with 10 nM nocodazole, and incubated
730 further for 60 min before fixation. After permeabilization the cells were stained with rabbit
731 antibodies against endogenous Rab6 and mouse antibodies against the inclusion membrane
732 protein CT813. Representative images are shown, scale bar: 10 μm. The Box and whiskers

733 plot displays the median intensity of the Rab6 staining at the inclusion periphery, relative to its
734 cytosolic intensity (N>50 cells from 3 independent experiments, see Methods for details).
735 Statistical analysis was performed using Student's t test.

736

737 **Figure 5: TaiP disrupts the ATG16L1-controlled traffic of Rab6-positive vesicles towards**
738 **TMEM59-positive compartments.**

739 **A)** Model.*a.* In uninfected cells, ATG16L1's binding to TMEM59 facilitates the supply of
740 Rab6 positive vesicles to TMEM59 positive compartments. *b.* In cells infected with *Ctr*^{WT}, TaiP
741 secretion by the bacteria prevents ATG16L1/TMEM59 interaction, and Rab6 positive vesicles
742 are hijacked by the inclusion. *c.* In the absence of TaiP, the bacteria lose access to this pool of
743 vesicles. *Ctr*^{Δ*taiP*} inclusions are smaller, as they only rely on alternative membrane sources. *d.*
744 Silencing ATG16L1 or TMEM59 expression reverts this phenotype.

745 **B)** Wild type and *atg16l1* KO HeLa cells were co-transfected with the indicated plasmids.
746 One day later, the cells were lysed, and immunoprecipitation (IP) was performed with anti-HA
747 coupled beads. Proteins were separated on SDS-PAGE, transferred to a PVDF membrane
748 and probed with the indicated antibody. GFP-Rab6 co-immunoprecipitated with TMEM59 only
749 in cells expressing ATG16L1. The use of cells that do not express HA-TMEM59 allows to
750 measure the level of non-specific association of GFP-Rab6 to the beads.

751

Figure 1

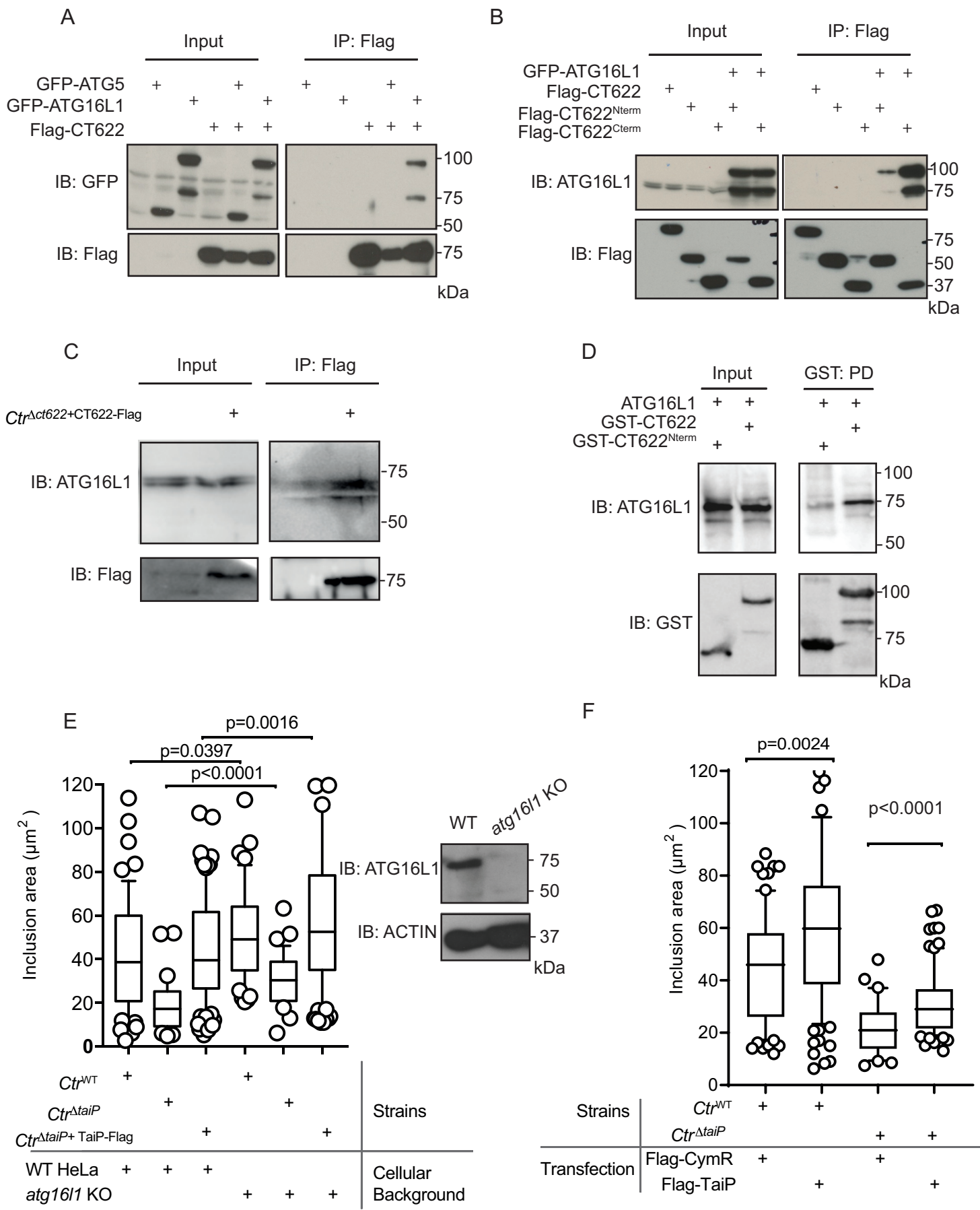


Figure 2

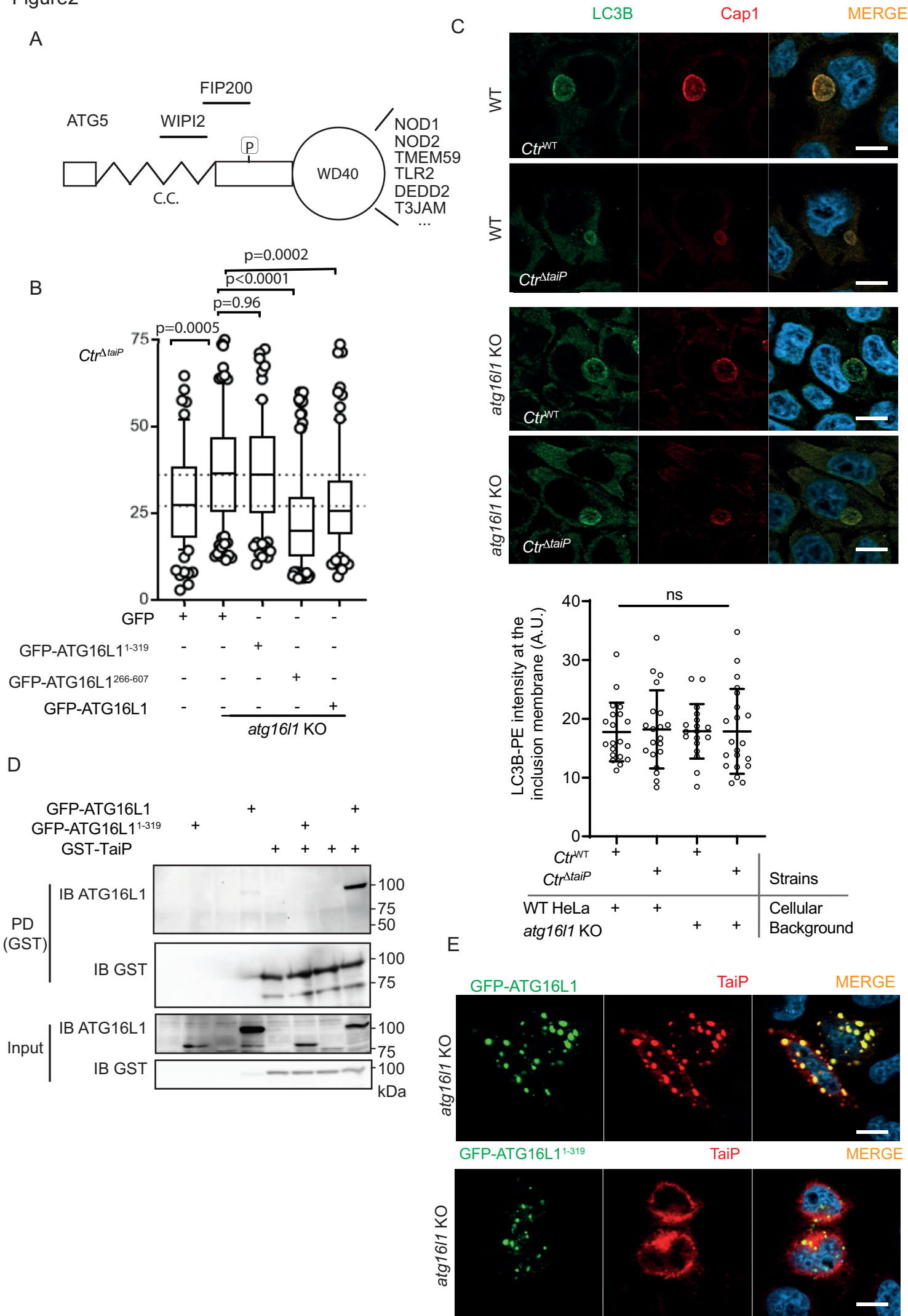


Figure 3

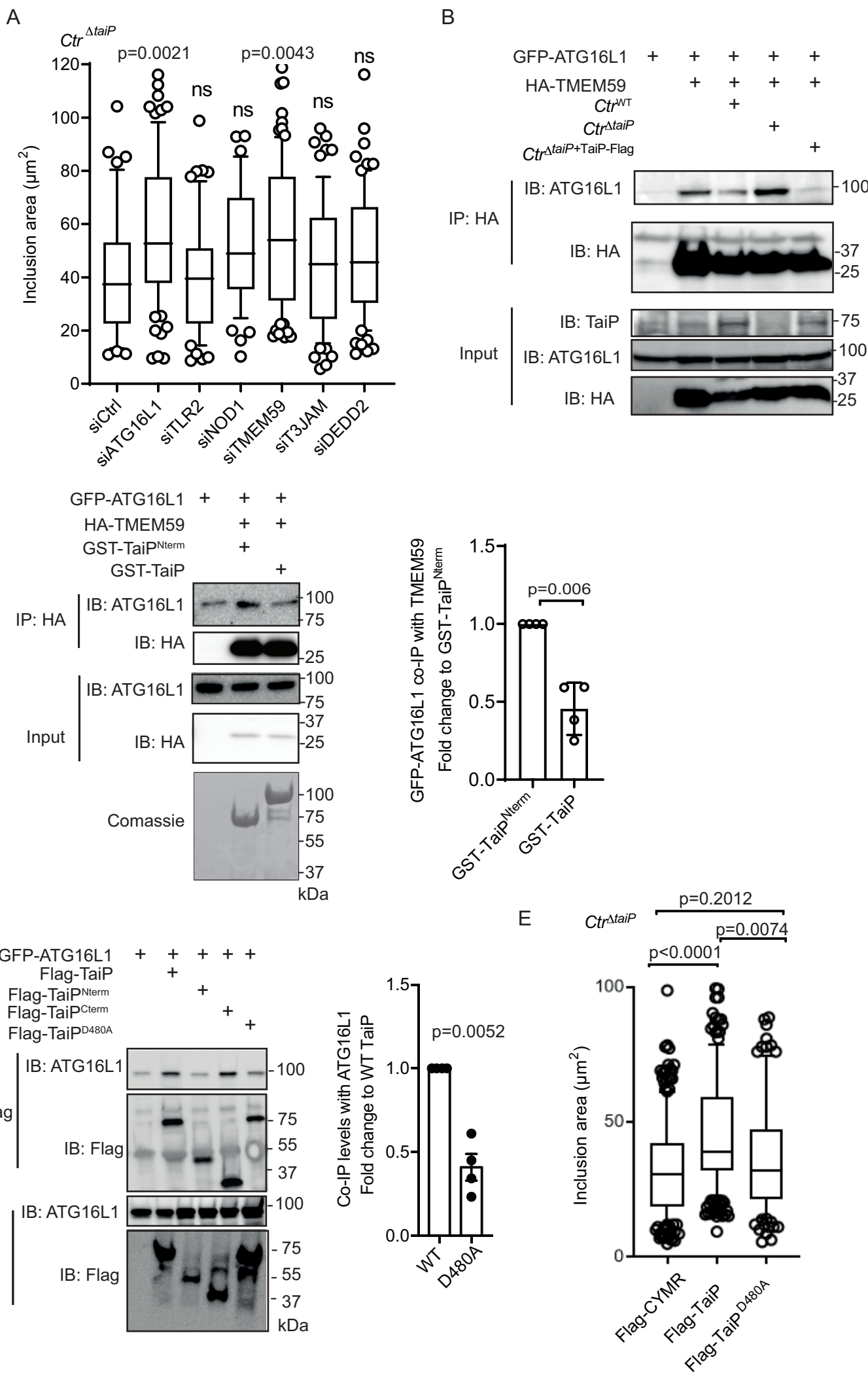


Figure 4

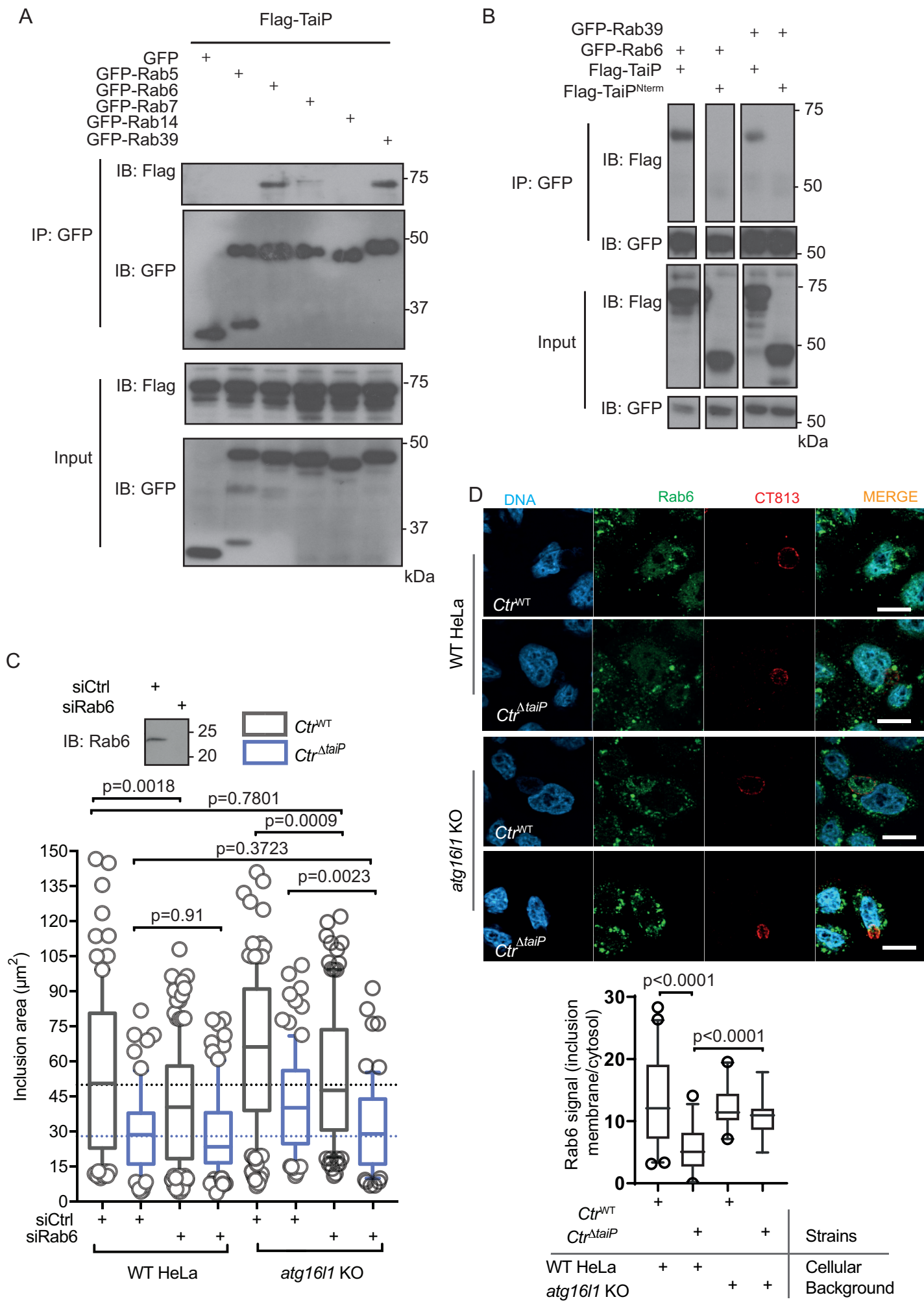
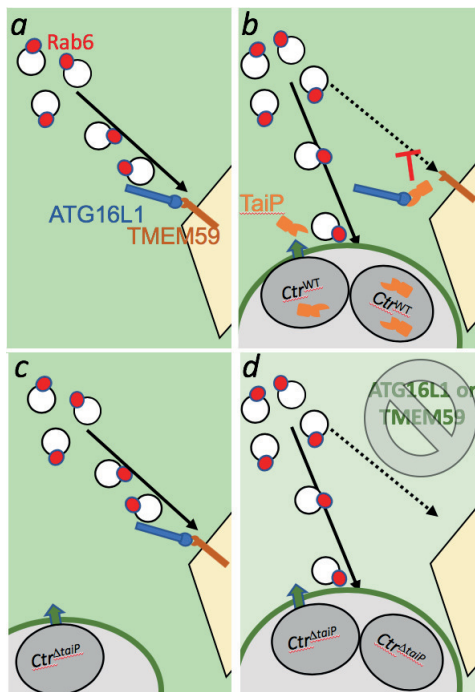


Figure 5

A



B

