

1 **Post-translational modifications of Beclin 1 provide multiple strategies**  
2 **for autophagy regulation**

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13 **Abstract**

14 Autophagy is a conserved intracellular degradation pathway essential for protein  
15 homeostasis, survival and development. Defects in autophagic pathways have been  
16 connected to a variety of human diseases including cancer and neurodegeneration. In  
17 the process of macroautophagy, cytoplasmic cargo is enclosed in a double-membrane  
18 structure and fused to the lysosome to allow for digestion and recycling of material.  
19 Autophagosome formation is primed by the ULK complex, which enables the  
20 downstream production of PI(3)P, a key lipid signalling molecule, on the phagophore  
21 membrane. The PI(3)P is generated by the PI3 kinase (PI3K) complex, consisting of  
22 the core components VPS34, VPS15 and Beclin 1. Beclin 1 is a central player in  
23 autophagy and constitutes a molecular platform for the regulation of autophagosome  
24 formation and maturation. Post-translational modifications of Beclin 1 affect its stability,  
25 interactions and ability to regulate PI3K activity, providing the cell with a plethora of  
26 strategies to fine-tune the levels of autophagy. Being such an important regulator,  
27 Beclin 1 is a potential target for therapeutic intervention and interfering with the post-  
28 translational regulation of Beclin 1 could be one way of manipulating the levels of  
29 autophagy. In this review, we provide an overview of the known post-translational  
30 modifications of Beclin 1 that govern its role in autophagy, and how these modifications  
31 are maintained by input from several upstream signalling pathways.

32

33 **Facts**

- 34 • Beclin 1 is a core component of the PI3K complex, important for producing  
35 PI(3)P on the phagophore membrane in order to recruit downstream  
36 autophagy effectors.
- 37 • Beclin 1 is a multi-domain protein, with a large interactome that facilitates  
38 regulation of autophagy in both a positive and negative manner.
- 39 • Post-translational modifications of Beclin 1 affect protein stability,  
40 confirmation, activity and its interactome, and can be used as a molecular  
41 rheostat to fine-tune autophagic activity.
- 42 • Targeting Beclin 1 modifiers to regulate Beclin 1 post-translational  
43 modifications could provide a possible therapeutic intervention for  
44 upregulating autophagy.

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### 47 **Open Questions**

- 48
- 49 • Understand the full repertoire of Beclin 1 post-translational modifications  
50 relevant to autophagy
  - 51 • Understand the relevance of Beclin 1 post-translational modifications to the  
52 activities of the different Beclin 1 complexes
  - 53 • Can regulation of Beclin 1 post-translational modifications be exploited as a  
54 therapeutic strategy to counteract accumulation of aggregate-prone proteins  
55 in neurodegenerative diseases?

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### 58 **Introduction**

59 Autophagy is an essential and widely conserved cellular degradation process that  
60 plays a crucial role in housekeeping and stress survival. In the process of  
61 macroautophagy (henceforth referred to as autophagy), a portion of cytoplasmic  
62 content, including proteins and organelles, is sequestered into a double-membrane  
63 structure called the phagophore. The phagophore membrane expands to fully enclose  
64 its cargo, forming the autophagosome, and the autophagosome is trafficked along  
65 microtubules to ultimately fuse with the lysosome<sup>1,2</sup>. Fusion with this lytic compartment  
66 ensures the enzymatic degradation of the cargo and subsequent release of recycled  
67 material. Autophagy was initially believed to be a non-selective bulk degradation  
68 process, but recent data indicate that it is often selective using specific receptors for  
69 cargo recognition<sup>3</sup>. Autophagy has been shown to aid in clearing intracellular  
70 aggregate-prone proteins underlying neurodegenerative diseases, including

71 Alzheimer and Parkinson disease, and upregulating autophagy induction may be a  
72 promising therapeutic approach for these types of diseases<sup>4</sup>.

73

#### 74 **PI(3)P production on the phagophore membrane**

75 Under normal conditions, cells maintain basal levels of autophagy to maintain  
76 homeostasis. In addition, autophagy can be triggered by a variety of stimuli such as  
77 nutrient deprivation, metabolic imbalance, protein aggregation and oxidative stress.  
78 The process of autophagosome biogenesis consists of three main stages: initiation,  
79 nucleation and expansion of the isolation membrane (Figure 1). Key initiating events  
80 in autophagosome biogenesis are dictated by the Unc-51-like autophagy-activating  
81 kinase (ULK) complex, which consists of ULK1 (or ULK2), ATG13, ATG101 and  
82 FIP200<sup>5</sup>. The ULK complex facilitates the production of phosphatidylinositol-3-  
83 phosphate (PI(3)P) by recruiting and activating the kinase VPS34, which is found in  
84 the class III PI3 Kinase (PI3K) complex together with a number of proteins, including  
85 VPS15, ATG14L and Beclin 1, the mammalian ortholog of yeast Atg6<sup>6</sup> (Figure 1). The  
86 pool of PI(3)P produced at the site of the phagophore, creates a platform for the  
87 recruitment of subsequent autophagy machinery effectors, like WIPI family proteins  
88 and ATG16L. These effectors facilitate conjugation of ATG8 proteins, such as LC3, to  
89 the phagophore membrane, which in turn mediates cargo recruitment, and membrane  
90 extension of the phagophore<sup>1, 7, 8</sup>.

91

#### 92 **The main signalling cues for autophagy initiation converge on PI3K regulation**

93 The early initiation steps of autophagy are regulated by diverse signal-sensing proteins,  
94 including four protein kinases: mammalian target of rapamycin complex 1 (mTORC1),  
95 ULK1, AMP-activated protein kinase (AMPK), and AKT (Figure 2). Nutrient starvation  
96 is one of the most studied autophagy inducers and the serine/threonine mTOR kinase  
97 plays a major role in sensing nutrient availability<sup>9, 10, 11</sup>. Lack of nutrients, mainly amino  
98 acids, triggers an intracellular signalling cascade, which inhibits mTORC1 activity<sup>12</sup>.  
99 Upon starvation, inactivated mTORC1 dissociates from ULK1, resulting in  
100 dephosphorylation and activation of the ULK1 complex<sup>13, 14</sup>. Activated ULK1  
101 phosphorylates several proteins involved in autophagy initiation and progression,  
102 including the ULK complex components ATG13, ATG101 and FIP200<sup>15</sup>, and

103 downstream effectors like the VPS34, ATG14L, Beclin 1, and AMBRA1 components  
104 of the PI3K complex<sup>16, 17, 18</sup>.

105 Autophagy can also be triggered by low energy-conditions through activation of AMPK.  
106 AMPK responds to low energy conditions, detected as changes in the ATP-to-ADP or  
107 ATP-to-AMP ratios, by inhibiting ATP-consuming anabolic processes and by  
108 promoting catabolic processes to restore the proper metabolic balance<sup>19</sup>. Activated  
109 AMPK triggers autophagy by coordinated activation of ULK1 and negative regulation  
110 of the mTOR kinase<sup>20, 21, 22</sup>. As described in the following sections, AMPK also exerts  
111 a direct role in regulating components of the PI3K complex.

112 Another important input for nutrient status is the presence of extracellular growth  
113 factors. The serine threonine kinase AKT (also called Protein kinase B, PKB) is  
114 recruited to active growth factor receptors at the plasma membrane, and binding  
115 initiates its downstream signalling cascade<sup>23</sup>. AKT phosphorylation activates mTORC1  
116 and inhibits autophagy, and AKT also suppresses autophagy via mTOR-independent  
117 pathways by direct phosphorylation of key autophagy proteins, including components  
118 of the PI3K complex<sup>24, 25, 26</sup>.

### 119 **Beclin 1 is a key regulator of autophagy**

120 Beclin 1 was one of the first mammalian autophagy proteins identified<sup>27</sup>, and *BECN1*  
121 is an essential gene required for embryonic survival and development<sup>28</sup>. *BECN1* also  
122 functions as a tumour suppressor gene and has been found to be monoallelically  
123 deleted in many cancer types, and Beclin 1 deficiency is also associated with several  
124 neurodegenerative diseases<sup>6, 28, 29, 30, 31, 32</sup>. Beclin 1 regulates both autophagosome  
125 synthesis and autophagosome maturation, by forming three distinct PI3K complexes  
126 together with the core lipid kinase VPS34 and the regulatory component VPS15<sup>33, 34,</sup>  
127 <sup>35</sup>. Complex I includes the core proteins together with ATG14L and is involved in  
128 autophagy initiation and autophagosome formation, whereas in complex II ATG14L is  
129 replaced by UVRAG and this complex regulates autophagosome maturation and  
130 endocytosis<sup>33, 36</sup>. In the third PI3K complex, RUBICON interacts with Beclin 1, VPS34  
131 and UVRAG of complex II to inhibit lipid kinase activity and reduce autophagic flux<sup>34,</sup>  
132 <sup>35, 37</sup>.

133

134 Beclin 1 interacts with the PI3K core component VPS34 and lipid membranes via its  
135 C-terminal  $\beta/\alpha$ -repeated, autophagy related (BARA) domain (previously denoted  
136 evolutionary conserved domain)<sup>38, 39, 40</sup> (Figure 3A-B), and binds ATG14L or UVRAG  
137 in a mutually exclusive manner via its coiled-coiled domain (CCD)<sup>36</sup>. Additionally,

138 Beclin 1 has an unstructured N-terminal domain, followed by a BCL2-homology-3  
139 (BH3) domain and a flexible helical domain (HD) (Figure 3A-B)<sup>41,42</sup>. The many domains  
140 of Beclin 1 mediate communications with multiple interaction partners that can alter its  
141 conformation and binding accessibility, thus making Beclin 1 an important molecular  
142 platform for the regulation of PI3K activity and autophagy<sup>43</sup>. For example, the BH3  
143 domain of Beclin 1 mediates its interaction with the anti-apoptotic protein BCL2 and  
144 this interaction causes a steric block inhibiting PI3K complex formation<sup>27, 44</sup>. Thus,  
145 BCL2 binding to Beclin 1 inhibits autophagy, and nutrient-dependent regulation of this  
146 interaction modifies the pool of Beclin 1 molecules available for VPS34 interaction<sup>44, 45</sup>.  
147 Another example of how the Beclin 1 interactome can be utilized as a way of fine-  
148 tuning autophagic activity is demonstrated by the interaction with AMBRA1. AMBRA1  
149 is a positive regulator of autophagy and competes with BCL2 for binding to the Beclin  
150 1 BH3 domain<sup>46</sup>. Binding of AMBRA1 promotes Beclin 1 interaction with VPS34, and  
151 regulates the positioning of the PI3K complex to the site of phagophore formation,  
152 thereby mediating autophagy initiation<sup>47, 48</sup>. Consequently, modifications that alter the  
153 interaction between Beclin 1 and the PI3K components, or the interaction with  
154 AMBRA1 and BCL2, allow cells to modulate autophagic activity in response to a  
155 multitude of internal and external cues and could also provide potential targets for  
156 pharmaceutical interventions.

157

158 The functions of many proteins directly involved in autophagosome biogenesis are  
159 regulated by their post-translational modifications. These modifications include  
160 phosphorylation, ubiquitination, acetylation, lipidation and redox regulation. The  
161 initiation of autophagy relies on the coordinated spatio-temporal regulation of the levels  
162 of modification of key players constituting the core machinery. In this review, we  
163 provide an overview of the known post-translation modifications of Beclin 1 that govern  
164 its role in autophagy, and how these modifications are maintained by inputs from  
165 upstream signalling pathways. The modifications of Beclin 1 mentioned in this paper  
166 are listed in table 1 together with information on supporting experimental evidence,  
167 and the physiological relevance in terms of effect on autophagy. Post-translational  
168 modifications of other autophagy-related proteins are reviewed elsewhere<sup>49</sup>.

169

### 170 **Activational phosphorylation of Beclin 1**

171 Phosphorylation is the most intensively studied post-translational modification in the  
172 autophagy process, where a phosphate group is reversibly attached onto serine,

173 threonine, or tyrosine residues in the substrate protein<sup>50</sup>. This post-translational  
174 modification changes protein conformation and can affect accessibility for interactions  
175 and enzymatic activity, and many of the events in autophagy initiation rely on kinase  
176 signalling. Upon amino acid depletion, mTORC1 is inactivated and its inhibitory effect  
177 on the ULK1 complex is relieved<sup>13</sup>. ULK1 then phosphorylates Beclin 1 on Ser15  
178 (Ser14 in mouse) to induce the activity of ATG14L-containing VPS34 complexes and  
179 stimulate autophagy initiation<sup>18</sup>. This phosphorylation site is in the N-terminal domain  
180 of Beclin 1 (Figure 3A, 3C; Table 1), which is dispensable for interaction with the PI3K  
181 components. Indeed, mutating the ULK1 phosphorylation site did not affect the Beclin  
182 1 interactions with PI3K components, indicating that this phosphorylation site is  
183 important for the regulatory role of Beclin 1 on VPS34 kinase activity, rather than  
184 affecting complex assembly<sup>18, 51</sup>.

185 The activating phosphorylation of Ser15 of Beclin 1 in autophagy initiation is dependent  
186 on ATG14L, which acts as an adaptor for recruiting ULK1 to Beclin 1 at the phagophore  
187 by binding Beclin 1 and ATG13 of the ULK1 complex<sup>17</sup>. Indeed, expression of a mutant  
188 ATG14L ( $\Delta$ BATS) that binds Beclin 1 but is unable to localize to the phagophore  
189 severely compromised ULK1-mediated phosphorylation of Beclin 1<sup>18, 52</sup>. In addition,  
190 the recruitment of Beclin 1 to the phagophore is dependent on ULK1-mediated  
191 phosphorylation of AMBRA1<sup>48</sup>. Thus, ULK1-dependent phosphorylation activates  
192 autophagy via two steps: Beclin 1 recruitment and activation. ULK1-dependent  
193 phosphorylation of Beclin 1 Ser15 is also thought to be important during later steps of  
194 autophagosome formation, where UVRAG has been suggested to play a role similar  
195 to ATG14L in stimulating ULK1 activity<sup>18</sup>.

196 The activity of ULK1 is also governed by AMPK, another major nutrient-sensing kinase.  
197 During glucose depletion and in low energy states, AMPK phosphorylates ULK1 and  
198 stimulates ULK1-dependent autophagy initiation (Figure 2)<sup>21</sup>. AMPK also directly  
199 phosphorylates Beclin 1 on Ser90 and Ser93 (Ser91 and Ser94 in mouse) (Table 1)<sup>53</sup>.  
200 Similar to the activating phosphorylation by ULK1, phosphorylation at these sites do not  
201 alter PI3K complex formation or membrane association, but rather regulate PI3K kinase  
202 activity<sup>39, 54</sup>. Interestingly, AMPK exerts a dual regulation of the PI3K activity governed  
203 by ATG14L, where AMPK mediates an inhibitory phosphorylation on VPS34 unless  
204 ATG14L is present to inhibit this phosphorylation and instead stimulates the activational  
205 phosphorylation of Beclin 1<sup>53</sup>. In vitro studies show that UVRAG might play a similar role  
206 in directing AMPK activity to the PI3K complex II, but further investigations of this are  
207 required<sup>53</sup>. Remarkably, the regulation of AMPK activity by ATG14L seems independent  
208 of phagophore localization of ATG14L, as phosphorylation was still stimulated by an

209 ATG14L  $\Delta$ BATS mutant<sup>34, 53</sup>. Consequently, the timing and location of AMPK-mediated  
210 phosphorylation of Beclin 1 in respect to autophagy initiation remains unclear. Another  
211 study confirmed that AMPK phosphorylates Beclin 1, but identified Thr388 as the target  
212 residue<sup>55</sup>. As opposed to Ser90 phosphorylation, this modification affected PI3K  
213 complex formation by shifting Beclin 1 affinity, disrupting the Beclin 1-BCL2 interaction  
214 and favouring the Beclin 1-VPS34 interaction<sup>55</sup>. While the importance of AMPK  
215 phosphorylation for inducing autophagy during glucose-deprivation was evident in the  
216 two separate studies, it is not known if and how phosphorylation at the two different sites  
217 are co-regulated. In addition, an autophagy-independent role for Beclin 1  
218 phosphorylation by AMPK has been shown, where phosphorylation of Ser90, Ser93 and  
219 Ser96 enables Beclin 1 to interact with SLC7A11 and initiate ferroptosis, a type of  
220 programmed cell death<sup>56</sup>.

221

222 Phosphorylation at Ser90 seems to be a key event for Beclin 1 activation and is  
223 considered to be the initial event that mediates further phosphorylation at Ser93<sup>39</sup>.  
224 Monophosphorylation at S90 is considered sufficient to have an activating effect upon  
225 autophagy induction, and this modification appears to be mediated by different kinases,  
226 depending on cellular context and tissue-type (Figure 3; Table 1). In addition to AMPK-  
227 mediated phosphorylation upon glucose starvation, Ser90 is phosphorylated by the  
228 stress-responsive kinases MAPKAPK2/3 upon amino acid starvation, and by Death-  
229 associated protein kinase 3 (DAPK3) during serum starvation in mouse skeletal muscle  
230 tissue<sup>54, 57</sup>. Upon insulin addition, the same site is dephosphorylated by the protein  
231 phosphatase 2A (PP2A). When autophagy is induced by treatment with ionomycin, the  
232 calcium/calmodulin dependent protein kinase II (CaMKII) phosphorylates Beclin 1 at  
233 Ser90 in a manner that increases interaction with VPS34 and activates Beclin 1 via  
234 TRAF6-mediated K63-linked ubiquitination<sup>58, 59</sup>. Furthermore, the Ser90  
235 phosphorylation site is located within the BCL2-binding domain of Beclin 1, where  
236 phosphorylation and BCL2 binding occurs in a competitive manner, providing another  
237 important point of regulation<sup>54, 58</sup>. The importance of the Ser90 phosphorylation site is  
238 further emphasized by studies showing that this site is important for the tumour  
239 suppression function of Beclin 1<sup>54</sup>.

240

241 PI3K activity can also be enhanced by phosphorylation of Beclin 1 Ser30, and this  
242 seems to be the preferred mechanism for autophagy activation upon glutamine  
243 deprivation and hypoxia in cancer cell lines, where Ser15/Ser90/Ser96  
244 phosphorylation by ULK1 and AMPK seem less essential<sup>60</sup>. This phosphorylation is

245 accomplished by phosphoglycerate kinase 1 (PGK1) and contributes to cancer cell  
246 proliferation during hypoxic conditions.

247

248 Phosphorylation of Beclin 1 is not only a way to positively regulate autophagy, it can  
249 also be used to reduce PI3K activity and decelerate autophagic flux. In the presence  
250 of extracellular growth factors, AKT phosphorylates Beclin 1 Ser295 and Ser234<sup>26</sup>.  
251 This phosphorylation bridges Beclin 1 and 14-3-3 proteins and sequesters the protein  
252 complex to intermediate filaments, inhibiting the activation of Beclin 1 in nutrient-rich  
253 conditions to suppress autophagy.

254

### 255 **Phosphorylation regulates the Beclin 1 interactome**

256 Several phosphorylation events can directly affect protein interactions, and shift the  
257 Beclin 1 interactome to induce or inhibit autophagy. In growth-factor rich conditions,  
258 active epidermal growth factor receptor (EGFR) binds Beclin 1, leading to multisite  
259 phosphorylation on residues Tyr229, Tyr233, and Tyr352, which decreases the Beclin  
260 1 interaction with VPS34 and increases its interaction with negative regulators such as  
261 RUBICON and BCL2<sup>61</sup>. Inhibitory phosphorylation of Beclin 1 Tyr233 occurs also  
262 during phenylephrine-stimulated repression of autophagy, where focal adhesion  
263 kinase (FAK) seems to be the responsible modifier<sup>62</sup>. Another growth factor receptor  
264 that regulates autophagy by tyrosine phosphorylation on Beclin 1 was identified in  
265 human breast cancer cells<sup>63</sup>. Here, the activated human epidermal growth factor  
266 receptor 2 (HER2) was shown to bind Beclin 1 and phosphorylate Tyr233, a  
267 modification that leads to a decrease in both basal and starvation-induced autophagy<sup>63</sup>.  
268 However, the direct phosphorylation of these sites has not been verified in vitro, and  
269 therefore the possible involvement of other kinases and regulators cannot be excluded.

270

271 As mentioned previously, the anti-apoptotic protein BCL2 affects the interaction  
272 between Beclin 1 and VPS34, and also sterically hinders accessibility for activating  
273 kinases<sup>54, 58</sup>. The interaction between Beclin 1 and BCL2 is itself highly regulated by  
274 phosphorylation, and can be prevented via direct phosphorylation of the BH3 domain  
275 in either Beclin1 or BCL2 to induce autophagy. Beclin 1 can be phosphorylated at  
276 Thr119 by DAPK or Rho kinase 1 (ROCK1)<sup>64, 65</sup>. ROCK1 may also prevent the  
277 inhibitory Beclin 1/BCL2 dimer formation upon nutrient stress by activating the c-Jun  
278 aminoterminal kinase 1 (JNK1) pathway, where JNK1 phosphorylates BCL2<sup>65, 66</sup>.  
279 However, it is not clear which kinase is directly phosphorylating Thr119 of Beclin 1.  
280 There may be differences in this process between cell types and conditions, and it



281 should be noted that Thr119 phosphorylation was not observed upon starvation in an  
282 MCF7 cell line<sup>57</sup>. The phosphorylation that inhibits the Beclin 1-BCL2 interaction does  
283 not seem to interfere with Beclin 1 binding to AMBRA1, even though this positive  
284 autophagy regulator also binds Beclin 1 via the BH3 domain. This might be due to  
285 slightly different binding motifs, and it is possible that AMBRA1 interacts with Beclin 1  
286 also via other domains<sup>46</sup>.

287

288 Phosphorylation can also stabilize the interaction of Beclin 1 and BCL2 to inhibit  
289 autophagy, and possibly initiate apoptosis by limiting the amount of free BCL2 that can  
290 interact with and inactivate the pro-apoptotic protein BAX<sup>67</sup>. This is the case in  
291 cardiomyocytes, where stress-induced mammalian sterile 20-like kinase 1 (MST1)  
292 phosphorylates Beclin 1 in its BH3 domain at Thr108, promoting binding of Beclin 1 to  
293 BCL2<sup>68</sup>.

#### 294 **Activational ubiquitination of Beclin 1**

295 Ubiquitination of protein is a key regulatory step targeting proteins for degradation, but  
296 also could alter its localisation or composition and activity of multiprotein complexes.<sup>69</sup>  
297 In the process of ubiquitination, ubiquitin is covalently attached to a lysine residue of a  
298 target protein by a cascade of enzymatic reactions involving E1, E2 and E3 enzymes.  
299 The action of ubiquitin ligases can be reversed by deubiquitinating enzymes (DUBs),  
300 a family of proteases that remove ubiquitin chains from substrate proteins by peptide  
301 cleavage.

302 Beclin 1 is regulated by ubiquitination in numerous ways that affect its stability and  
303 function in the autophagy initiation process. The E3 ubiquitin ligase NEDD4 (neural-  
304 precursor-cell-expressed developmentally down-regulated 4) was reported to interact  
305 with Beclin 1 via a PY-motif located downstream of the BARA domain (aa 349–352),  
306 to mediate Beclin 1 ubiquitination and degradation (Figure 3, Table 1)<sup>70</sup>. Levels of  
307 Beclin 1 correlate inversely with the levels of NEDD4, and enhanced expression of  
308 NEDD4 results in increased Lys11- and Lys63-linked polyubiquitination of Beclin 1  
309 both *in vivo* and *in vitro*, further establishing the role of NEDD4 in Beclin 1 regulation.  
310 While the role of NEDD4-mediated Beclin 1 ubiquitination in autophagy was not  
311 established in the initial studies, it was assumed that it would inhibit autophagy by  
312 promoting Beclin 1 degradation<sup>70</sup>. Controversially, other studies found the opposite to  
313 be true, claiming that NEDD4 is a positive regulator of autophagy<sup>71, 72</sup>. The  
314 contradictory results suggest that NEDD4 mediates mainly Lys6- and Lys27-linked  
315 ubiquitination of Beclin 1, which functions to protect Beclin 1 against Lys48-linked  
316 ubiquitination and stabilise protein levels during autophagy induction<sup>71</sup>. This study

317 further showed that NEDD4 also interacts with proteins of the ATG8 family and ULK1,  
318 extending the role of NEDD4 in autophagy regulation beyond its modification of Beclin  
319 1.

320

321 Lys63-linked polyubiquitination of Beclin 1 has been linked to increased activity of the  
322 PI3K complex, and is considered to be a modification that positively regulates  
323 autophagy. During the inflammatory response in murine macrophage cells stimulated  
324 with the TLR4 agonist lipopolysaccharide (LPS) and interferon- $\gamma$ , the E3 ligase TRAF6  
325 modifies Beclin 1 with Lys63-linked ubiquitin chains<sup>59</sup>. This posttranslational  
326 modification occurs on Beclin 1 Lys117, which is strategically located in the BH3  
327 domain of Beclin 1 (Figure 3A), and the modification was opposed by the activity of the  
328 deubiquitinating enzyme A20. Both TRAF6 and A20 were shown to bind Beclin 1 and  
329 the binding of TRAF6 was mapped to two sites, one in the Beclin 1 N-terminal domain  
330 (aa 54-58) and one in the C-terminal BARA domain (aa 297-301). The TRAF6-  
331 mediated polyubiquitination of Beclin 1 affected oligomerisation of Beclin 1, inhibiting  
332 homodimerization and promoting complex formation with VPS34, thereby leading to  
333 autophagy induction<sup>59, 73</sup>. Additionally, Lys63-linked ubiquitination of Beclin 1 was  
334 enhanced by coexpression of the E3 ligase TRIM16, suggesting that this protein might  
335 be an additional regulator of Beclin 1 stability<sup>74</sup>. However, a direct interaction was not  
336 shown, and it is possible that this effect is mediated indirectly via other E3 ligases.

337

338 During mouse embryonic development, Lys63-linked ubiquitination of Beclin 1 is  
339 necessary to maintain autophagic flux and ensure embryonic survival<sup>75</sup>. In this  
340 scenario, Lys437 is the main target residue and ubiquitination is suggested to be  
341 mediated by the DDB1–Cullin 4 E3 ubiquitin ligase complex containing AMBRA1 as  
342 the substrate receptor to mediate the interaction with Beclin 1 (Figure 3A, 3C, Table  
343 1)<sup>75, 76</sup>. The WASH (Wiskott–Aldrich syndrome protein (WASP) and SCAR Homologue)  
344 protein was identified as a negative regulator of autophagy by binding Beclin 1 and  
345 inhibiting Beclin 1 Lys63-linked ubiquitination to suppress PI3K activity. WASH was  
346 shown to bind the central region of Beclin 1, competing with AMBRA1, and this  
347 interaction was decreased upon starvation-induced autophagy. Consistently, WASH  
348 deficiency increased Beclin 1 Lys63-linked ubiquitination and enhanced autophagy  
349 induction in mouse embryos<sup>75</sup>.

350

351 A more recent study identified Tripartite motif protein 50 (TRIM50) as another E3 ligase  
352 responsible for Beclin 1 Lys63-linked ubiquitination, showing that TRIM50 dependent  
353 ubiquitination of Beclin 1 increases during autophagy-inducing conditions, such as

354 starvation or treatment with rapamycin<sup>77</sup>. The ubiquitination of Beclin 1 did not affect  
355 interactions with VPS34, but did increase the interactions between the PI3K complex  
356 and ULK1, which could account for the activation of autophagy. In addition, TRIM50  
357 activity was dependent on acetylation by the acetyltransferase EP300, placing Beclin  
358 1 activating ubiquitination downstream of regulation governed by acetylation<sup>77</sup>.

359

360 The activating effect of Beclin 1 Lys63-linked ubiquitination is counteracted by the  
361 deubiquitinating enzyme USP14<sup>78</sup>. USP14 interacts directly with the Beclin 1 CCD, and  
362 depletion of USP14 or treatment with its inhibitor increases Beclin 1 Lys63-linked  
363 ubiquitination and promotes the Beclin 1 interaction with ATG14L and UVRAG, but not  
364 with VPS34. Thus, absence of USP14 increases activation of the VPS34 complex  
365 without changing the levels of the key PI3K complex components, and promotes both  
366 autophagy initiation and autophagosome maturation by enhancing the activity of PI3K  
367 complexes I and II. Moreover, USP14 deubiquitinating activity is suggested to be  
368 regulated by AKT-mediated phosphorylation, providing evidence for the upstream  
369 signalling events governing Beclin 1 ubiquitination<sup>78</sup>.

370

#### 371 **Ubiquitination and deubiquitination regulate Beclin 1 levels**

372 Beclin 1 is marked for degradation via modification by Lys48-linked ubiquitin. Thus,  
373 any proteins that increase this modification would be predicted to be negative  
374 regulators of autophagy. In macrophages, the E3 ligase RNF216 (ring finger protein  
375 216) was identified as the protein responsible for Beclin 1 degradation<sup>79</sup>, and  
376 overexpression of RNF216 was shown inhibit TLR-mediated autophagy induction in  
377 bacteria-infected mice, indicating that it is an important regulator of autophagy in the  
378 pathogen response<sup>79</sup>.

379

380 The CUL3-KLHL20 ubiquitin ligase complex is another E3 ligase that governs  
381 autophagy by facilitating degradation of several autophagy proteins during prolonged  
382 starvation-induced autophagy<sup>80</sup>. KLHL20 was shown to directly bind and ubiquitinate  
383 ULK1, VPS34 and Beclin 1 at the phagophore to promote their degradation and  
384 prevent unrestrained autophagic activity during prolonged starvation<sup>80</sup>. Consequently,  
385 KLHL20 depletion caused an increase in the amplitude and duration of starvation-  
386 induced autophagy and reduced cell survival.

387

388 Deubiquitinating enzymes (DUBs) that remove Lys48-linked ubiquitin chains  
389 counteract the degradation signal from E3 ligases and rescue proteins from  
390 degradation. USP10 and USP13 were identified as two deubiquitinases that target and

391 stabilise Beclin 1<sup>81</sup>. Depletion of USP10 and USP13 reduced levels of VPS34, Beclin  
392 1, ATG14L, VPS15 and UVRAG, replicating the effects observed when treating cells  
393 with Spautin-1, a compound known to inhibit the two deubiquitinating enzymes.  
394 Intriguingly, the interaction between Beclin 1 and USP10/USP13 does not only  
395 stabilise Beclin 1 levels, but also reciprocally stabilises the deubiquitinases. Thus,  
396 Beclin 1 could be considered a regulator of other USP10 and USP13 targets, one of  
397 which is the tumour suppressor protein p53<sup>82</sup>. All together, these results provide one  
398 mechanism to explain the tumour suppressor function of Beclin 1, and illustrate that  
399 PI3K components can regulate their own levels via feedback control of USP10 and  
400 USP13<sup>81</sup>. Another mechanism that may contribute to the tumour suppressor properties  
401 of Beclin 1, in addition to its role in autophagy, is mediated by the USP9X  
402 deubiquitinase, which is another modifier of Beclin 1<sup>83</sup>. Beclin 1 competes with the  
403 antiapoptotic protein MCL1 for binding to USP9X and thus the levels of Beclin 1 and  
404 MCL1 are reciprocally regulated, providing cells with a way of upregulating autophagy  
405 at the same time as inducing apoptosis to prevent unrestricted cell growth<sup>83</sup>.

406

407 Most recently, the deubiquitinase ATAXIN3 was also reported to control Beclin 1  
408 stability upon starvation induced autophagy<sup>31</sup>. Depletion of ATAXIN3 caused a  
409 decrease in autophagy flux as well as a decrease in the Beclin 1 levels. The drop in  
410 Beclin 1 levels was correlated with an increase in Lys48-linked ubiquitination and the  
411 Lys402 site within Beclin 1 BARA domain was shown to be a major site for ATAXIN3-  
412 mediated deubiquitination (Figure 3, Table 1). Furthermore, these proteins were shown  
413 to directly interact through Beclin 1 BARA domain and ATAXIN3 polyQ region.  
414 Interestingly, this interaction was compromised in the presence of neurodegenerative  
415 disease-causing polyQ-expanded proteins, like mutant huntingtin, resulting in the  
416 decrease in Beclin 1 levels and impaired starvation-induced autophagy<sup>31</sup>.

417

#### 418 **Proteolytic cleavage of Beclin 1**

419 Specific proteolytic events often regulate protein activity and function. Beclin 1 can be  
420 regulated by a direct cleavage of its polypeptide chain into fragments, thereby  
421 providing additional means for regulating the autophagic response. Beclin 1 cleavage  
422 by caspases has been demonstrated, and provides a direct link between autophagy  
423 and apoptosis<sup>84, 85, 86</sup>. Upon prolonged withdrawal of growth factors, treatment with pro-  
424 apoptotic compounds or overexpression of pro-apoptotic BAX, Beclin 1 was cleaved  
425 into three major fragments of 50, 37 and 35 kDa<sup>85, 86</sup>. Beclin 1 was specifically cleaved  
426 in the presence of Caspase 3, 7 and 8 and the generated fragments resulted from  
427 Caspase-3-mediated cleavage after TDVD<sup>133</sup> and DQLD<sup>149</sup> sites (Figure 3, Table 1).

428 Generated N-terminal fragments of Beclin 1 localise to the nucleus or mitochondria,  
429 enhancing cellular apoptotic response<sup>85, 86, 87</sup>. The caspase-3 mediated Beclin 1  
430 cleavage can be inhibited by treatment with spermidine, revealing a neuroprotective  
431 effect through restoration of Beclin 1-dependent autophagy<sup>88</sup>. Additionally, Beclin 1  
432 was shown to be a substrate for calpain-mediated cleavage following retinal ischemic  
433 injury in rats, although the exact cleavage site was not identified<sup>89</sup>.

434

435

#### 436 **Other post-translational modifications of Beclin 1**

437 Acetylation is an additional important post-translational modification of proteins, and  
438 proteomic studies have identified thousands of acetylated proteins in mammalian cells,  
439 including Beclin 1, where this modification appears to be an important regulatory  
440 mechanism governing Beclin 1 function in autophagosome maturation<sup>90</sup>. Beclin 1 was  
441 found to be acetylated at Lys430 and 437 by EP300 and this modification was further  
442 shown to promote the binding of Beclin 1 to RUBICON, providing a mechanism for  
443 negative regulation of autophagy<sup>90</sup>. Beclin 1 interaction with EP300 and subsequent  
444 acetylation is enhanced when Beclin 1 is phosphorylated at Ser409 by CK1 $\gamma$ 2 (Casein  
445 kinase 1 gamma 2). Finally, Beclin 1 acetylation and its increased interaction with  
446 RUBICON was shown to inhibit intracellular trafficking of autophagosomes and  
447 endocytic cargo to lysosomes for degradation<sup>90</sup>.

448

449 The ubiquitin-like type modifier, ISG15, is one of the major interferon I stimulated gene  
450 products (ISGs) and can be conjugated to the target protein (ISGylation) as part of the  
451 antiviral immune response. The ISG-conjugating enzyme, HERC5, and ISG-  
452 deconjugating enzyme, USP18, were shown to interact directly with the BH3 domain  
453 and CCD of Beclin 1 in cells stimulated with type I interferon<sup>91</sup>. The ISGylation of Beclin  
454 1 was shown to decrease the activity of the PI3K complex by competing with Beclin 1  
455 Lys63 ubiquitination which is essential for Beclin 1 activation, thereby decreasing  
456 autophagy response<sup>91</sup>. However, these findings have not been validated in other  
457 systems, and might correspond to a cell type-specific response.

458

459 The O-Linked  $\beta$ -N-acetylglucosamine (O-GlcNAc) modification is a dynamic, distinct  
460 form of protein glycosylation that can occur within the nucleus, cytoplasm or  
461 mitochondria as a response to cellular stress. Beclin 1 and BCL2 appear to be targets  
462 of O-GlcNAc modification in cardiomyocytes isolated from diabetic mice, however the  
463 impact of this modification on autophagy process remains elusive<sup>92</sup>.

464

465

466 **Future perspectives**

467 Post-translational modification of proteins has been revealed as an essential  
468 regulatory mechanism for controlling autophagy. By regulating the assembly and  
469 activity of the PI3K complex and recruitment of critical autophagy effectors, Beclin 1  
470 has emerged as a critical regulatory node and a possible point for intervention to  
471 modify autophagic flux. Pharmacological stimulation of autophagy appears to be  
472 desirable, as an increase in autophagy flux has been shown to reduce levels of harmful  
473 aggregated protein species involved in neurodegeneration<sup>93, 94, 95, 96</sup>, and targeting  
474 Beclin 1 modifications could constitute a way of achieving this. The feasibility of this  
475 approach was indicated by mouse model studies where the inhibitory interaction of  
476 Beclin 1 and BCL2 was obstructed, and these mice showed signs of increased  
477 autophagic flux, increased neuroprotection and extended lifespan<sup>45, 97</sup>. While this study  
478 was based on a genetic disruption of the Beclin1-BCL2 complex, other studies are  
479 investigating the viability of BH3 mimetics for this approach<sup>98, 99</sup>. Additional studies  
480 have developed small peptides to alter Beclin 1 interactions, and these have been  
481 proved to be sufficient to regulate autophagy in *in vivo* settings relevant for disease<sup>63,</sup>  
482 <sup>100, 101, 102, 103</sup>.

483

484 As phosphorylation of Beclin 1 Ser90 seems to be a key event for autophagy activation  
485 in many tissues, blocking or inhibiting this modification might provide a conceivable  
486 approach for developing an autophagy-modulating drug. Another approach to modify  
487 Beclin 1-dependent autophagy could be to alter levels of Beclin 1 by targeting E3  
488 ligases or deubiquitinating enzymes<sup>104, 105</sup>. The example of using the DUB inhibitor  
489 Spautin-1 have already been mentioned and, as another example, small molecule  
490 inhibitors targeting USP14 have been developed and are showing promising results in  
491 neuroprotection<sup>81, 106</sup>. However, specific targeting of modifying enzymes can be  
492 challenging as enzymes of a particular family often share similar domains and structure.  
493 Furthermore, as many enzymes have a wide range of substrates, inhibition may impact  
494 other cellular pathways. An alternative approach could be to specifically inhibit certain  
495 modifications by developing peptides or small-molecules that target a specific site on  
496 the substrate, similar to what was recently reported for blocking the phosphorylation of  
497 the apoptotic protein BAD<sup>107</sup>.

498

499 Future studies of Beclin 1 function are needed to uncover additional layers of regulation  
500 by post-translational modifications. There are several post-translational modifications  
501 such as methylation, SUMOylation, and oxidation that have been identified as

502 regulators of other key autophagy proteins<sup>108, 109, 110, 111</sup>, and prediction tools reveal that  
503 Beclin 1 might be a target for several of these modifications<sup>112</sup>. However, it remains to  
504 be determined whether any of these predicted sites are utilised *in vivo*. Another future  
505 direction in the research on post-translational regulation of autophagy would be to  
506 identify the proteins that are responsible for reversing the modifications, such as  
507 phosphatases, deubiquitinating enzymes and deacetylases. It also remains to be  
508 determined how different modifications might be affecting each other, and how  
509 regulation might diverge in different tissues and conditions.

510

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519

520

### 521 **Conflict of Interest**

522 The authors declare that they have no competing interests.

523

### 524 **Table 1: Post-translational modifications of Beclin 1.**

525 Known residues of Beclin 1 targeted for modification, the modifying enzyme and the  
526 relevance of that modification in terms of autophagy (activation/inhibition). The table  
527 also lists the autophagy-inducing conditions used and what systems/ cell models were  
528 used to establish the modification of Beclin 1, and whether the modification was  
529 confirmed *in vitro*.

530

Site	Modification	Modifier	Consequence	Conditions, cell system	Reference
<b>Ser15</b>	Phosphorylation	ULK1	Activation	Amino acid starvation, MEFs, HEK293, <i>C. elegans</i> , <i>in vitro</i> .	18
<b>Ser90</b> <b>Ser93</b>	Phosphorylation	AMPK	Activation	Glucose starvation, HeLa, MEFs, <i>in vitro</i>	53 36
<b>Thr388</b>	Phosphorylation	AMPK	Activation	Glucose starvation, HEK293T, MEFs, treatment with AMPK stimulator/inhibitor, <i>in vitro</i>	55
<b>Ser90</b>	Phosphorylation	MAPKAPK2/3	Activation	starvation (HBSS), HeLa, MEFs, <i>in vitro</i>	54
<b>Ser90</b>	Phosphorylation	DAPK3	Activation	Serum starvation, mouse tissues, HeLa, MCF7, <i>in vitro</i>	57
<b>Ser90</b>	Dephosphorylation	PP2A	Inhibition	Insulin addition, okadic acid treatment, mouse tissues, HeLa, MCF7, <i>in vitro</i>	57
<b>Ser90</b>	Phosphorylation	CaMKII	Activation	Ionomycin treatment, HEK293, <i>in vitro</i>	58
<b>Ser30</b>	Phosphorylation	PGK1	Activation	Glutamine starvation, Hypoxia, U87, BxPC-3, MDA-MB-231, <i>in vitro</i>	60
<b>Ser234</b> <b>Ser295</b>	Phosphorylation	AKT1	Inhibition	Starvation (EBSS), HeLa, <i>in vitro</i>	26
<b>Tyr229</b> <b>Tyr233</b> <b>Tyr352</b>	Phosphorylation	EGFR	Inhibition	Serum starvation, HeLa, A549	61
<b>Tyr233</b>	Phosphorylation	FAK	Inhibition	Phenylephrine treatment, Cardiomyocytes, Cos	62
<b>Tyr233</b>	Phosphorylation	HER2	Inhibition	Basal conditions, serum starvation, HER2-positive breast cancer cell lines (BT-474, SK-BR3, and MDA-MB-361)	63
<b>Thr119</b>	Phosphorylation	DAPK	Activation	Basal condition, HEK293, <i>in vitro</i>	64
<b>Thr119</b>	Phosphorylation	ROCK1	Activation	starvation (HBSS), HeLa, MEFs, <i>in vitro</i>	65
<b>Thr108</b>	Phosphorylation	MST1	Inhibition	Glucose starvation, cardiomyocytes, <i>in vitro</i>	68
-	Lys <sup>11</sup> - and Lys <sup>63</sup> -linked ubiquitination	NEDD4	Inhibition?	Basal conditions, HeLa. Role in autophagy not established.	70
-	Lys <sup>6</sup> - and Lys <sup>27</sup> -linked ubiquitination	NEDD4	Activation	Basal conditions, Torin treatment, HeLa, HEK293, MEFs	71
<b>Lys117</b>	Lys <sup>63</sup> -linked ubiquitination	TRAF6	Activation	LPS and IFN- $\gamma$ treatment, starvation (HBSS), mouse macrophages (RW264.7), primary human monocytes	59
<b>Lys437</b>	Lys <sup>63</sup> -linked ubiquitination	AMBRA1	Activation	Starvation (HBSS), MEFs, HeLa	75
-	Lys <sup>63</sup> -linked ubiquitination	TRIM50	Activation	Basal condition, starvation (EBSS), rapamycin treatment, HEK293, HeLa, MEFs	77
-	Lys <sup>63</sup> -deubiquitination	USP14	Inhibition	Serum starvation, H4	78
-	Lys <sup>48</sup> -linked ubiquitination	RNF216	Inhibition	LPS treatment, starvation (HBSS), mouse macrophages (RW264.7), HEK293T	79



-	Lys <sup>48</sup> -linked ubiquitination	KLHL20	Inhibition	Starvation (HBSS), HeLa, mouse muscle cells, HEK293T	80
-	Lys <sup>48</sup> -deubiquitination	USP10, USP13	Activation	Basal conditions, MEFs, HeLa, HEK293T, H4, Bcap-37	81
-	deubiquitination	USP9X	Activation	Basal conditions, HEK293T	83
<b>Lys402</b>	Lys <sup>48</sup> -deubiquitination	ATAXIN3	Activation	Starvation (HBSS), HeLa, primary neurons, mouse striatal-derived cells	31
-	cleavage	CASP3,6,9,10		Basal conditions, HeLa	84
<b>TDVD133</b> <b>DQLD149</b>	cleavage	CASP3,7 and 8	Inhibition	Growth-factor withdrawal (interleukin-3), pro-apoptotic compounds, mouse (Ba/F3, FDCP1) and human (U937) cell lines, HeLa	86
<b>DQLD149</b>	cleavage	CASP3	Inhibition	BAX overexpression, pro-apoptotic compounds, HeLa, SK-N-SH, MEFs	85
-	cleavage	Calpain	Inhibition	Ischemia injury, Rat retina	89
<b>Lys430</b> <b>Lys437</b>	acetylation	EP300	Inhibition	Basal conditions, HEK293T, MCF7, HeLa,	90
<b>Ser409</b>	Phosphorylation	CK1 $\gamma$ 2	Inhibition	Serum starvation, HEK293	90
<b>Lys430</b> <b>Lys437</b>	deacetylation	SIRT1	Activation	Basal conditions, HEK293T, MCF7	90
<b>Lys117</b> <b>Lys263</b>	ISGylation	HERC5/USP18	Inhibition	Type I interferons treatment, H4 cells, HepG2, HEK293T,	91
-	O-GlcNAc	-	-	Cardiomyocytes	92

531

532

533 **Figure legends**

534

535 **Figure 1: Autophagy initiation depends on ULK1 priming and PI(3)P production.**

536 Upon autophagy initiation signals, the ULK1 complex (containing ULK1 or ULK2)  
537 activates the PI3K complex, which directs PI(3)P production to a membrane site. The  
538 PI(3)P-rich membrane recruits downstream autophagy effectors, like WIPI proteins  
539 and ATG16L, which mediates the membrane conjugation of LC3. LC3 then stimulates  
540 membrane expansion and cargo recruitment into the forming phagophore.

541

542 **Figure 2: Upstream modulation of autophagy responds to nutritional cues and  
543 converges on regulation of the Beclin 1-containing PI3K complex**

544 When amino acids are abundant, mTORC1 is active and phosphorylates and inhibits  
545 the autophagy-priming complex ULK1. This prevents ULK1 from activating the PI3K  
546 complex to initiate PI(3)P production. During amino acid starvation, the inhibitory effect  
547 of mTORC1 is released and autophagy can be induced. The presence of growth  
548 factors is sensed by the AKT kinase, which activates mTORC1 and inhibits PI3K  
549 activity. Cellular energy status can also be detected via the AMP/ATP ratio, where  
550 increase in AMP activates the kinase AMPK, which activates autophagy via mTORC1  
551 inhibition, ULK1 activation, and PI3K activation.

552

553 **Figure 3: Positions of Beclin 1 post-translational modifications**

554 A-B) Linear domain structure of Beclin 1 indicating the BCL2 homology 3 domain  
555 (BH3), Helical domain (HD), coiled-coiled domain (CCD), and  $\beta/\alpha$ -repeated, autophagy  
556 related (BARA) domain. Known post-translational modifications are indicated by  
557 position and type: P = phosphorylation, Ub = ubiquitination, Ac = Acetylation ISG =  
558 ISGylation. Modifications known to induce autophagy are illustrated in A) and  
559 modifications known to inhibit autophagy are shown in B). Further details on  
560 modifications can be found in table 1.

561 C) Positions of post-translational modifications of Beclin 1. Beclin 1 is visualized  
562 oriented in the PI3K complex II (in light grey) in a structure model based on the crystal  
563 structure of the yeast PI3K complex, with the Beclin 1 homologue Atg6<sup>113</sup>. Domains  
564 important for Beclin 1 interactors are denoted in dashed lines with labels for interactors.  
565 The orientation of Beclin 1 in the complex is indicated by denotation of the C- and N-  
566 terminals. The N-terminal of Beclin 1 is intrinsically disordered and has no determined  
567 crystal structure; therefore this part is represented only by a gray surface  
568 representation without the overlay of a ribbon model. Protein structure downloaded  
569 from iCn3D web viewer<sup>114</sup>

570

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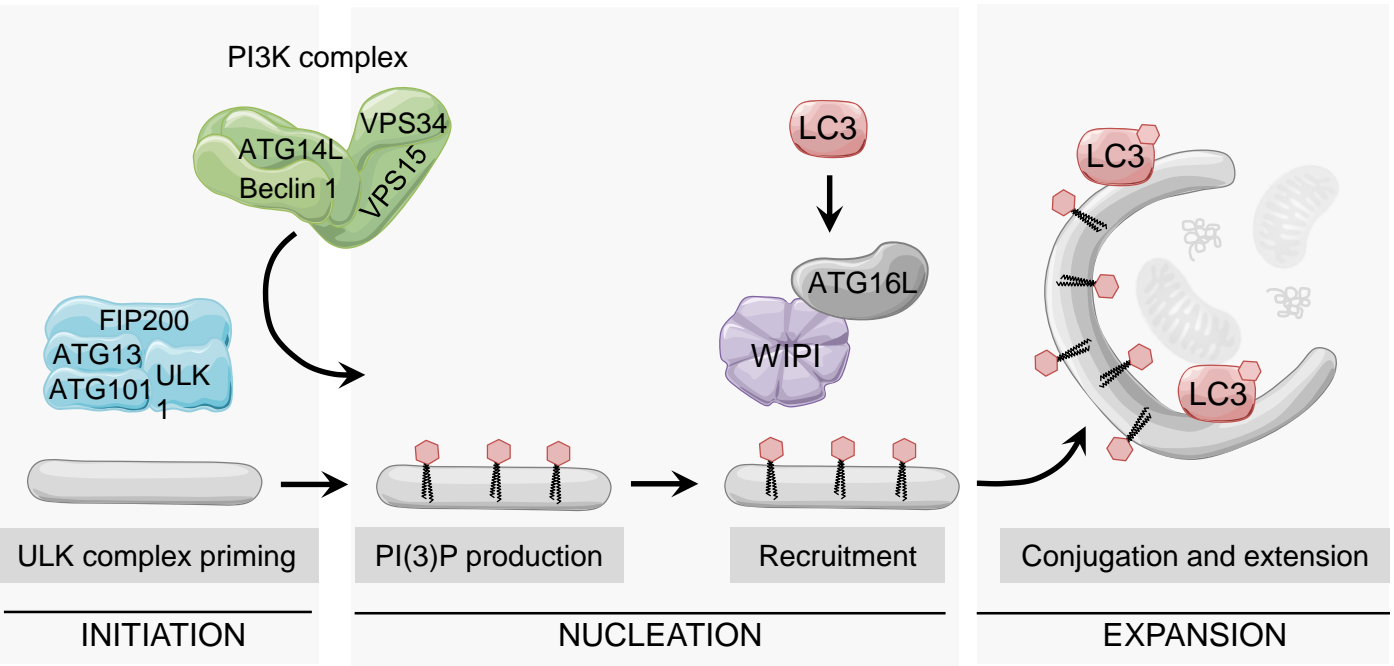


Figure 1

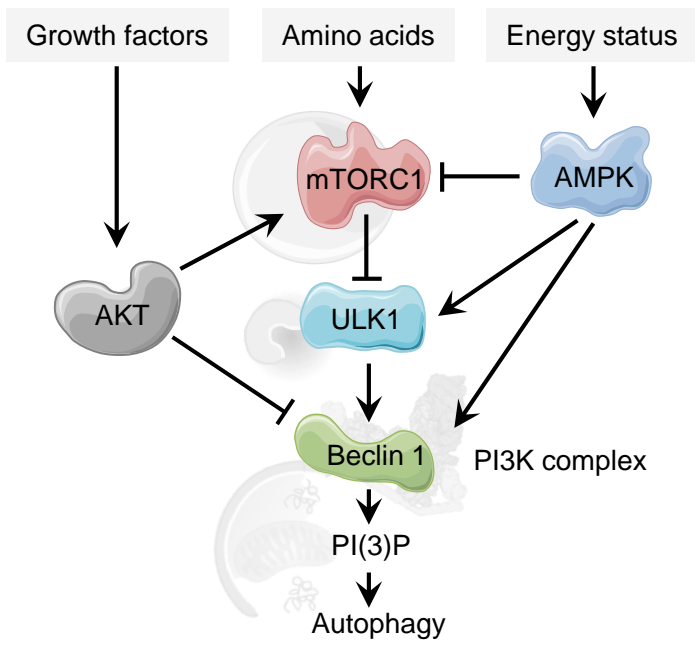
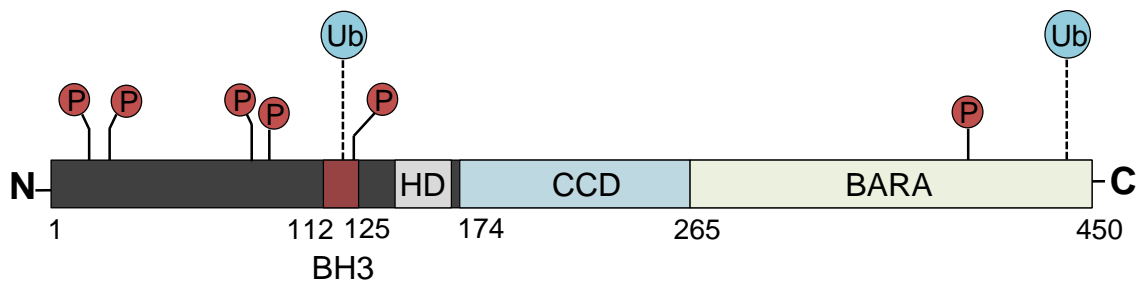
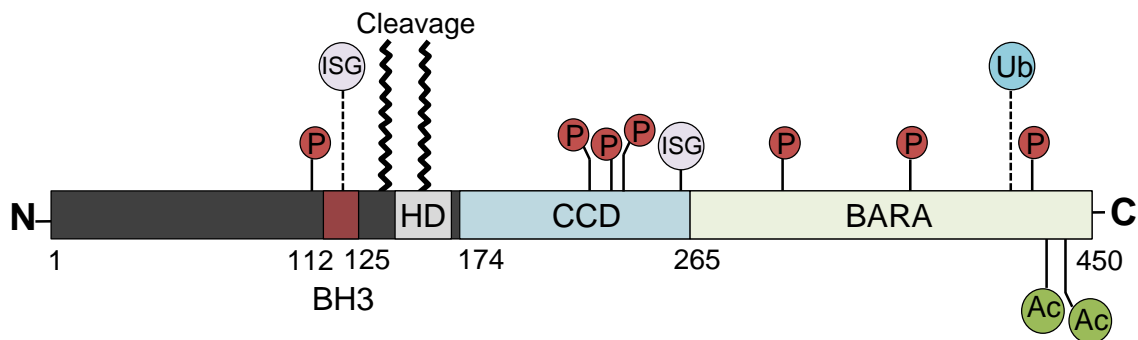


Figure 2

# A Autophagy activating PTMs



# B Autophagy inhibiting PTMs



# C

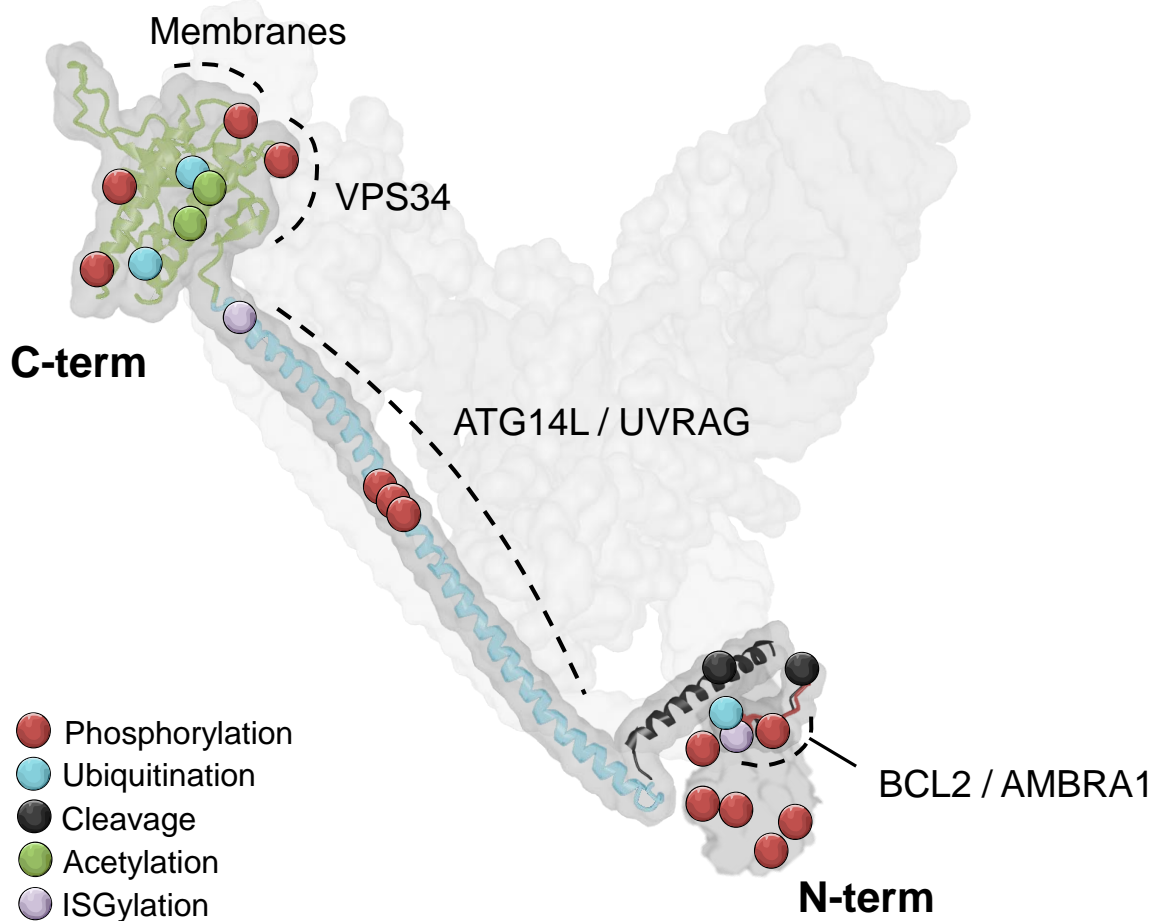


Figure 3