

1 **HIV-1 antisense protein of different clades induces autophagy and**
2 **associates to the autophagy factor p62**

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5 **Short title: Association between HIV-1 ASP and p62-driven autophagy**

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21

22 **Abstract**

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24 Over recent years, strong support argues for the existence of an HIV-1 protein encoded by
25 antisense transcripts and termed Antisense Protein (ASP). Furthermore, a recent *in silico*
26 analysis has provided evidence for its recent appearance in the genome of HIV-1. We have
27 previously detected ASP in various mammalian cell lines by Western blot (WB), flow
28 cytometry and confocal microscopy analyses and reported that it induced autophagy,
29 potentially through multimer formation. The aim of the current study was to examine
30 autophagy induction by testing ASP from different clades, and to identify the implicated
31 autophagy factors. We firstly confirmed that NL4.3-derived ASP was interacting with itself
32 and that multimer formation was dependent on its amino region. Removal of this region was
33 associated with reduced level of induced autophagy, as assessed by autophagosome formation
34 but deletion of the most amino cysteine triplet did not totally abrogate multimer and
35 autophagosome formation. Expression vectors of ASP from different clades were next tested
36 and led to detection of monomers and varying levels of multimers with concomitant induced
37 autophagy, as determined by increased LC3-II and decreased p62 (SQSTM1) levels. Through
38 confocal microscopy, ASP was noted to co-localize with p62 and LC3-II in autophagosome-
39 like cellular structures. CRISPR-based knock-out of ATG5, ATG7 and p62 genes led to
40 increased stability in the levels of ASP. Furthermore, co-immunoprecipitation experiments
41 demonstrated the interaction between p62 and ASP, which was dependent on the PB1 domain
42 of p62. Interestingly, immunoprecipitation experiments further supported that ASP is
43 ubiquitinated and that ubiquitination was also responsible for the modulation of its stability.

44 We are thus suggesting that ASP induces autophagy through p62 interaction and that its
45 abundance is controlled by autophagy- and Ubiquitin/Proteasome System (UPS)-mediated
46 degradation in which ubiquitin is playing an important role. Understanding the mechanisms
47 underlying the degradation of ASP is essential to better assess its function.

48 **Author Summary**

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50 In the present study, we provide the first evidence that a new HIV-1 protein termed ASP
51 when derived from different clades act similarly in inducing autophagy, an important cellular
52 process implicated in the degradation of excess or defective material. We have gained further
53 knowledge on the mechanism mediate the activation of autophagy and have identified an
54 important interacting partner. Our studies have important ramification in the understanding of
55 viral replication and the pathogenesis associated with HIV-1 in infected individuals. Indeed,
56 autophagy is implicated in antigen presentation during immune response and could thus be
57 rendered inefficient in infected cells, such as dendritic cells. Furthermore, a possible link with
58 HIV-1-associated Neurological Disorder (HAND) might also be a possible association with
59 the capacity of ASP to induce autophagy. Our studies are thus important and demonstrate the
60 importance in conducting further studies on this protein, as it could represent a new
61 interesting target for antiretroviral therapies and vaccine design.

63 **Introduction**

64

65 The genome of HIV-1 expresses essential genes for its replication, which include *gag*, *pol*
66 and *env*, common to all replication-competent retroviruses in addition to the genes encoding
67 regulatory proteins (Tat and Rev) crucial for virus replication. Furthermore, HIV-1 is known
68 to encode a number of accessory proteins, i.e. Vpu, Vpr, Vif, and Nef, all of which are
69 suggested to be implicated in the inactivation of restriction factors and the modulation of
70 immune functions (1-6). A former study had however demonstrated that HIV-1 isolates
71 contained a conserved open reading frame (ORF) on the antisense strand of the *Env* gene,
72 thereby hinting toward the existence of a tenth gene with a encoding potential for a 189 amino
73 acid protein (7, 8). A recent study has further underscored the high degree of conservation of
74 this ORF in most HIV-1 clades, but further argued that this gene correlated with the spread of
75 the virus, being less present (or absent) in most SIV, HIV-1 groups N, O and P and less
76 prevalent HIV-1 clades (9). Importantly, a number of previous and more recent studies have
77 further confirmed that antisense transcription overlapping this ORF sequence was detected in
78 transfected and chronically infected cells (10-19).

79 The existence of the presumed encoded protein termed ASP (Antisense Protein) in
80 infected patients has been further suggested by several groups through detection of specific
81 antibodies and CD8+ T cell-mediated immune responses (10, 20-22). However, ASP
82 detection itself has proven to be more difficult and was first analyzed by *in vitro* translation
83 and electron microscopy in transfected and chronically infected cells (10, 15). More recent
84 studies have provided a better understanding of this protein in terms of its potential membrane

85 localization and its biased expression in monocyte-derived macrophages and dendritic cells
86 (7, 17). In another recent report, we have been able to clearly detect the ASP protein for the
87 first time by Western blot and various other approaches (confocal microscopy and flow
88 cytometry) in transfected cell lines (23). Furthermore, our work further suggested that ASP
89 was unstable, formed multimers and induced autophagy, likely through the formation of these
90 high molecular weight complexes.

91 Autophagy, a major cellular degradation pathway, plays an important role in
92 developmental processes, cellular stress responses, and immune pathways induced by
93 pathogens. The early step of what is known as macroautophagy initiates through mTOR
94 inhibition and ensuing formation of an active autophagy complex consisting of the
95 phosphorylated Beclin-1 factor, normally negatively inhibited by its binding to Bcl-2 (24-27).
96 Following this activation, a protrusion of two lipid-based structures termed the isolation
97 membrane and the omegasome is induced intracellularly to form the initial opened structure
98 termed the phagophore (28). The phagophore further elongates circularly and finalizes the
99 maturation process of the autophagosome, in which are trapped cytosolic elements targeted
100 for degradation (27). Final fusion of the autophagosome with lysosomes leads to degradation
101 of its content and recycling of amino acids and other constituents (29, 30). Different proteins
102 play an active role in autophagy, such as phagophore-associated Autophagy-related-genes
103 (ATG) ATG5, ATG7, ATG10 ATG12 and ATG16 (31). One of the classical autophagy
104 marker is LC3-II, a phosphatidylethanolamine-modified form of LC3-I form, which is
105 embedded in the autophagosome membrane and contribute to its formation. Another
106 implicated autophagy marker is p62/SQSTM1, a scaffold protein, which links aggregated

107 complexes to LC3 and consequently becomes itself degraded.

108 Autophagy can either be beneficial or detrimental toward viral replication; the outcome of
109 induced autophagy depends on the virus, the cell type, and the cellular environment (32). In
110 fact, for HIV-1, initial studies in CD4+ T cells had shown that the envelope gp41 subunit
111 induced autophagy-dependent apoptosis of uninfected cells (33, 34). However, a genomic
112 screen has also identified certain autophagy-related host factors, which were rather essential
113 for HIV-1 infection (35). Furthermore, in macrophages specifically, HIV-1-induced
114 autophagy greatly improved gag pr55 processing and particle production (36). Importantly,
115 Nef has been reported to block the process of autophagy at late stages (fusion with lysosomes)
116 and avoid intracellular degradation of viral particles (5, 37). More recent data has also
117 demonstrated a potential role for other HIV-1 proteins in regulating autophagy, such as Vif
118 (38, 39).

119 Although previously associated to the proteasome degradation pathway, ubiquitination,
120 the covalent conjugation of ubiquitin to proteins, has become of high relevance to autophagy
121 (40-43). The C-terminus of ubiquitin is covalently link to the target protein by specific lysine
122 residues (e.g. K48, K63), which subsequently leads to polyubiquitination. While K48- and
123 K29-linked polyubiquitination has been shown to be optimal for degradation through the
124 proteasome, other types of lysine-linked polyubiquitinations (e.g. on K63, K11, K6) and
125 monoubiquitination may regulate processes such as autophagy, translation and DNA repair
126 (43). In fact, ubiquitinated proteins can be recognized by the autophagy pathway through
127 specialized adaptor proteins, also called sequestosome-1 like receptors (including
128 p62/SQSTM1), Optineurin and NDP52, among others). The p62 protein can multimerize via

129 its PB1 domain (NH₂-terminal Phox and Bem1p domain) and bind LC3-II via its LC3-
130 interacting region (LIR) and ubiquitinated proteins through the phosphorylation of its
131 ubiquitin-associated domain (UBA) (42-44).

132 Based on our previous report linking ASP to autophagy, in the current study, we aimed at
133 examining the mechanism behind ASP-induced autophagy. Our results demonstrate that
134 multimerization of ASP and induced autophagy partly involves its amino end. We show that
135 ASP from various HIV-1 clades all induced autophagy, as determined by LC3-II levels and
136 p62 degradation. We further highlight that ASP co-localized and interacted with p62 through
137 its PB1 domain and finally present data supporting that ASP is ubiquitinated, further
138 contributing to its targeting toward degradation pathways.

139 **Results**

140

141 **Analyses of ASP multimers.** A limited number of studies have shown that ASP could be
142 detected in infected and transfected cells (15, 23). Using a codon-optimized ASP expression
143 vector, we have successfully detected the ASP protein in transfected mammalian cells by
144 several technical approaches, and concomitantly, demonstrated that it was forming multimers
145 and could induce the formation of autophagosomes, thereby providing an explanation for its
146 difficult detection (23). Herein, we sought to further define the mechanism leading to
147 autophagy induction by ASP and to extend these analyses to ASP from different HIV-1
148 clades.

149 We were first interested in confirming the self-multimerization potential of ASP using
150 expression vectors for Myc-tagged and chimeric GFP-optimized ASP (Fig. 1). Extracts from
151 co-transfected 293T cells were immunoprecipitated with anti-Myc antibodies and
152 subsequently analysed in parallel with total extracts by Western blot. As depicted in Fig. 1A,
153 a specific signal was detected corresponding to the GFP-tagged ASP in immunoprecipitated
154 extracts, which could not be accounted by significant difference in expression of the fusion
155 protein in the different analysed samples. Similar co-transfection experiments were conducted
156 in COS-7 cells and again revealed that both ASP proteins were associated (Fig. 1B). As
157 cysteine-rich region of the protein might mediate these high molecular complexes, we
158 pretreated the extract from Myc-tagged-ASP expressing COS-7 cells with high concentrations
159 of reducing agents, DTT and β -mercaptoethanol. Upon treatment, the high molecular signals
160 were strongly diminished with either agent and resulted in increased intensity of the 20 and

161 200kDs signals. Very high concentration of DTT led to disappearance of the high molecular
162 weight signals (Fig. 1C)

163 In light of these results, a series of deletion mutants were next generated, in which the
164 first 15 and 30 amino acid residues from the amino or carboxyl end of Myc-tagged ASP were
165 deleted. Importantly, pMyc-optimized-ASP Δ N1-15 and pMyc-optimized-ASP Δ N1-30 vectors
166 permitted the expression of an ASP mutant, from which 4 and 7 conserved cysteine residues
167 were removed, respectively (Fig. 2A). Upon transfection of COS-7 cells, flow cytometry
168 analyses showed that mutants and wild-type ASP were detected at comparative levels, except
169 for mutant pMyc-optimized-ASP Δ N1-30, which showed a reduced signal (Fig. 2B). The
170 multimerization potential of these mutants were next analysed by Western blot (Fig. 2C).
171 Interestingly, the loss of the first 15 residues had an important effect on the presence of high
172 multimers, and led to a modest increase in the abundance of the monomer, while no such
173 effect was observed with mutants bearing deletion at their COOH end. The ASP Δ N1-30
174 mutant presented reduced signal for both monomeric and high molecular weight signals when
175 compared to wild-type-expressing cells, which was likely due to reduced overall abundance
176 of the protein. Confocal microscopy analyses were next performed in transfected cells (Fig.
177 2D). Deletion of the first 15 amino acids reduced (but not completely abolished) the presence
178 of punctuated ASP signals, previously having been identified as autophagosomes. Deletion of
179 the carboxyl end did not lead to differences in the distribution of the resulting ASP mutant
180 from the wild-type version (Fig. 2D). Another mutant in which the conserved PXXP domain
181 (position 47-53) was also tested in transfected 293T cells and revealed no clear change in
182 multimer formation or induction of autophagy (data not shown).

183 Since a cysteine triplet is present in the first 15 amino acid region, we specifically
184 deleted these cysteine residues (Fig. 3A). Flow cytometry indicated that the resulting mutant
185 was detected at levels equivalent to those of optimized-ASP WT in transfected COS-7 cells
186 (data not shown). Expression of the optimized ASP Δ CCC mutant in COS-7 cells resulted in a
187 typical punctuated distribution in the cytoplasm (Fig. 3C), while Western blot analyses
188 demonstrated that multimerization capacity of ASP Δ CCC appeared less pronounced with a
189 concomitant increase in the monomeric signal, suggesting that this cysteine triplet was
190 contributing to ASP multimer formation but that other cysteine (and possibly non-cysteine
191 residues) were implicated in ASP multimerization and autophagy induction (Fig. 3B). We
192 further tested the amino deletion mutants and the cysteine deletion mutants in 293T cells and
193 confirmed in a clearer manner that the amino end and cysteine residues were affecting ASP
194 multimer formation and protein levels (Fig. S1).

195 In light of these results, we conclude that the amino terminus (and cysteines) of ASP
196 contribute to its capacity to multimerize, and is partly implicated in its autophagy-inducing
197 properties.

198
199 **Expression and detection of ASP from various clades.** In order to better assess and
200 understand the mechanism leading to ASP-mediated autophagy in a more representative
201 manner, we next generated expression vectors for His-tagged ASP representing different
202 HIV-1 clades. Importantly, these ASP ORFs were directly derived from the original proviral
203 DNA sequence and were not codon-optimized, as we had previously performed in our earlier
204 study (23). Sequence comparison of the ASP genes tested in this study is presented in Fig. 4A

205 and highlights the previously observed absence of the first 25 amino acid in clade A ASP (9).
206 This thereby allowed us to assess the multimer formation and the autophagy-inducing
207 capacity of an ASP version naturally missing the typical amino end. Following transfection in
208 293T cells, we first used our monoclonal anti-ASP antibody derived against the epitope
209 indicated in Fig. 4A to determine if ASP could be detected by Western blot. As depicted in
210 Fig. 4B, despite variation in the amino acid sequence of the epitope in between tested ASP,
211 we demonstrated the presence of the expected 20 kDa signal (albeit with some variation in
212 molecular weight and intensity between clades). Clade A ASP was detected at a lower
213 molecular weight signal in comparison to other clade ASP, except for the 92NG083 ASP.
214 This signal was not due to preferential usage of the internal methionine residue, as
215 demonstrated by the similar monomeric signals observed for all tested ASP upon analyses
216 with an anti-His antibody (Fig. 4C) but could be accounted by lower amino acid number of
217 this ASP in the variable COOH end region. In addition, high molecular weight signals
218 including important aggregates were detected in cells transfected with the different ASP
219 expression vectors. Interestingly, clade A showed a more abundant monomeric form when
220 compared to the intensity of the multimers versus ASP from other clades. A similar behavior
221 was noted for the clade A/G ASP representative (92NG083).

222 These results hence showed that His-tagged ASP from different clades can be detected
223 with anti-ASP and anti-His antibodies. Furthermore, these results confirmed that multimer
224 formation likely involved the amino end, but that, based on the variation of multimer
225 intensities among various tested ASP (including results with the clade A/G representative),
226 other regions of ASP are affecting the extent of multimer formation.

227

228 **Induction of autophagy by ASP from different HIV-1 clades.** Since the expression of all
229 tested ASP could be detected and led to variation in the extent of multimer formation, we
230 were next interested in determining if these different ASP proteins could indeed induce
231 autophagy. Based on our previous results showing that levels of the lipid-modified
232 microtubule-associated protein 1 light chain 3 (LC3-II), a well-known autophagy marker, was
233 increased in 293T and COS-7 cells expressing ASP (23), we used this same marker to
234 evaluate the autophagy-inducing capacity of ASP from different clade representatives. As
235 depicted in Fig. 5A, the presence of ASP was again confirmed for all transfected ASP vectors
236 and was detected as monomers and multimers. Importantly, ASP from the different clades all
237 increased levels of LC3-II, albeit to different extent in transfected 293T cells. Furthermore,
238 based on these Western blot analyses, clade A ASP 94CY032 seemed equally capable of
239 inducing autophagy, when compared to other ASP expression vector. We then sought to
240 validate that this increase in LC3-II levels was related to autophagy induction and not
241 inhibition of the late step of the autophagy, both of which would lead to increase in the
242 abundance of LC3-II levels. Hence, transfected 293T cell were treated with Bafilomycin A1,
243 a blocking agent of lysosomal degradation. In these conditions, ASP-dependent induced
244 levels of LC3-II should be maintained if ASP led to induced autophagy, while no such
245 increase should be noted if ASP inhibited the last stage of autophagy. In Figure 5B, Western
246 blot analyses revealed that LC3-II levels were indeed increased upon Bafilomycin A1
247 treatment, as expected but that ASP-driven induction was also evidenced. LC3-I/LC3-II ratios
248 in ASP-expressing cells were calculated and shown to be higher than for similarity treated

249 cells (-/+ Bafilomycin A1) transfected with the empty vector.

250 Since p62 degradation can also be used as a marker of autophagy, we conducted
251 additional Western blot analyses in 293T cells transfected with increasing quantity of the
252 clade B Bal ASP expression vector. A dose-dependent decrease in p62 levels was confirmed
253 upon ASP expression, further confirming induced autophagy (Fig. 5C). Expression vectors of
254 ASP from different clades further revealed reduced levels of p62 in transfected cells when
255 compared to empty vector-transfected cells (Fig. S2). As a further indication of autophagy
256 being induced by these different ASP-expressing vectors, transfected COS-7 cells were
257 analyzed by confocal microscopy using anti-Myc antibodies. Analyses revealed the
258 characteristic presence of cytoplasmic punctuated ASP (including cells expressing clade A
259 ASP), which is reminiscent of autophagosomes (Fig. 5D).

260 These results confirmed that ASP from different clades induced autophagy and that no
261 striking differences were noted in between clades, especially with respect to clade A ASP.
262 They further confirmed that ASP induced autophagy, and do not suggest an inhibitory action
263 of ASP on late stage o autophagy leading to degradation of autophagosome content.

264

265 **Knockout of autophagy factors increase the abundance of ASP.** Using inhibitors of early
266 and late steps of autophagy, we have previously demonstrated that the abundance of codon-
267 optimized NL4.3 ASP increased (23). In order to further confirm the involvement of
268 autophagy in the regulation of ASP, we first conducted targeted knock-out of the ATG5 and
269 ATG7 genes using the CRISPR/Cas9 system by lentivirus-mediated transduction (Fig. 6).
270 293T cells were first stably transduced with lentivirus expressing selective sgRNA and

271 following puromycin selection, two clones per knock-out were subsequently transfected with
272 the His-tagged NL4.3 ASP expressing vector. Resulting extracts were analyzed for ASP,
273 ATG5 and ATG7 expression. Knock-out of ATG5 or ATG7 through targeting of two
274 different regions resulted in the reduced levels of the ATG5-ATG12 complex, although more
275 limited for clone sgATG5-1 and, of ATG7, respectively (Fig. 6A and B). Importantly, His-
276 tagged ASP levels increased in these clones, with a more increased abundance associated with
277 lower expression levels of the targeted gene.

278 The results thereby confirmed that ASP levels in transfected cells were modulated by
279 autophagy, and that typical autophagy factors, i.e. ATG5- ATG12 and ATG7, were
280 implicated.

281

282 **ASP interacts with autophagy factor p62.** Given that all tested ASP could induce
283 autophagy, we next investigated on the possible mechanism of action. We first focused our
284 attention on determining whether ASP could associate and co-localize with specific
285 autophagy factors by confocal microscopy in different cell lines (Fig. 7). As we have
286 previously reported (23), we first confirmed that expression of NL4.3 ASP in 293T cells
287 showed association to LC3 upon co-immunoprecipitation with anti-ASP followed by Western
288 blot analyses (Fig. 7A). In addition, ASP was confirmed to partially co-localize with LC3-II
289 in both COS-7 and HeLa cells (Fig. 7B and data not shown). We next performed confocal
290 microscopy experiment to identify potential co-localization with the important autophagy
291 protein p62. Our results revealed that a clear co-localization between ASP and p62/SQSTM1
292 was observed in both HeLa and 293T cells (Fig. 7C).

293 Since ASP forms multimers and that p62 is an important cargo transporter of
294 multimerized proteins toward the autophagy pathway, we next tested whether ASP and
295 p62/SQSTM1 could indeed associate. 293T cells were transfected with expression vectors of
296 His-tagged ASP of different clades and resulting extracts were used for co-
297 immunoprecipitation with an anti-His antibody (Fig. 7D). Upon Western blot analyses, p62
298 indeed could co-immunoprecipitate with ASP in transfected 293T cells. To confirm these
299 data, immunoprecipitation was performed in similarly transfected 293T cells with anti-p62
300 antibodies and analyzed with anti-His antibodies (Fig. 7E). These analyses confirmed the
301 association between ASP and p62. The specificity of this interaction was further demonstrated
302 in transfected 293T cells by the lack of interaction of ASP with the other autophagy factors,
303 ATG5 and ATG7 implicated in the ASP-induced autophagy cascade (Fig. S3). The impact of
304 this association between ASP and p62 was further tested through knockout of the p62 gene in
305 293T cells. Although two different targeted region were tested, only one strategy (sgp62-1)
306 provided sufficient reduction in p62 abundance. Knockout cells were thus transfected with an
307 ASP expression vector and analyzed by Western blot. These analyses indicated that reduction
308 in p62 levels led to a marked increase in ASP abundance (Fig. 7F).

309 These data thus indicated that p62 co-localized and associated to the different ASP clade
310 representatives, and that this association might consequently lead to induction of autophagy.

311

312 **Interaction domain of p62 and ASP ubiquitination.** We next sought to identify the
313 domain of p62, which was responsible for its association with ASP. These domains have been
314 previously shown to interaction with ubiquitinated protein, LC3 and p62 itself, and termed

315 UBA, LIR and PB1, respectively (Fig. 8A). Various p62 mutants deleted for these various
316 domains and fused to the GST protein were thus co-transfected with Myc-tagged NL4.3 or
317 94CY032 (clade A) ASP expression vector in 293T cells and resulting extracts were co-
318 immunoprecipitated with anti-GST antibodies (Fig. 8B). Upon Western blot analysis of
319 immunoprecipitated extracts, interaction of ASP (monomeric and multimeric) was shown to
320 be importantly reduced in cells expressing the PB1 domain-deleted p62 mutant (Fig. 8B).
321 Expression of other mutants (deleted for LIR or UBA regions) led to retention of ASP in
322 extracts co-immunoprecipitated with anti-GST antibodies, thereby suggesting that these other
323 domains were not crucial for the association between ASP and p62. Similar results were
324 observed in immunoprecipitation experiments following co-transfection of the various p62
325 mutants and a His-tagged NL4.3 ASP expression vector in 293T cells upon Western blot
326 analyses (Fig. S4).

327 Since p62/SQSTM1 has been often implicated in ubiquitination-dependent autophagy, it
328 was surprising that the association between ASP and p62 did not involve the ubiquitinating
329 interacting domain UBA. We were thus interested in determining if ASP was nonetheless
330 ubiquitinated. Immunoprecipitation experiments were thus conducted in 293T cells co-
331 transfected with two different His-tagged ASP expression vectors (from proviral DNA clade
332 B NL4.3 and clade A 94CY032) and pRK5-HA-Ubiquitin-KOR (expressing an HA-tagged
333 ubiquitin protein limiting targeted proteins to monoubiquitination). Upon transfection of 293T
334 cells and immunoprecipitation with anti-His antibodies, Western blot analyses revealed that
335 ASP was indeed ubiquitinated, as revealed by the expected size of a ubiquitinated monomeric
336 ASP form in both clade A and clade B expressing cells (Fig. 8C). In addition, high molecular

337 weight multimers were detected in immunoprecipitated extracts. Interestingly, for both tested
338 ASP expression vectors, levels of the non-ubiquitinated form was also importantly increased
339 in transfected cells expressing ubiquitin KOR, likely suggesting that these might represent
340 non-ubiquitinated ASP protein composing ASP multimers targeted for ubiquitin-dependent
341 degradation. In fact, high molecular signals were also importantly more abundant in KOR
342 expressing cells. Further experiments using HA-tagged ubiquitin expression vectors
343 selectively producing K48 (UPS-prone) or K63 (autophagy-prone) polyubiquitination revealed
344 that ASP was targeted by both of these modifications (data not shown).

345 Together, these data demonstrated that, despite the implication of the homodimerization
346 domain of p62 in its association with ASP, ASP is ubiquitinated, which impacts its abundance
347 in its monomeric and multimeric forms.

348

349 **Discussion**

350

351 Antisense transcription in HIV -1 has been described and characterized in a number of studies
352 (18). However, the encoded ASP protein remains a limited focus of investigation. We have
353 formerly detected ASP in different cell types by various technical approaches and further
354 demonstrated that this viral protein led to the induction of autophagy (7, 17, 23). In the
355 current study, we demonstrate that ASP-associated autophagy is induced in all tested clade-
356 derived ORF and is associated to p62 via its oligomerization domain. Furthermore, ASP was
357 shown to be ubiquitinated, which impacted on its sensitivity toward degradation.

358 Based on our earlier studies linking ASP-induced autophagy to its multimerization
359 capacity, we pursued our analysis of the ASP multimers and confirmed that ASP is indeed
360 part of multimers by showing the interaction between two differently tagged ASP. As cysteine
361 residues can mediate multimerization, denaturing agents were tested and indeed reduced the
362 formation of the aggregates. Furthermore, removal of the cysteine-rich amino region of ASP
363 led to similar lower abundance of detected high molecular signals with an increase in the
364 monomeric version, especially noted in COS-7 cells. Interestingly, as expected, the amino-
365 truncated version was also less prone to autophagy induction. It is not clear why increase in
366 the abundance of amino-truncated ASP mutants was not as clearly seen in 293T cells,
367 although higher selective degradation of the codon-optimized NL4.3 ASP monomers targeted
368 by either degradative pathways in this cell type might account for these differences. However,
369 differences in the levels of monomeric form of ASP deleted at their amino end was further
370 supported by the lower abundance of multimers in cells expressing clade A ASP lacking the

371 first 25 amino acid cysteine-rich region. These results argue that cysteine residues are
372 implicated in the formation of these ASP multimers, which is reminiscent of other HIV-1
373 proteins (45, 46). Although our results argue that the cysteine triplet (position 10-12) are
374 implicated in ASP multimer formation, we have not fully investigated on which cysteines are
375 important but it is highly probable that various cysteine residues are involved, some of which
376 could also lie outside of the tested regions. Furthermore, since autophagy was not completely
377 abrogated in cells expressing amino deleted version of NL4.3 ASP, we speculate that other
378 highly conserved regions (9) are likely to be involved in the multimerization of ASP.

379 In this study, a more representative assessment of the autophagy-inducing capacity was
380 assessed through expression vectors of various ASP from various clade representatives of
381 clinical isolates and laboratory strains. We first demonstrated for the first time that we can
382 detect ASP from those different isolates at the expected molecular weight in transfected cells.
383 Again, high molecular weight signals and multimers were prevalent in these analyses, albeit
384 at different abundance, a variation potentially accountable by the absence of the cysteine-rich
385 amino end in the case of clade A. Despite variation in multimer formation, all tested ASP
386 induced autophagy, as determined by variation in the levels of p62 and LC3-II levels and by
387 the presence of autophagosome-like signals by confocal microscopy. Although we have not
388 tested all known clades (having limited out analyses to most frequent ones), our data argue for
389 a conserved capacity of ASP from the different clade families to induce autophagy and that
390 low abundance of multimers for certain ASP is nonetheless sufficient for this induction.

391 Our results thereby strongly suggest that ASP multimers in the form of potential
392 aggregates induces autophagy and acts upon ASP abundance. This parallels similar

393 aggregating proteins, known to be targeted for degradation by this pathway (47, 48). We have
394 further confirmed the implication of autophagy on the abundance of ASP by targeting known
395 autophagy factors through CRISPR-mediated deletion. Although certain selected clones still
396 expressed varying levels of target protein, (likely reminiscent of either more than one clone
397 and/or single allele inactivation), resulting ASP levels (monomer and multimers) were
398 strongly affected and their increase in abundance inversely correlated with the remaining
399 levels of the targeted protein. We have further looked at the potential mechanisms of
400 autophagy induction by ASP and through localisation and immunoprecipitation experiments,
401 an association between ASP and p62 was detected, being dependent on the PB1
402 homodimerization domain, but not affected in a p62 mutant deleted for its ubiquitin-
403 interacting UBA region. Interestingly, this was comparable to previous studies, in which viral
404 proteins could interact with p62 via a ubiquitin-independent manner (49, 50). In fact, HIV-1
405 Tat is one of these proteins and is selectively degraded through its association to p62 via
406 autophagy in an ubiquitin-independent manner.

407 These latter results are unexpected given that other experiments have revealed that ASP is
408 ubiquitinated and that such post-translational modification contributes to ASP degradation, as
409 demonstrated by the use of the ubiquitin mutant preventing polyubiquitination. Expression of
410 this polyubiquitination-blocking ubiquitin protein indeed led to an important increase in
411 abundance of the non-ubiquitinated monomeric and multimeric forms of ASP, the former
412 likely resulting from their its in multimer complex and subsequent release upon cell extract
413 preparation. Hence, these multimers being composed of non-ubiquitinated and ubiquitinated
414 ASP are thus subject to degradation by autophagy through their interaction with p62.

415 Although it is not clear how ASP triggers autophagy, the recent publication of Lu *et al.* might
416 shed light on the exact mechanism behind ASP-dependent induction of autophagy. The
417 authors indeed argued that association of ubiquitinated multimeric proteins to p62 with
418 subsequent induced autophagy is more dependent on the formation of aggregates than
419 ubiquitination itself (51). In this perspective, it is likely that the association of ASP with p62
420 depends on its oligomeric form and that the interaction with the ubiquitin-interacting UBA
421 domain might not be detectable in our immunoprecipitation experiments as the association is
422 more dependent on the multimeric form of ASP than the affinity toward ubiquitin conjugates.
423 This is thus different from the previously suggested view that autophagy vs. UPS is mainly
424 dependent on the type of linked ubiquitin in the polyubiquitination chain (K48 vs. K63) (52) .
425 Our results in fact argue that both types of modification are present in monomeric and
426 multimeric forms of ASP (data not shown).

427 It is well known that autophagy is primarily responsible for the degradation of most long-
428 lived proteins in cells, but also targets aggregated proteins as well as cellular organelles and
429 infectious organisms (53). The importance of autophagy in modulating the abundance of ASP
430 might be crucial to maintain its abundance at low level, as this protein is likely detrimental to
431 cell survival (if in high excess), especially given its propensity to form important multimers.
432 In addition, the capacity of ASP to function as an inducer of autophagy also depends on other
433 concomitantly expressed viral proteins, which have been associated to this degradation
434 pathway (39). As previously reported, Nef can inhibit autophagy at late steps of viral
435 replication in macrophages and could thus block ASP-induced autophagy (5, 36). Another
436 interesting aspect of our studies relate to the essential role played by autophagy in antigen

437 presentation in cells, such as dendritic cells. Infected DC might thus be altered in their
438 capacity to adequately process and present antigen, especially knowing that our previous
439 study showed that ASP presented the highest expression in this cell type when compared to
440 macrophages and activated CD4⁺ T cells (17).

441 Overall, our results demonstrate that the autophagy-inducing properties of ASP are
442 observed with different clades ASP and that multimerization acts on autophagy induction.
443 Our results further indicate that the association of p62 to ASP is likely essential for ASP and
444 that ASP is ubiquitinated, also affecting the stability of the monomeric and multimeric forms
445 of ASP. The body of results thereby helps to better appreciate the unstable nature of ASP in
446 mammalian cells. The function of ASP in HIV-1 infection *in vivo* has yet to be defined but
447 our results suggest that autophagy could link ASP to HIV-mediated pathogenesis and the
448 chronic infection state. Future studies will be determinant in providing more information on
449 these potential roles.

450 **Materials and Methods**

451

452 **Plasmids**

453 Expression vectors for the Myc-tagged optimized ASP and GFP-ASP optimized fusion
454 proteins have been previously described (23). A series of deletion mutants (15 and 30 amino
455 acids at amino or carboxyl ends) and internal deletion of cysteine triplet or PXXPXXP motif
456 were generated by PCR from Myc-tagged optimized ASP expression vector using primers
457 presented in Table S1. These resulted in the constructs termed pMyc-optimized-ASP Δ N1-15,
458 pMyc-optimized-ASP Δ N1-30, pMyc-optimized-ASP Δ C174-189, pMyc-optimized-
459 ASP Δ C159-189, pMyc-optimized-ASP Δ ¹⁰CCC¹² and pMyc-optimized-ASP Δ PXXP. Proviral
460 DNA p94CY032 (clade A), NL4.3 (clade B), JR-FL (clade B), 89.6 (clade B), MJ4 (clade C),
461 p92NG003 (clade G) and p92NG083 (clade G) were obtained through the NIH AIDS Reagent
462 Program (Germantown MD) while proviral DNA Mal (clade D) and IndieC1 (clade C) were
463 provided by Dr. Anne Gatignol (McGill University, Montreal, Canada). DNA fragments
464 encoding His-tagged antisense protein (ASP) were amplified by PCR from these proviral
465 DNA or the Bal (clade B) envelope expression vector (provided by Dr. Michel J. Tremblay,
466 Laval University, Quebec city, Canada) using specific forward and reverse primers bearing
467 NotI and BamHI restriction site, respectively at their 5' end. After NotI/ BamHI digestion,
468 PCR products were inserted into the vector pTT5 (Youbio Inc. China: #VT2202,), which was
469 similarly digested. CRISPR-Cas9 knockout plasmids (LentiCRISPRv2, Addgene: #98290 and
470 psPAX2, AddGene: #12260) were constructed as previously described (54, 55). Pairs of
471 oligonucleotides were designed for specificity toward targeted genes and are listed in Table

472 S2. Annealing of each pair further led to the generation of BsmBI restriction sites, which were
473 used to insert the resulting dimerized oligonucleotide in the BsmBI-digested LentiCRISPRv2
474 vector. Plasmids pDEST-GST-P62, pDEST-GST-P62- Δ LIR, pDEST-GST-P62- Δ UBA and
475 pDEST-GST-P62- Δ PB1 were kindly provided by Dr. Lucile Espert (Université Montpellier,
476 Montpellier, France) and express wild-type and various deletion mutants of p62 (56). The
477 plasmids pRK5-HA-Ubiquitin-WT (Addgene: #17608), pRK5-HA-Ubiquitin-KOR, pRK5-
478 HA-Ubiquitin-K48 and pRK5-HA-Ubiquitin-K63 and the empty vector (Addgene #17605)
479 were gifts from Dr. Ted Dawson (Johns Hopkins University School of Medicine, Baltimore
480 MD) (57). The pRK5-HA-Ubiquitin WT vector expresses HA-tagged wild-type ubiquitin,
481 while pRK5-HA-Ubiquitin-KOR expresses a ubiquitin version in which all lysines were
482 mutated to arginine. pRK5-HA-Ubiquitin-K48 and pRK5-HA-Ubiquitin-K63 encode for
483 ubiquitin-mutated versions in which lysine 48 and 63 respectively have been preserved, the
484 other lysine residues having been mutated to arginine.

485

486 **Cell lines and transfection**

487 Human embryonic kidney 293T, African green monkey kidney COS-7 and human cervical
488 HeLa cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented
489 with 10% fetal bovine serum (FBS) (Wisent Bioproducts, St-Bruno, Canada). Cell lines were
490 obtained from the American Type Culture Collection (ATCC) (Manassas VA). All DNA
491 plasmids were transfected using Polyethylenimine (PEI) (Polysciences, Warrington PA) at a
492 7:1 ratio of PEI (μ g): total DNA (μ g) in FBS-free DMEM: empty vectors were added to
493 normalize DNA quantity in between transfection and for transfection control samples. After

494 an incubation of 20 min at room temperature, the PEI/DNA mixture was added to cells for 6
495 h, after which medium was removed and replaced by fresh supplemented DMEM medium. In
496 certain transfection, cells were treated with Bafilomycin A1 (100 nM) for 6 h at 24 h post-
497 transfection.

498

499 **Generation of CRISPR-Cas 9 stable cell lines**

500 Pseudotyped lentiviruses were produced by co-transfecting 293T cells with lentiCRISPRv2 or
501 lentiCRISPRv2-sgRNA (specific to ATG5, ATG7 or p62), the HIV-1 backbone-containing
502 psPAX2 and the VSV envelope encoding-pVSVg vector (Addgene: #8454) using the PEI
503 agent. At 36 h post-transfection, viral supernatants were filtered with 0.22um filters
504 (Millipore Corporation, Billerica, MA) and added to 293T and COS-7 cells. CRISPR-Cas9
505 stable knockout cell lines were selected and maintained in DMEM supplemented with 10%
506 FBS and 0.2ug/ml puromycin. Clones were generated by 10X serial dilution and single clone
507 wells were further expanded in puromycin-containing medium. Selected and amplified clones
508 were analyzed by PCR for the targeted region.

509

510 **Antibodies**

511 Anti-GST antibodies were purchased from GE Healthcare Life Science (Chicago IL). Anti-
512 Flag, anti-tubulin, anti-GAPDH, anti-LC3, anti-ATG5, anti-ATG7, anti-ATG12, anti-Beclin-1
513 and anti-p62 antibodies were purchased from Sigma-Aldrich (St-Louis MO). Antibodies
514 against Myc, His and HA tags, HRP-conjugated anti-GFP and HRP-conjugated goat anti-
515 mouse and anti-rabbit IgG antibodies were ordered from Santa Cruz Biotechnology Inc.

516 (Dallas TX). Mouse TrueBlot® ULTRA(Anti-Mouse Ig HRP) was purchased from Rockland
517 Inc. (Limerick PA). Goat anti-mouse IgG coupled to Alexa Fluor 488 or to Alexa Fluor 568
518 were obtained from Thermo Fisher Scientific (Waltham MA). Monoclonal anti-ASP
519 antibodies generated by Eurogentec (Liege, Belgium) were kindly provided by Dr. Jean-
520 Michel Mesnard (Université Montpellier, Montpellier, France) and are specific to the amino
521 acid sequence indicated in Fig. 4A.

522

523 **Western blot analyses and immunoprecipitation**

524 Cells were washed in PBS 1X and then lysed in lysis buffer (Tris 50mM pH8.0, NaCl
525 100mM, EDTA 1mM, 1%Triton with proteinase inhibitor (Roche, Mississauga, Canada) on
526 ice for 15 min. In certain experiments, β -mercaptoethanol (2 %) and varying concentrations of
527 DTT (0.1 -0.5 M) were added to the lysis buffer. The lysates were centrifuged at $2000 \times g$ for
528 15 min at 4°C. Proteins (the supernatant) were denatured by mixing 20 μ l lysates with 4X
529 loading solution (12% sodium dodecyl sulfate (SDS) with 3 mM Tris pH 6.8 and 0.05%
530 bromophenol blue), followed by incubation at 70°C for 10 min. For immunoprecipitation,
531 SureBeads™ Protein G Magnetic Beads (BioRad, Hercules CA) were resuspended (20 μ l per
532 sample) and washed twice with 500 μ l cold 0.1% Tween20 in PBS (PBS-T). Antibodies
533 diluted in 500 μ l PBS-T (anti-Myc (1:200), anti-p62 (1:100), anti-His (1:200), and anti-GST
534 (1:100)) were then added to the beads for 1 hour at room temperature, and beads were next
535 washed three times with cold PBST. Total cell extracts (lysed in 50 mM Tris-HCl pH 8, 100
536 mM NaCl, 1 mM EDTA and 1% Triton) were then incubated with the antibody-bead complex
537 overnight at 4°C. After three washing with cold PBS-T, bound fractions were eluted with 20

538 μ l of 2X loading buffer heated at 100°C. Samples were run on 10-12% SDS-PAGE and
539 blotted onto an Immun-Blot™ PVDF membrane (Bio-Rad Laboratories, USA) in PBS-T.
540 Membranes were blocked with 5% milk in PBS-T at room temperature for 1 h and incubated
541 with anti-ASP (1:2000), anti-GFP (1:5000), anti-Myc (1:250), anti-HA (1:10000), anti-GST
542 (1:3000) anti-His (1:5000), anti-p62 (1:2000), anti-ATG5 (1:2000), anti-ATG7 (1:2000), anti-
543 LC3 (1:500), anti-Beclin-1 (1:500), anti-GAPDH (1:1000) or anti-tubulin (1:10000),
544 antibodies at 4°C overnight. Membranes were next incubated with the appropriate HRP-
545 conjugated secondary antibodies (1 μ g/ml) at room temperature for 2 h and visualized with
546 the ECL Western blotting detection kit ((Immobilon™ Western, Millipore Corporation,
547 Billerica MA). Image acquisition was performed with Fusion FX7 (Vilber Lourmat, Marne-
548 la-Vallée, France). For certain experiments, densitometric analyses were conducted on LC3-
549 related signals and a LC3-II/LC3-I ratio was calculated and normalized over cells transfected
550 with an empty vector (set at a value of 1).

551

552 **Flow cytometry**

553 Transfected COS-7 and 293T cells were washed with PBS, fixed with 4% formaldehyde for
554 10 min, and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Cells were
555 then washed three times with PBS and incubated with the anti-Myc antibody (dilution, 1:250)
556 overnight at 4°C. After three additional washes with PBS, cells were incubated with goat anti-
557 mouse IgG antibodies coupled to Alexa Fluor 488 (1/500) (Invitrogen Canada Inc) for 1 h at
558 4°C. Cells were fixed with 1% formaldehyde and incubated overnight at 4°C before analysis
559 with the FACScan device (BD Biosciences).

560

561 **Confocal microscopy**

562 COS-7 and HeLa cells were seeded in 24-well plates containing a 1.5-mm-thick coverslip for
563 12 h and then transfected, as described above. At 48 h post-transfection, cells were rinsed
564 twice with cold PBS and fixed with 4% formaldehyde/PBS for 10 min at room temperature on
565 a slow rotatory shaker. Cells were next washed with PBS/ 0.1% Triton X-100 4 times. Fixed
566 cells were incubated with a blocking solution (3% BSA, 5% milk and 50% FBS in 0.1%
567 Triton X-100, 0.05% NaN₃ PBS) overnight at 4°C, then rinsed with cold PBS three times and
568 incubated with anti-p62 (1:250), anti-ATG5 (1:250), anti-ATG7 (1:250), anti-LC3 (1:250),
569 anti-ASP (1:500), or anti-Myc (1:250) antibodies in a PBS solution containing 3% BSA, 0.1%
570 TritonX-100 and 0.05% NaN₃ at 4°C overnight. Cells were next washed with cold PBS/0.1%
571 Triton X-100 and incubated with a secondary antibody (1:500 goat anti-mouse IgG coupled to
572 Alexa Fluor 488 or goat anti-rabbit IgG coupled to Alexa Fluor 568) in the PBS solution
573 described above for 45 min. at room temperature. Cells were washed with PBS/ 0.1% Triton
574 X-100 and incubated in the immune-mount solution in the presence of 1% DAPI. All cell
575 samples were visualized with a Nikon A1 laser scanning confocal microscope (Nikon Canada,
576 Mississauga, Canada) through a 60X objective under oil immersion.

577

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579

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581

582

583 **Reference**

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733

734

735

736

737 **Figure legends**

738

739 **Figure 1: Multimerization of ASP involves disulfide bonds. A-B.** 293T (A) and COS-7 (B)

740 cells were transfected with expression vectors for GFP-optimized-ASP, Myc-tagged-

741 optimized-ASP and/or the empty vector pcDNA3.1. At 48 h post transfection,

742 immunoprecipitation was performed using the anti-Myc antibody and Western blot analyses

743 were conducted through anti-GFP and anti-Myc antibodies. Total cellular extracts were

744 similarly analysed in parallel with an anti-GFP antibody. C. Extracts from COS-7 cells

745 transfected with the Myc-tagged optimized ASP expression vector (vs. pcDNA3.1) were

746 treated with of DTT and/or β -mercaptoethanol prior to Western blot analyses with anti-Myc

747 and anti-GAPDH antibodies. Both stacking and resolving gels are depicted and high

748 molecular weight (including 200 kDa multimers) vs. monomeric 20kDa ASP signals are

749 indicated.

750

751 **Figure 2: The amino end of ASP is implicated in its multimerization, A.** Schematic

752 representation of the different domains of ASP and the generated deletion mutants targeting

753 the first 15 or 30 amino acids of either the N- or C-terminal of ASP. **B-D.** COS-7 cells were

754 transfected with expression vectors for these different ASP mutants and the wild-type version

755 vs. pcDNA3.1 (empty vector) and, at 48 h post-transfection, were analysed with an anti-Myc

756 antibody by flow cytometry (**B**) and confocal microscopy (60X objective and with numerical

757 aperture of 1.4) (**C**). Cells analysed by confocal microscopy were also stained for their nuclei

758 with Hoechst. Cellular extracts from these transfected cells were also analysed by Western

759 Blot using anti-Myc and anti-GAPDH antibodies (**D**). High molecular weight (including 200

760 kDa multimers) vs. monomeric 20kDa ASP signals are indicated.

761

762 **Figure 3: Deletion of the first cysteine triplet of ASP reduces multimer formation and**

763 **autophagosome signals. A.** Schematic representation of the different domains of ASP and

764 the deletion mutant of the first cysteine triplet (¹⁰CCC¹²). **B-C.** COS-7 cells were transfected

765 with expression vectors for wild-type or cysteine triplet-deleted ASP vs. pcDNA3.1 (empty

766 vector). After 48h of transfection, using anti-Myc antibodies, cellular extracts were analysed

767 by Western Blot (**B**) and confocal microscopy (60X objective and with numerical aperture of

768 1.4) (**C**). Cells analysed by confocal microscopy were also stained for their nuclei with

769 Hoechst. High molecular weight (including 200 kDa multimers) vs. monomeric 20kDa ASP

770 signals are indicated.

771

772 **Figure 4. ASP from different clade representatives form detectable multimers. A.**

773 Sequence alignment of the predicted amino acid of ASP from different HIV-1 isolates

774 representing different clades. The peptide region used to generate anti-ASP monoclonal

775 antibodies is highlighted over the sequence from amino acid 47 to 61. **B-C.** Expression

776 vectors of His-tagged ASP from various HIV clades were transfected in 293T cells and cell

777 lysates were subsequently analyzed by Western blot for ASP (**B**: anti-ASP and **C**: anti-His).

778 Depicted on the right side of the panels are multimers and high molecular weight and

779 monomeric 20kDa ASP signals. Clade type for each tested ASP is indicated on top of each

780 lane.

781 **Figure 5. ASP from different clade representatives induce autophagy. A-B.** Expression

782 vectors of His-tagged ASP from various HIV clades and the empty vector were transfected in
783 293T cells and cell lysates were analyzed by Western blot for the detection of ASP (anti-His),
784 LC3-II levels (anti-LC3) and tubulin. Signals for LC3-I, LC3-II and ASP are indicated on the
785 left of each panel. In panel B, after transfection, cells were treated with DMSO (-) or with 100
786 nM Bafilomycin A1 (+) (Baf A1) for 6 h. LC3-II/LC3-I ratios are indicated for each lane and
787 are representative of fold value over the calculated ratio for untreated cells transfected with
788 the empty vector. **C.** Increasing quantity of clade 89.6 ASP expression vector was transfected
789 in 293T cells and cell lysates were analyzed by Western blot for ASP, p62 and tubulin. **D.**
790 COS-7 cells were transfected with expression vectors of Myc-tagged ASP from various
791 clades. After fixation, cells were labeled with anti-Myc antibodies followed by goat anti-
792 mouse IgG coupled to Alexa Fluor 488, stained with DAPI and observed by confocal
793 microscopy.

794

795 **Figure 6. Suppressed expression of ATG5 and ATG7 leads to increased ASP expression.**

796 Two different 293T cell clones knocked out for either ATG5 (sgATG5-1 and sgATG5-2) (A)
797 or ATG7 (sgATG7-1 and sgATG7-2) (B) and control stably transfected clones were
798 transfected with an His-tagged NL4.3 ASP expression vector.. Cell lysates were prepared and
799 analyzed by Western blot using anti-His, anti-ATG-5, anti-ATG7 and anti-tubulin antibodies.
800 Monomeric and multimeric signals are representing different ASP signals. The signal for
801 ATG5 is shown as the typical covalently linked ATG5-ATG12 complex.

802

803 **Figure 7. Association between ASP and p62 with and effect of knock out p62 expression**
804 **on ASP levels.** **A.** 293T cells were transfected with expression vectors for optimized NL4.3
805 ASP or the empty vector pcDNA3.1. After 48 h post transfection, immunoprecipitation was
806 performed using the anti-ASP antibody and Western blot analyses were conducted through
807 anti-ASP and anti-LC3 antibodies. The Mouse TrueBlot® ULTRA (Anti-Mouse Ig HRP) (for
808 ASP) was used as secondary antibody for Western blot analyses. **B.** Expression vectors
809 pcDNA-opt-ASP and GFP-LC3 were transfected in COS-7 cells. After fixation and nuclei
810 staining with DAPI, ASP was detected with anti-ASP antibodies followed by a goat anti-
811 mouse IgG antibody coupled to Alexa Fluor 568, while LC3 detection was performed with an
812 anti-LC3 antibody followed by a goat anti-rabbit IgG antibody coupled to Alexa Fluor 568. **C.**
813 Expression vectors for NL4.3-ASP were transfected in COS-7 (upper panels) and HeLa
814 (bottom panels) cells. Nuclei were stained with DAPI, and ASP was detected with anti-ASP
815 antibodies followed by goat anti-mouse IgG antibodies coupled to Alexa Fluor 568 (COS-7)
816 or Alexa Fluor 488 (HeLa), while p62 was detected by an anti-p62 antibodies followed by
817 goat anti-rabbit IgG antibodies coupled to Alexa Fluor 488 (COS-7) or Alexa Fluor 568. **D-E.**
818 Expression vectors of His-tagged ASP of various HIV clades were transfected in 293T cells
819 and after 48 h, cellular extracts were used for immunoprecipitation with the anti-His (**D**) or
820 anti p62 antibodies (**E**). Immunoprecipitated samples and total extracts were analyzed by
821 Western blot using anti-p62, anti-His and anti-tubulin antibodies. **F.** 293T cell clone knocked
822 out for p62 (sgp62) and control stably transfected clone were transfected with His-tagged
823 NL4.3 ASP expression vector. Cell lysates were prepared and analyzed by Western blot using
824 anti-His, anti-p62 and anti-tubulin antibodies. Monomeric and multimeric signals are

825 representing different ASP signals.

826

827 **Figure 8. The interaction of p62 with ASP requires its homodimerization domain despite**

828 **ASP ubiquitination. A.** The various domains of the p62 protein are depicted and include the

829 ubiquitination-binding domain UBA, the LC3-binding domain LIR, NLS and NES regions

830 and the homodimerization domain PB1. **B.** 293T cells were co-transfected with expression

831 vectors for GST-tagged wild-type and deletion mutants of p62 and of Myc-tagged-NL4.3

832 ASP. At 48 h post transfection, immunoprecipitation was performed using the anti-GST

833 antibody and Western blot analyses of immunoprecipitated samples along with total extracts

834 were conducted through anti-GST, anti-Myc and anti-tubulin antibodies. **C.** 293T cells were

835 co-transfected with expression vectors for His-tagged NL4.3 or 94CY032 ASP and for the

836 HA-tagged Ubiquitin-KOR mutant (restricted to monoubiquitination). At 48 h post

837 transfection, immunoprecipitation was performed with anti-His antibodies and Western blot

838 analyses of immunoprecipitated samples along with total extracts were conducted through

839 anti-His, anti-HA and anti-tubulin antibodies. Arrows on the right side of the right panels

840 indicated the position and expected MW (25-30 kDa) of the ubiquitinated ASP from the

841 different tested proviral DNA.

842

843

844

845

846 **Table S1. Primers used for generation of ASP deletion mutants.** Primers are presented as
847 forward and reverse pairs suitable for the generation of the deletion mutants by inverse PCR,
848 as described in Material and Methods.

849

850 **Table S2: Primers for generation of expression vectors for CRISPR-mediated knockout**
851 **of ATG5, ATG7, ATG12 and p62 genes.** Complementary oligonucleotides are depicted for
852 each targeted gene and were inserted in the lentiviral vector, as indicated in Material and
853 Methods.

854

855 **Figure S1. Amino deletion and removal of a cysteine triplet in the amino end of ASP**
856 **increase abundance of its monomeric form.** COS-7 cells were transfected with expression
857 vectors for ASP mutants (see Fig. 2A and 3A) or the wild-type version vs. pcDNA3.1 (mock).
858 At 48 h post-transfection, cellular extracts were analysed by Western Blot using anti-ASP and
859 anti-tubulin antibodies.

860

861 **Figure S2. Expression of ASP from different clades leads to reduced levels of p62. .**
862 Expression vectors for His-tagged ASP from NL4.3, 94CY032, IndieC1 and Mal proviral
863 DNA (vs. empty vector) were transfected in 293T cells and cell lysates were analyzed by
864 Western blot for ASP (anti-His), p62 and tubulin. Clades of origin of the different tested ASP
865 representative are indicated above each lane.

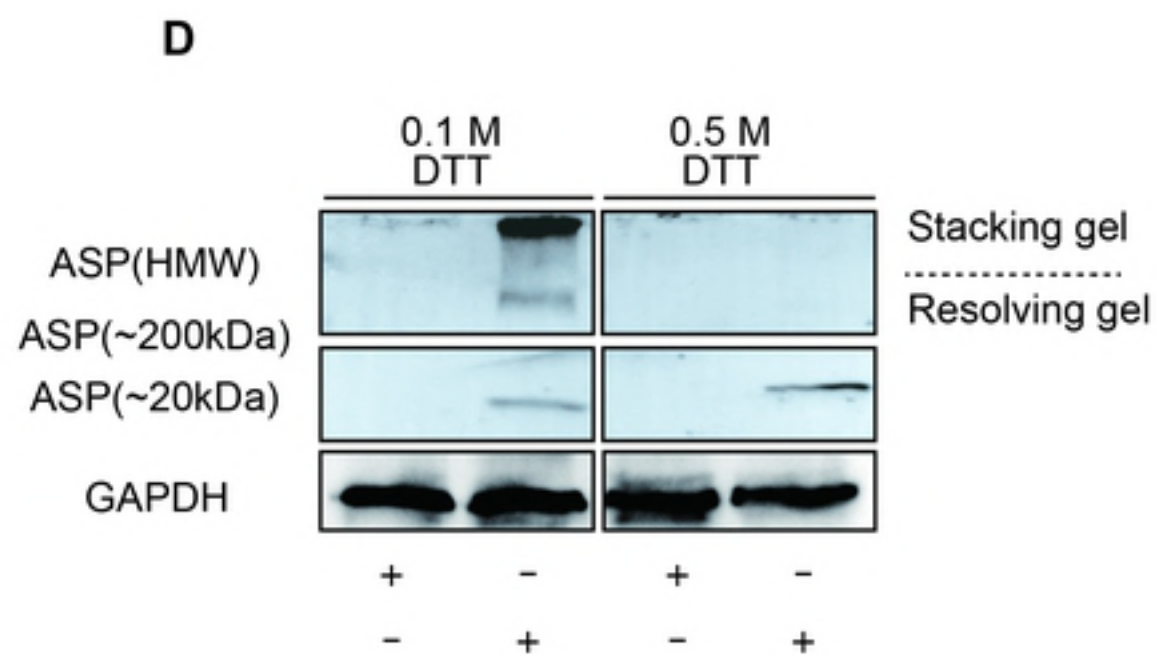
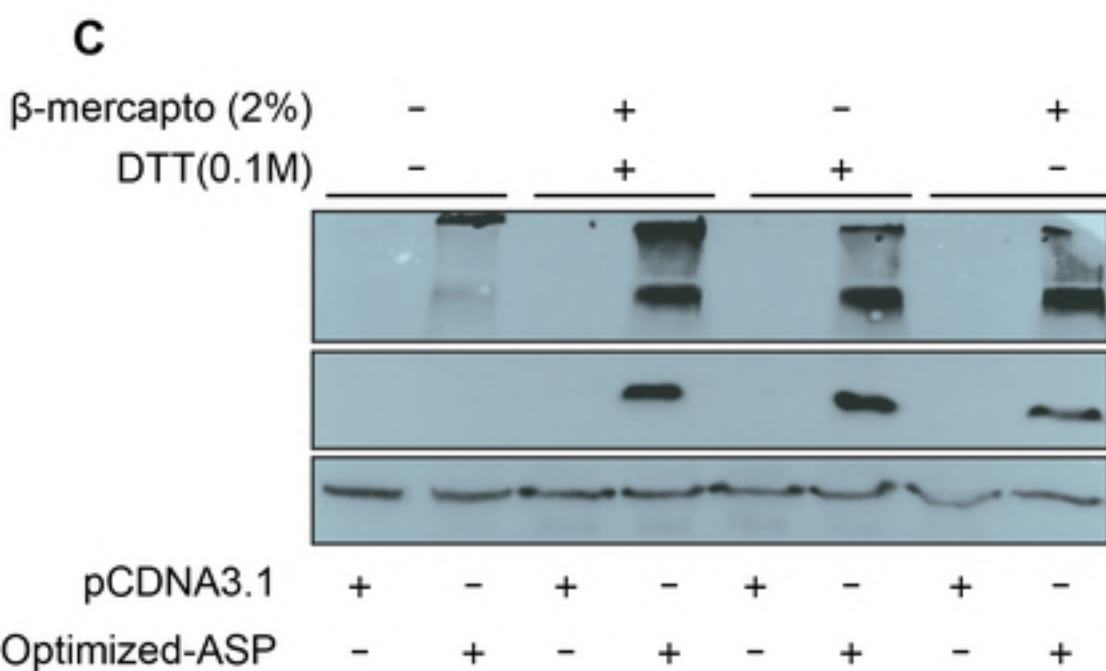
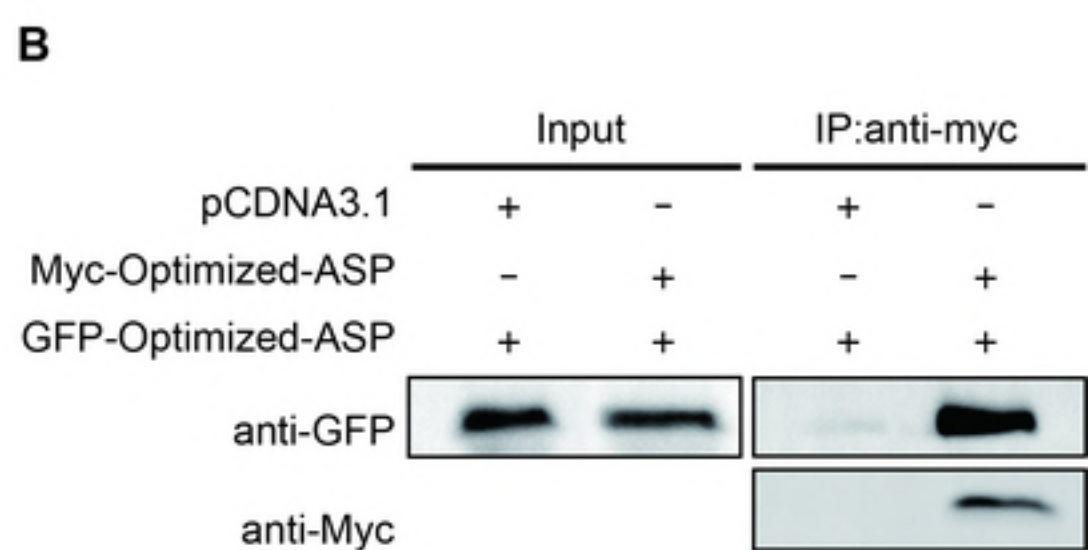
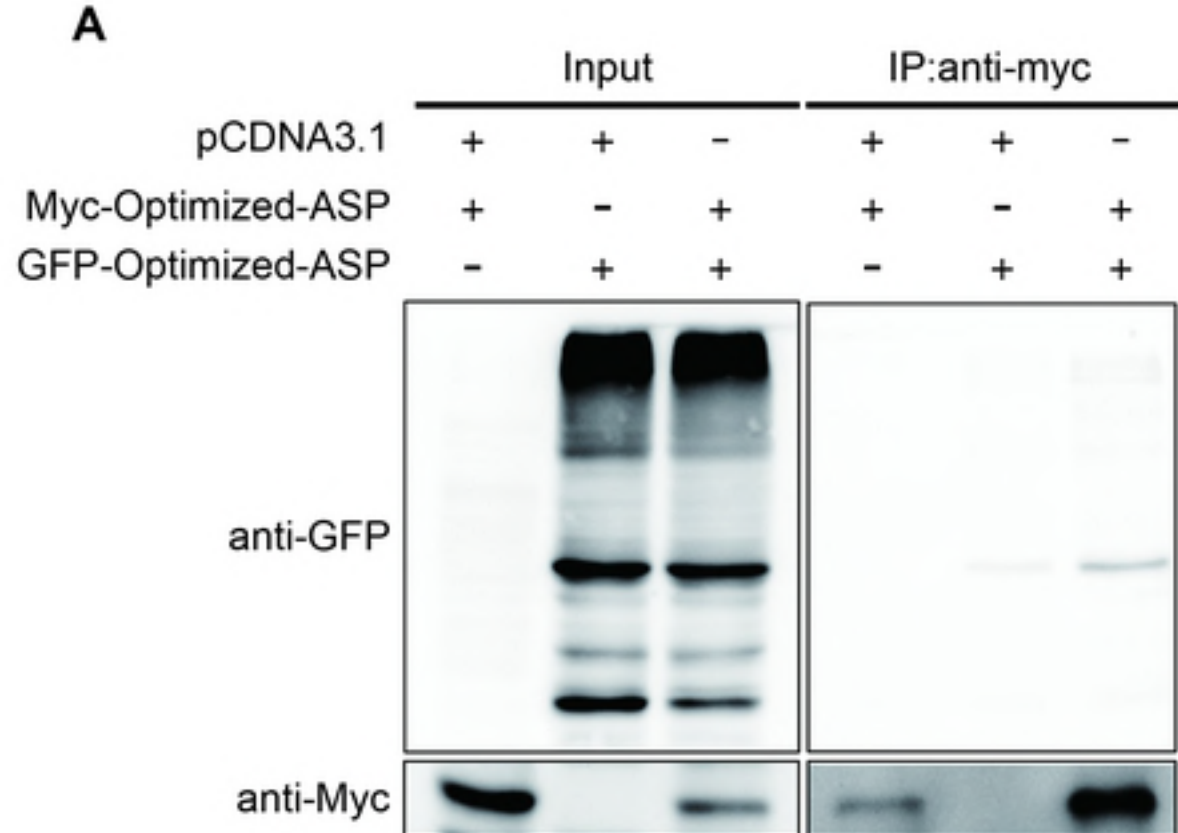
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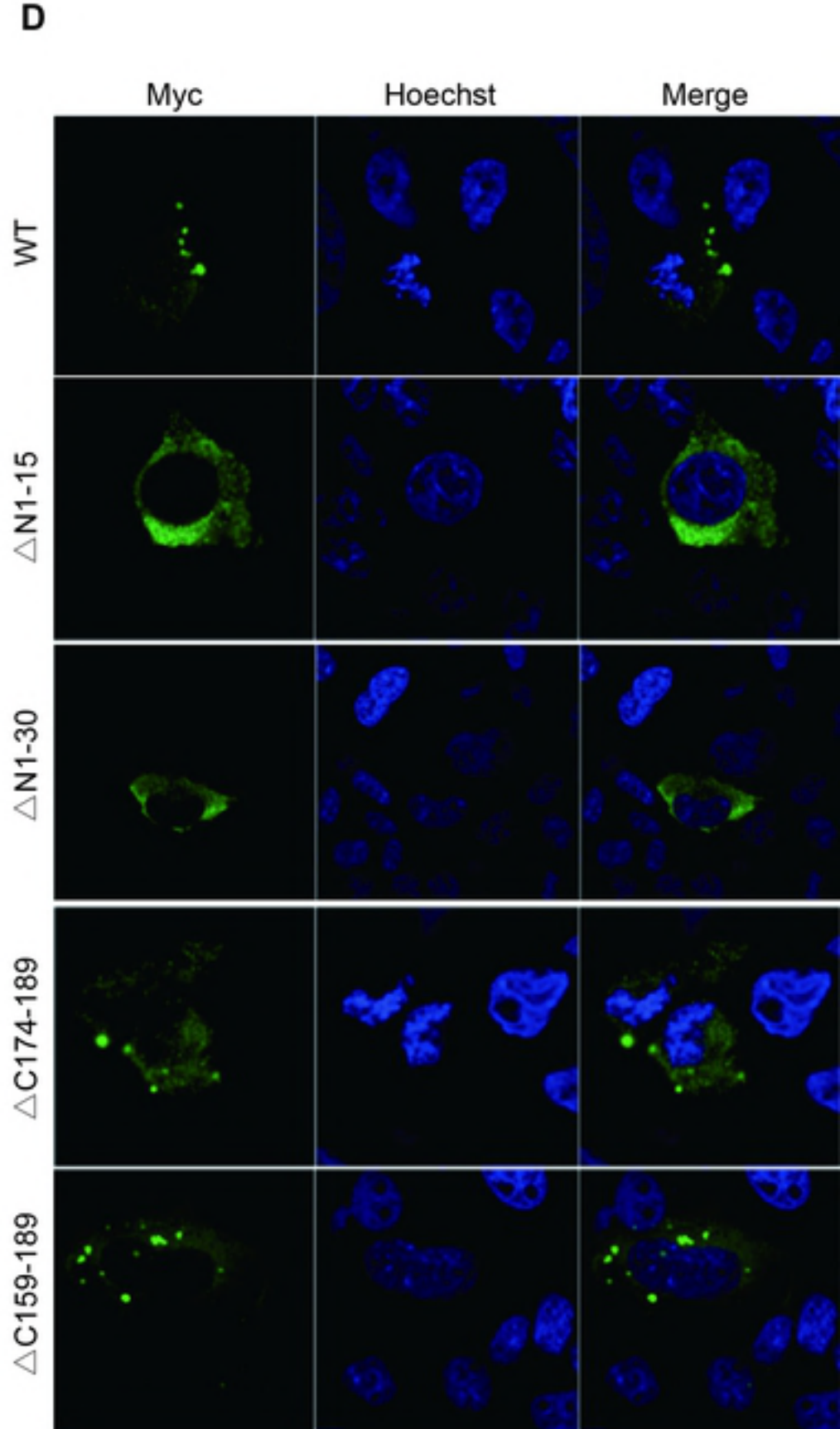
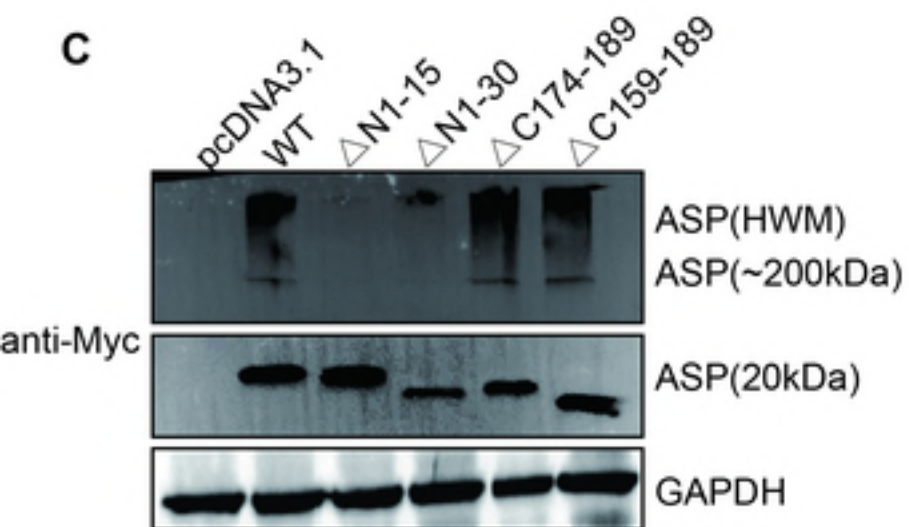
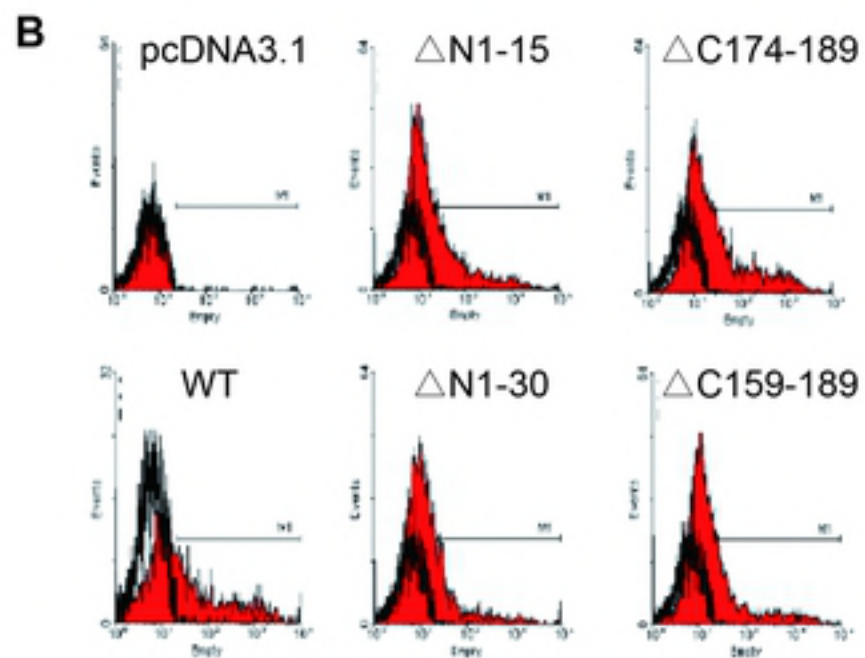
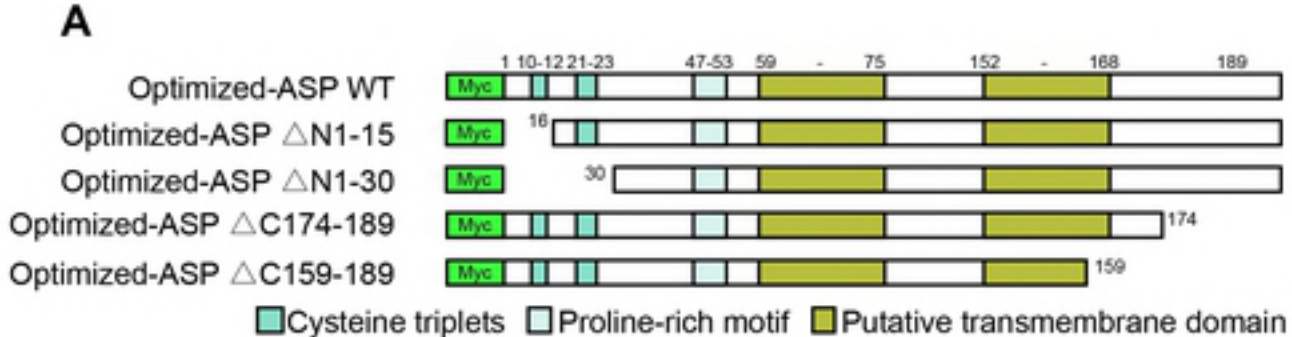
867 **Figure S3. Absence of association between ASP and the ATG5-ATG12 complex or**
868 **ATG7.** The expression vector for His-tagged NL4.3 ASP (vs. empty vector) was transfected
869 in 293T cells and at 48 h post-transfection, cellular extracts were used for
870 immunoprecipitation with anti-His antibodies Immunoprecipitated samples and total extracts
871 were analyzed by Western blot using, anti-His, anti-ATG5, anti-ATG7 and anti-tubulin
872 antibodies.

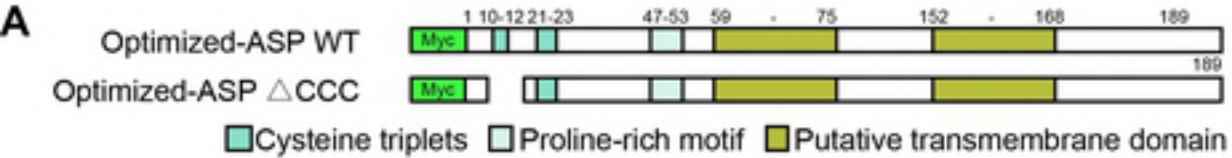
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874 **Figure S4. Interaction of p62 with ASP requires its homodimerization domain.** 293T
875 cells were co-transfected with expression vectors for GST-tagged wild-type and deletion
876 mutants of p62 and for His-tagged-NL4.3 ASP. At 48 h post transfection,
877 immunoprecipitation was performed using the anti-GST antibody and Western blot analyses
878 of immunoprecipitated samples along with total extracts were conducted through anti-GST,
879 anti-His and anti-tubulin antibodies.

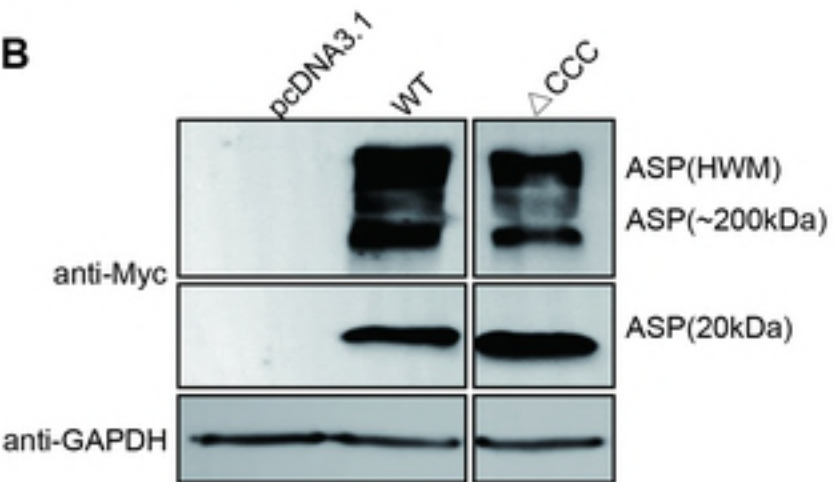
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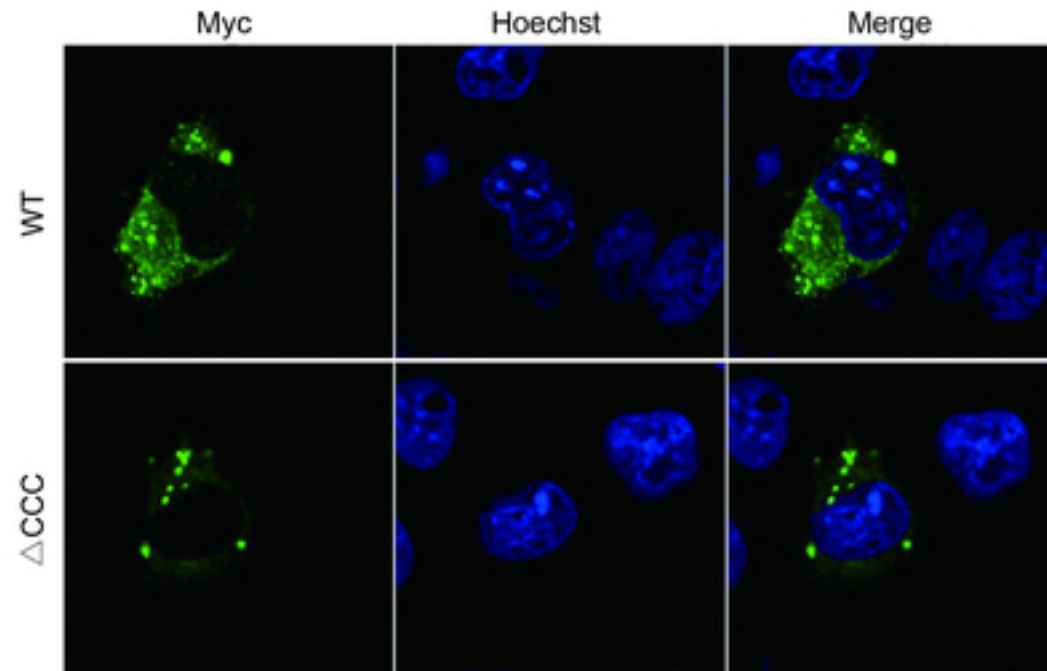




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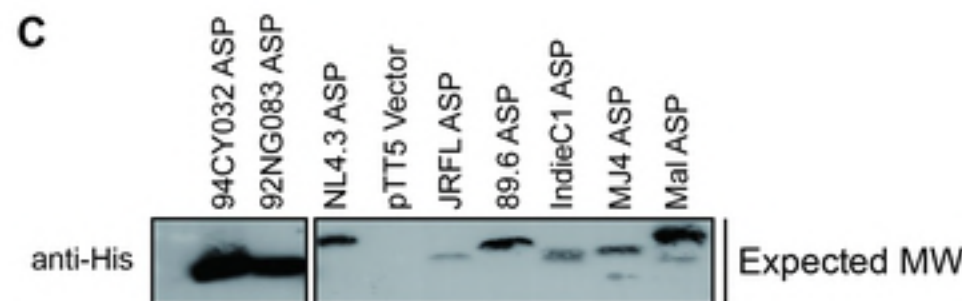
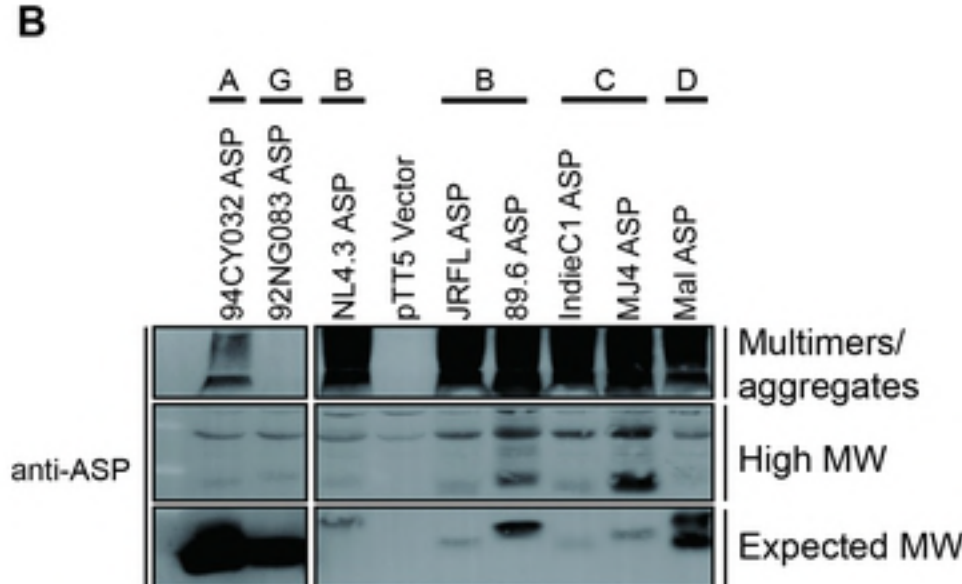


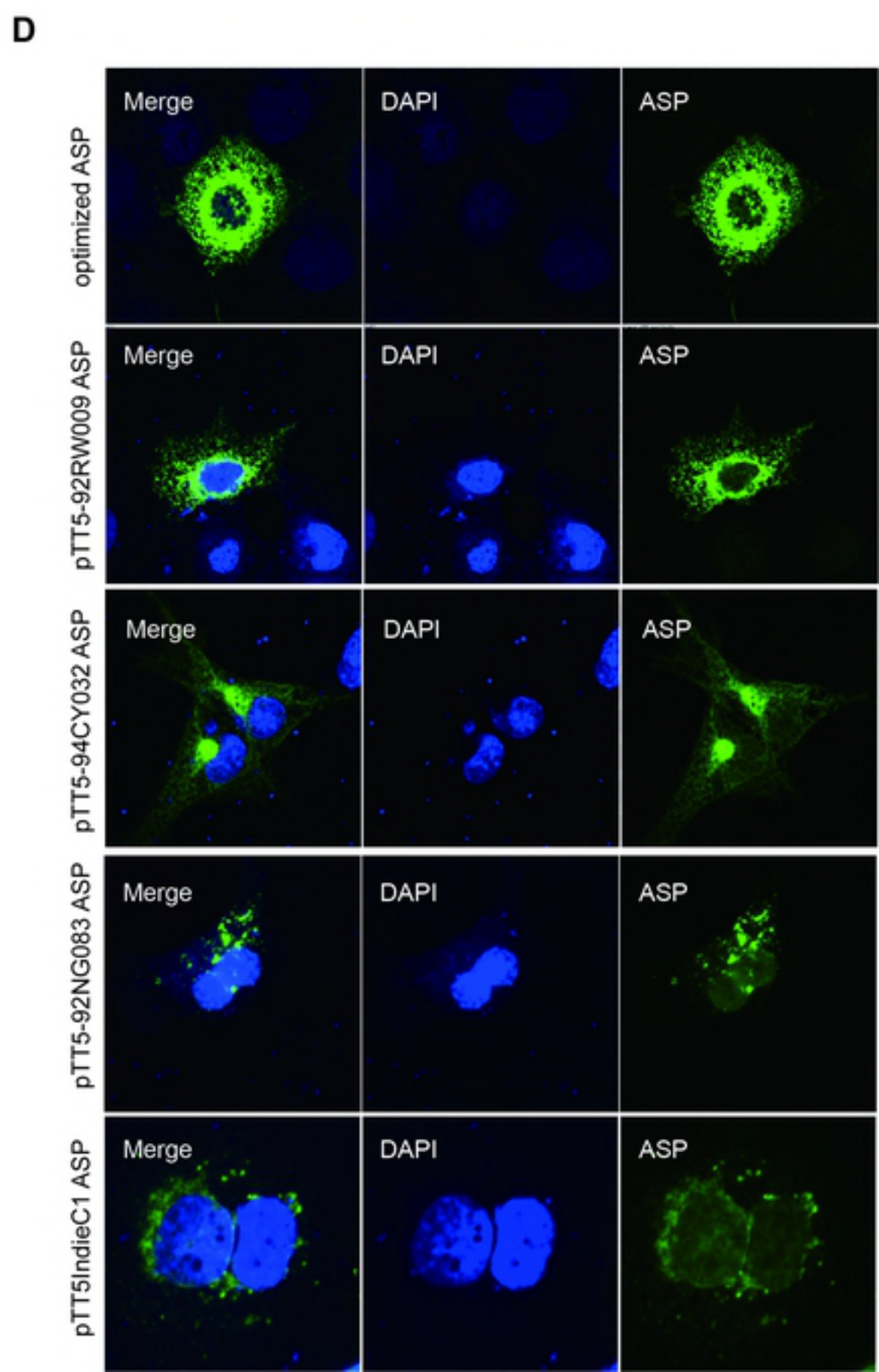
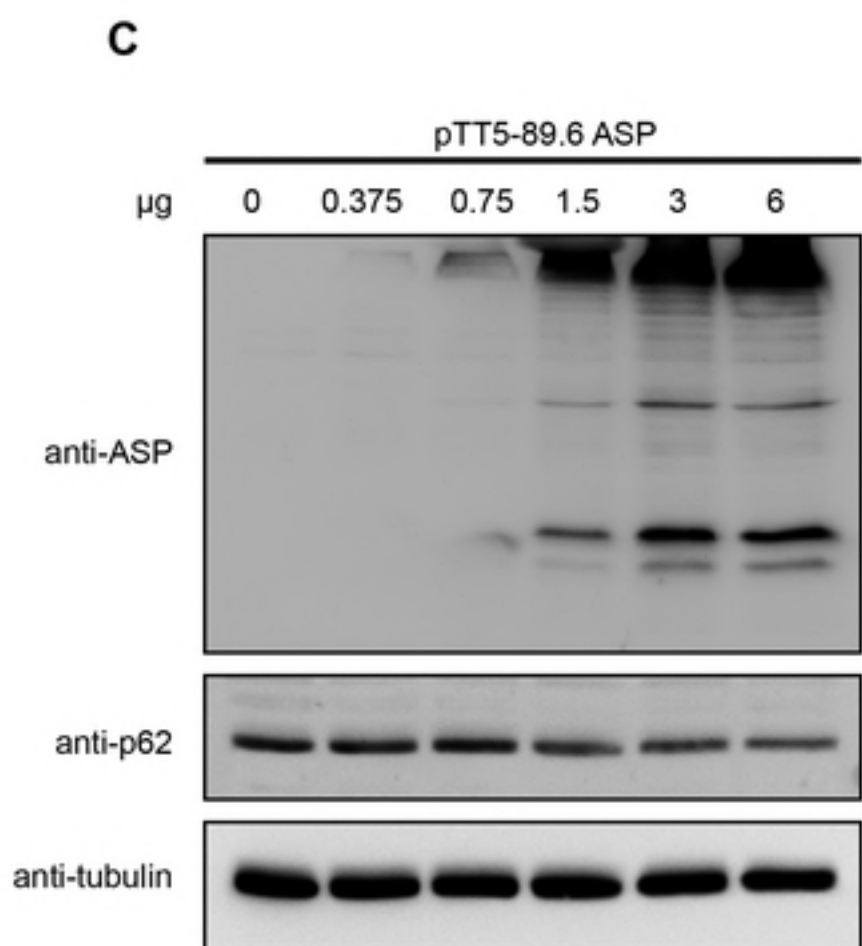
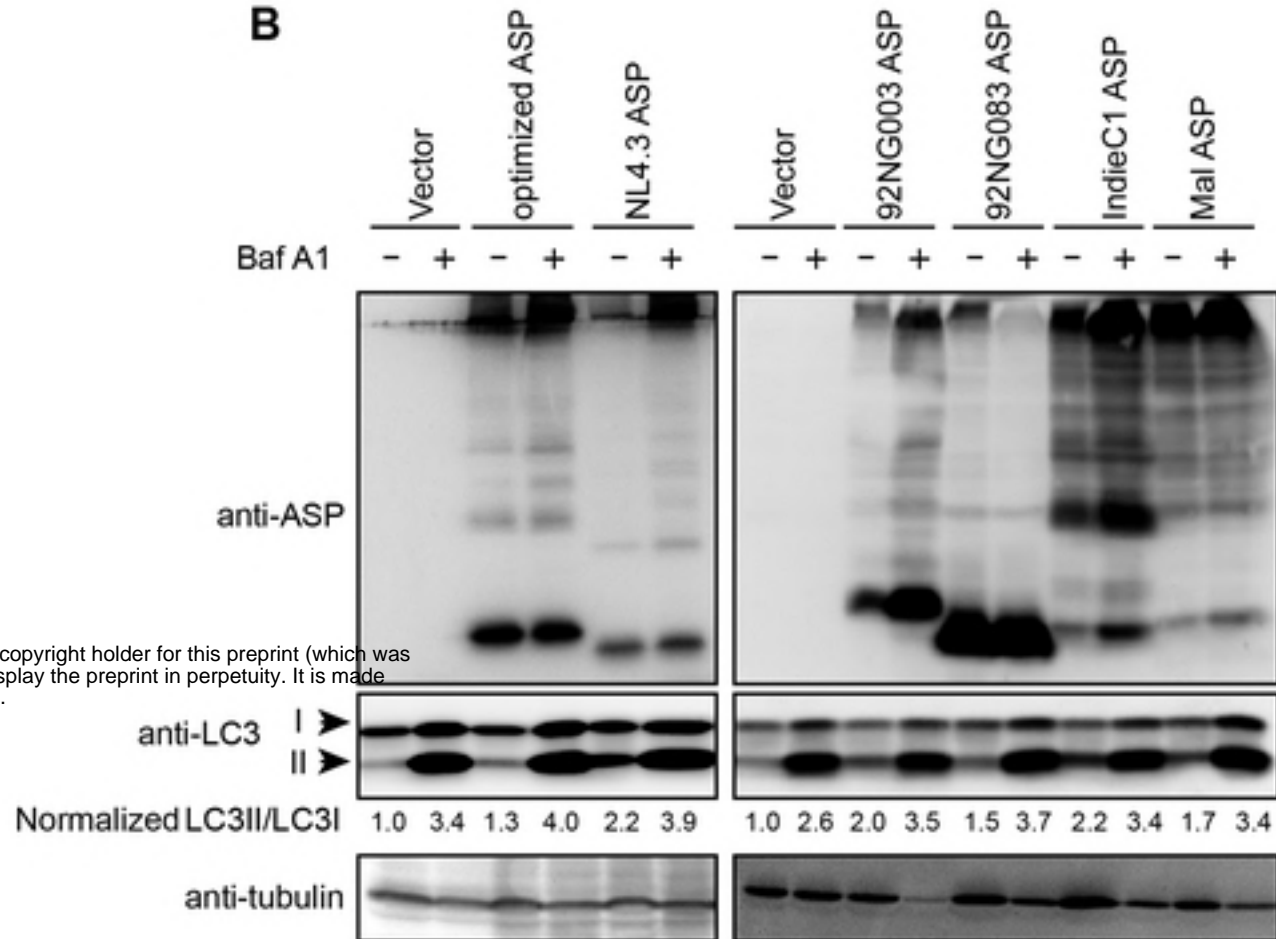
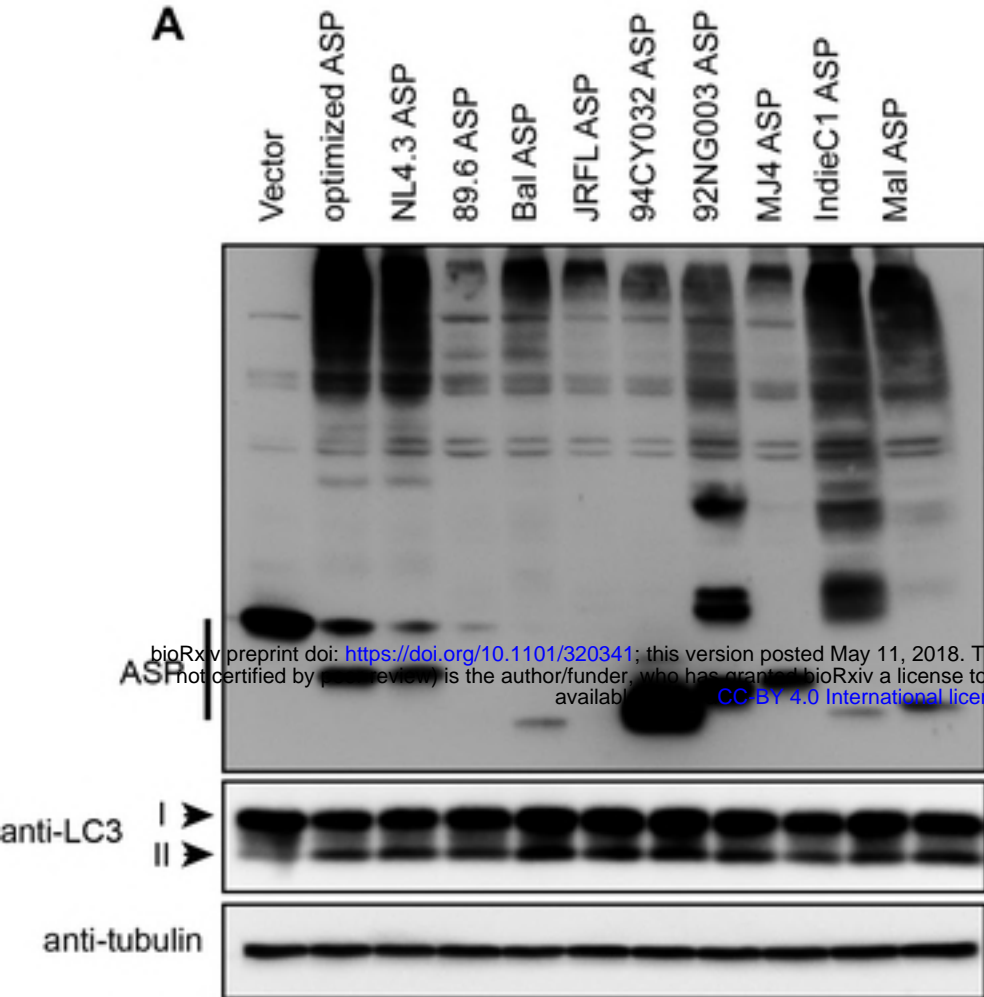
A

		Epitope sequence
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Ba1	MPQTVSCNRCCCASIALSRLFCCTIPDNNSLACTVSVIAAAPIVLPAAPKNPRNTAPIPTALFS	
JRFL	MPQTVSCNIRCCASIALSKLFCCTIPDNNSLACTVSVIDAAPIVLPAAPKNPRNTAPIPTALFS	
94CY032	-----MPDNNCLACTVSVIDAAPIVLPAAPKNPRNMAPIPTAHFS	
92NG003	MPQTVSCNRCCCASIALSKLLCCCTMPDNNCLTCTVSVIDPAPIVLPAAPKNPRKTAPSPTALFP	
92NG083	MPQTVSCNRCCCASIALSKLLCCCTMPDNNCLTCAVSVIDAAPIVLPAAPKNPRKTAPSPTALFS	
MJ4	MPQTVSCNICCCASIALSKLLCCCTIPDNSCLACTVSVMDAAPIVLPAAPKNPRNIAPNVTALFS	
IndieC1	MPQTVSCNRCCCASIALSKLLCCCTIPDNNCLCAVSVIDAAPIVLPAAPKNPRNTAPIPTALFS	
Ma1	MPQTVSCNRCCCASIALSKLFCCTIPDSNCLACTVSVSDAAPIVLPAAPKNPRNMAPSPIALFS	
		:. . . : *

NL4.3	LCTTLLFALVGATPNGSIFTTLYLYNSLLQLSLISPPPGLKISDPLL----LLPPSLVNSSPVIF
89.6	LCTVLLFALVGATPIGSILTTLTYLYNSLLQLSLISPPPGLKISVSVSV---LLPPSLVSSSPVIF
Ba1	LCTTLLFALVGATPNGSIFTTLYLYNSLLQLSLISPPPGLKTSVLLS----SGPPSLVNSSPVIF
JRFL	LCTTLLFALVGATPNGSIFTTLYLYNSLLQLSLISPPPGLKISVPFS----LIPPSLVNSSPVIF
94CY032	LCTTRLLALLGATPIGSIFTTLYLYSSLLQLSLMSPPPPGLKIS----V--LLVPPSLVNIIPVIS
92NG003	LSTTLLLALVGAIPSGLIIFTILTYLYNSLLQLSLISPPPGLKVSSFP--V--LLTPPSLVNNSPVMF
92NG083	LSTTLLLALVGATPSDLIFTVLYLYNSLLQLSLISPPPGLKVSSV-LS--LLFPPSLVNINPVMF
MJ4	LSTTLRFDLVGANPNGLISTTLYLYNSLLQLSLISPPAGLNISIPVLS----FPPSRVNSSPVIF
IndieC1	LSTTLLFAAVGATPNGLISTTLYLYNSLLQLSLISPPPGLNISVLFVSVSFSFIPPSTNNSPVIF
Ma1	LSTTLLFALVGATPRGSILTTLTYLYNSLIQLSLISPPPGLKVSLLS--LLFPPSLVNISPVIF
	* : *

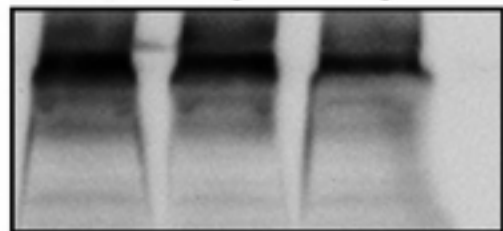
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89.6	DEHLICPVMGGAYIAFPTFCHIFIICFILHWSVIMSFPVFPVPPVT-----FQVLLNSCAVLQ*
Ba1	DEHLICPLMGGAYIALPTSCHMFIICFILHGSVIVLFSTVLFDSSVT-----FQVLLNSCVELQ*
JRFL	DEHLICPLMGGAYIAFPTSCHMFIICFILHGSVIV-FPSVLFDPVSL----LFQVLLNSCVELQ*
94CY032	ELQLMLPAMGEAYIACPTSCHILTNCFSLHWSVMVEFVESVLMFV---PFCMVCVLLNSGVVLQ*
92NG003	DLHVISPAMGGAYIACPTLCHIL TICFILHGSVIVVVSFP--PD-----SLNNDVLLQ*
92NG083	DLHTRFPAMGRAYIACPTFCHIL TICFILHGSVIVSLLILLIL-----LLNSPDVLLQ*
MJ4	DLHVMFPAMGEAYIARPTPCHMFIICFILHESVIVEFEVS-----PVLLYVPLNSFDVLLQ*
IndieC1	DVHVMFPAMGGAYIARPTSCHMFIICFILHGIVIVEFELLSVPLGMYV---GMYVPLNRPDVLQ*
Ma1	DKQLMTPAMGGAYIAFPVFCHIFIICFILHGSVILPVDSVLLLSLA---PLFCHVLLNSFDVLLQ*
	* : * * * * * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *





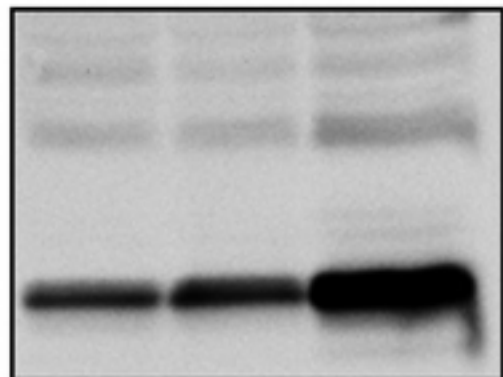
A

Ctrl sgATG5-1 sgATG5-2



anti-His

ATG5-ATG12 complex →



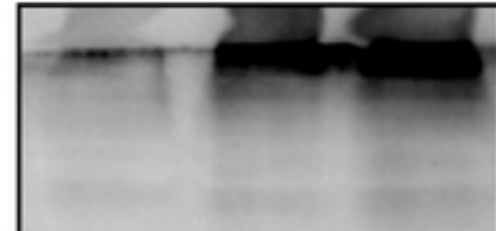
anti-ATG5



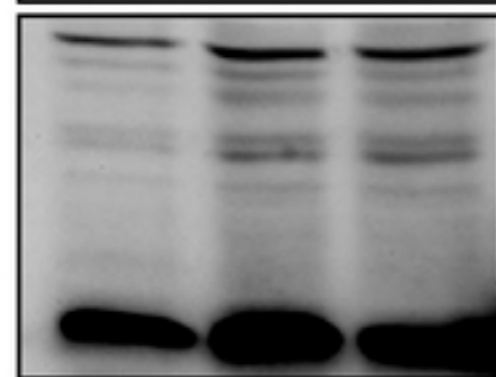
anti-tubulin

B

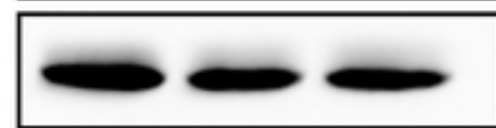
Ctrl sgATG7-1 sgATG7-2



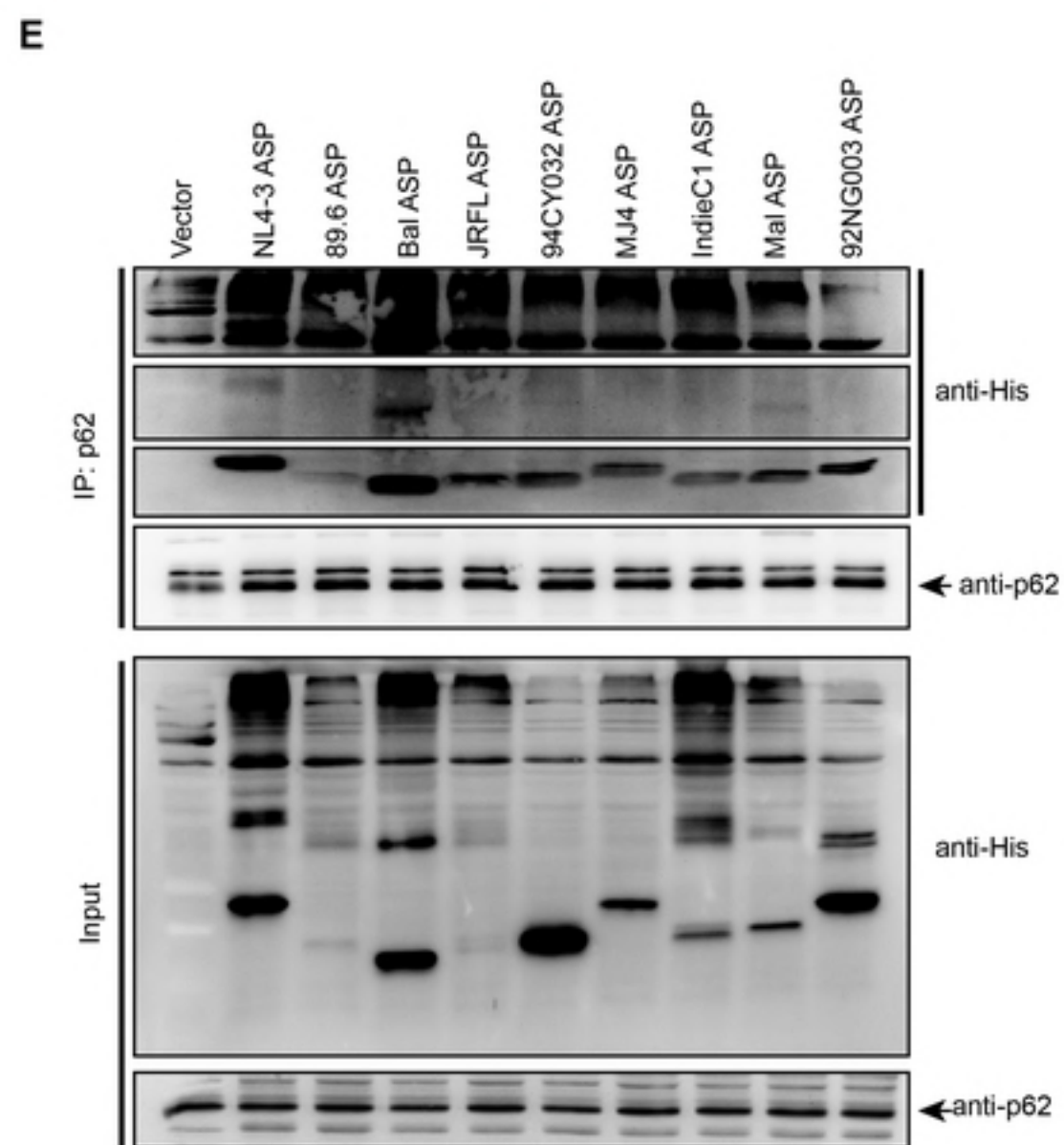
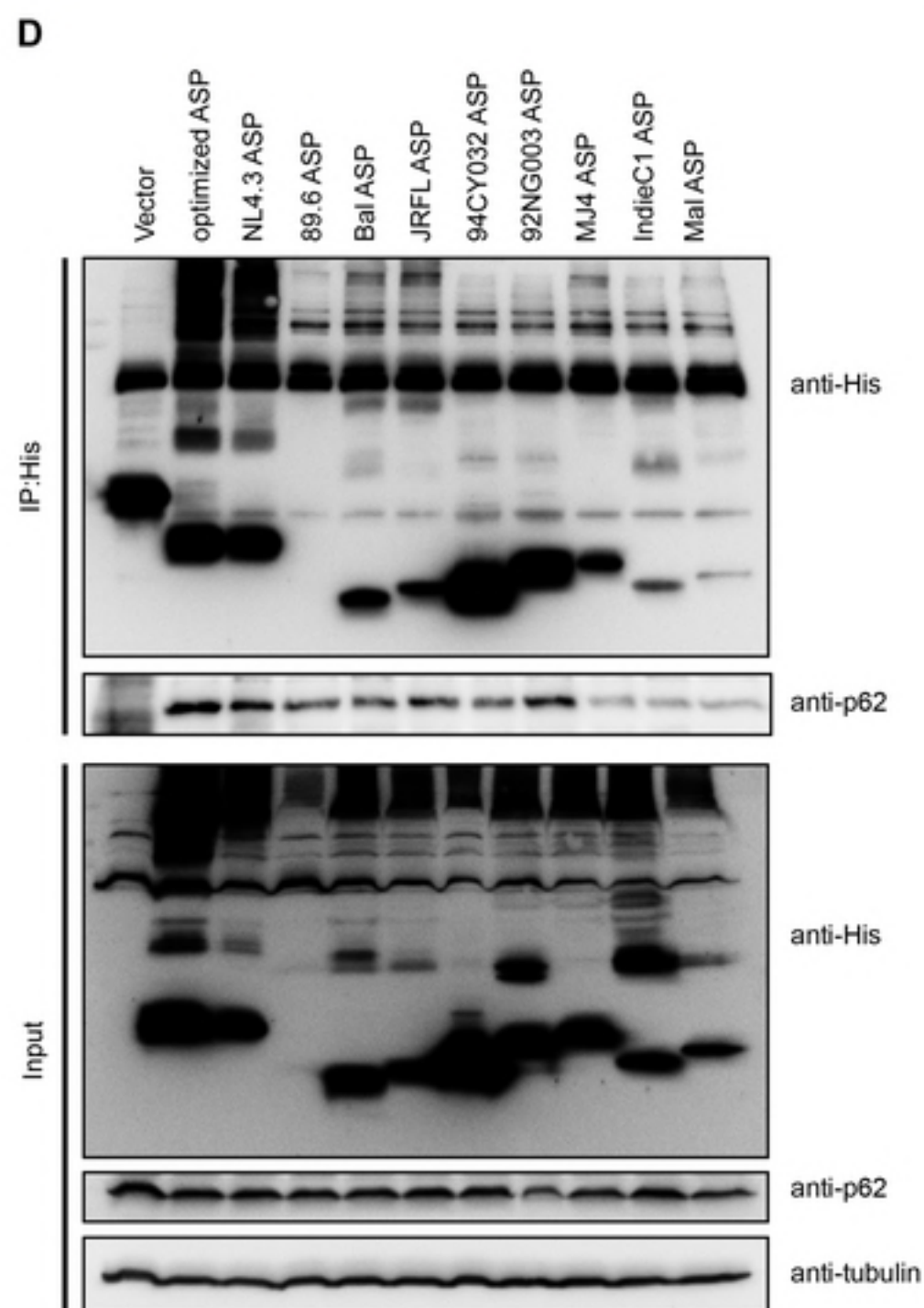
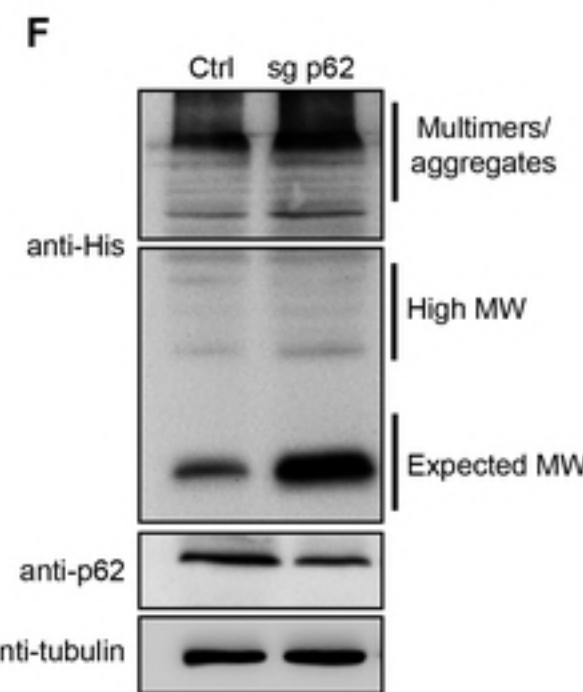
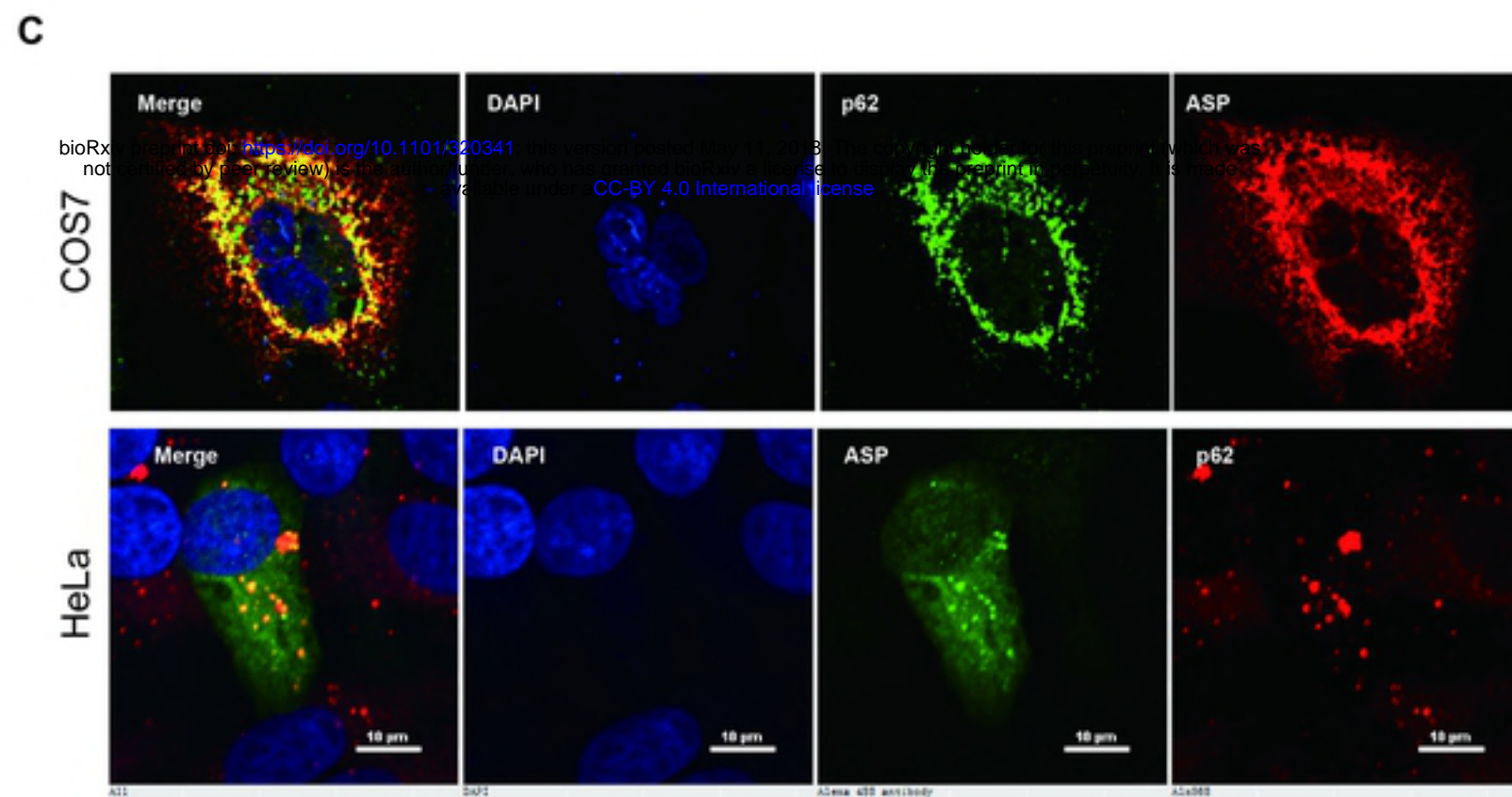
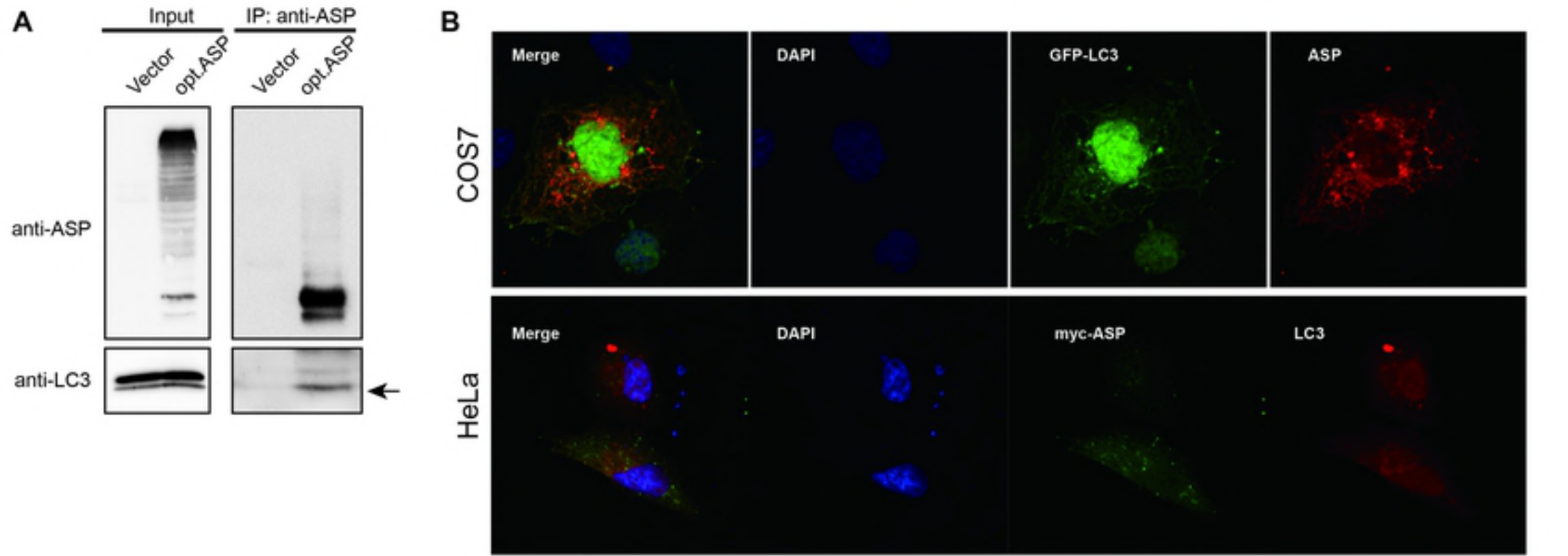
anti-His

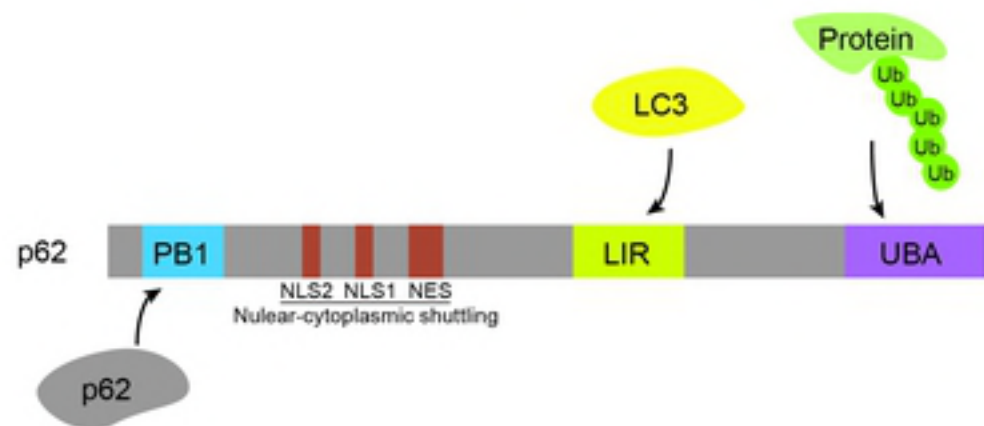
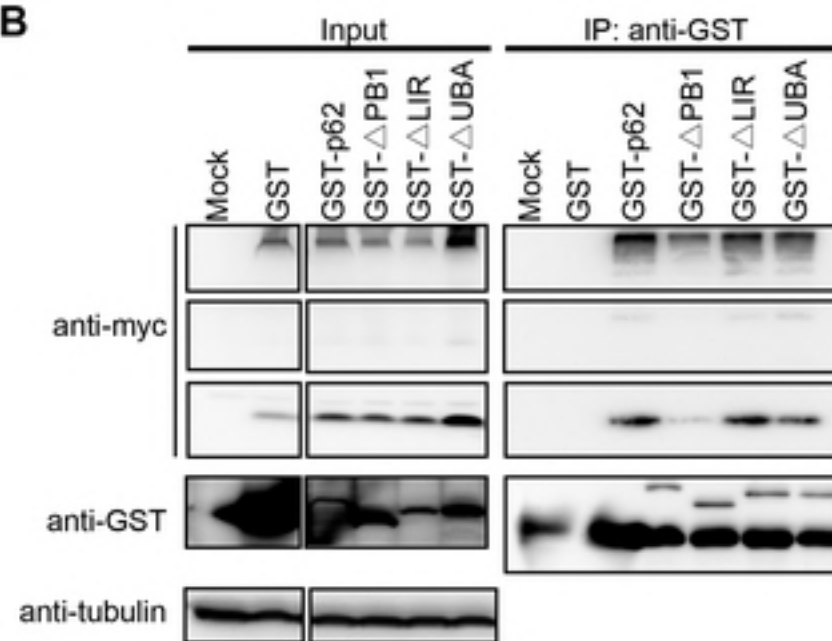


anti-ATG7



anti-tubulin



A**B****C**