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1 **Analysis of ATG8 family members using LC3-Interacting Regions (LIR)-based**
2 **molecular traps**

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16
17
18 **Abstract**

19 The ATG8 family of proteins regulates the autophagy process from the autophagosome
20 maturation and cargo recruitment up to degradation. Autophagy dysfunction is involved in
21 the development of multiple diseases. The LC3-Interacting Regions (LIR)-based molecular
22 traps have been designed to isolate endogenous ATG8 proteins and their interactors in
23 order to facilitate the study of selective autophagy events. Here, we summarise protocols
24 describing LC3 traps and sample preparation as well as adaptations for the analysis of
25 ATG8 proteins in different biological models. This protocol was optimised to prepare

26 affinity columns, reduce background and improve the protein recovery to be analysed by
27 immunodetection with antibodies recognising proteins of interest.

28

29 **Keywords**

30 LIR, ATG8-family, LC3, GABARAP, Autophagy, Isolation, Purification.

31 **Running head:** Analysis of ATG8 molecules using LIR-based traps

32

33 **1. Introduction**

34 Autophagy is a conserved mechanism that drives bulk degradation in mammalian, plants,
35 insects and fungi. This degradation process occurs within an organelle called
36 autophagosome that fuses with mammalian lysosomes or plant/yeast vacuoles after
37 substrate engulfment (1). Distinct autophagy mechanisms selectively eliminate different
38 types of cargoes through the lysosomes. Therefore, several types of selective autophagy
39 pathways have been identified depending on the nature of the cargo (2). For example,
40 mitophagy, xenophagy, proteaphagy are the names for selective autophagy of
41 mitochondria, pathogens or proteasomes, respectively.

42 Among the AuTophagy (ATG) proteins identified in yeast, ATG8 is essential for the
43 autophagy regulatory cascade (3). ATG8 homologs in other species have been distinctly
44 named LGG1 and LGG2 in *C. elegans* or LC3/GABARAP in mammals (4, 5). LC3B is
45 often used as autophagy activation marker in human cells. Lipidated ATG8 proteins are
46 tethered into the autophagosome membrane, hence driving autophagosome maturation and
47 cargo recruitment through a complex combination of autophagy receptor, cofactors and
48 ubiquitin signals (2). Consequently, ATG8 proteins have been extensively used as
49 biomarkers to monitor autophagosome formation, localisation and dynamics in
50 physiological and pathological conditions (6).

51 To easily isolate and analyse ATG8 proteins from various biological models, we developed
52 innovative molecular tools as an alternative to expensive antibodies or probes
53 overexpression. These tools named LC3 traps contain multiple LC3-Interacting Regions
54 (LIR) disposed in tandem that efficiently isolate endogenous ATG8 from cell lysates (7)
55 (see **Figure 1**). Distinct LC3-traps were developed based in LIR consensus sequences with
56 hydrophobic core residues W (Wx8-LC3 traps), F (Fx8-LC3 traps). These easy-to-use
57 tools enhance the attractiveness of the LC3 traps to monitor selective autophagy events.
58 Protocols detailing the production and use of LC3 traps for purification of ATG8-
59 associated complexes in several biological models are detailed below.

60

61 **2. Materials**

62 Prepare solutions with ultrapure water (8 M Ω x cm at 25°C) and use analytical grade
63 reagents. Store all reagents at 4°C unless indicated otherwise. If sodium azide is not added
64 to stock reagents, it is advised to prepare solutions as fresh as possible. To avoid
65 contaminations, solution can be filtered and conserved as frozen aliquots.

66

67 **2.1 Buffers and reagents**

- 68 1. GST expressing plasmid (i.e. pGEX-6P-1).
- 69 2. Bacteria culture media: supplement Luria Broth (LB) and LB-agar with 100 μ g/ml of
70 carbenicillin or 100 μ g/ml of ampicillin when indicated.
- 71 3. 1 M isopropyl β -D-1-thiogalactopyranoside (IPTG) stock solution.
- 72 4. Sterile cold 1x Phosphate Buffered Saline (PBS).
- 73 5. Empty plastic 30 ml columns for affinity chromatography.
- 74 6. Glutathione agarose beads
- 75 7. Dialysis tubing with a 12.4 kDa cut-off

- 76 8. Bacteria Lysis buffer (BL): 1x PBS, 0.8 M NaCl, 1 mM phenylmethylsulfonyl
77 fluoride (PMSF) and 2 mM benzamidine (*see Note 1*).
- 78 9. Fx8-LC3 and Wx8-LC3 traps
- 79 10. Cell lysis/Binding buffer (CLB): 20 mM phosphate buffer pH 7.5 (Na₂HPO₄,
80 NaH₂PO₄) containing 1% Igepal, 2 mM EDTA, 50 mM sodium fluoride, 5 mM tetra-
81 sodium pyro-phosphate, 10 mM β-glycerol-2-phosphate, 1.2 mg/ml complete protease
82 inhibitor cocktail. Filter through 0.22 μm membrane and store at 4°C (*see Note 1*).
- 83 11. Binding washing buffer (WB): 1x PBS, 0.05% Tween-20.
- 84 12. Elution/Boiling buffer (EB): 50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS,
85 Bromophenol Blue, 5% β-mercaptoethanol.
- 86 13. Nematode growth media (NGM): 0.25% bacto-peptone, 2% bacto agar, 50 mM NaCl,
87 1 mM CaCl₂, 1 mM MgSO₄, 20 mM KH₂PO₄, 3.3 mM K₂HPO₄, 0.013 mM cholesterol.
- 88 14. *Escherichia coli* strain BL21 and OP50.
- 89 15. M9 buffer: 85 mM NaCl, 40 mM Na₂HPO₄, 20 mM KH₂PO₄, 1 mM MgSO₄ 7H₂O.
- 90 16. Glass beads 600 μm.
- 91 17. Mini Spin chromatography columns
- 92 18. Wild type yeast cells (W303 or BY4741).
- 93 19. pGFP-ATG8 plasmid.
- 94 20. Yeast minimal media (SD-Ura or SG-Ura): 0.67% (weight/volume, w/v) yeast
95 nitrogen base (YNB) without amino acids supplemented with 0.1 g/l of each amino acid
96 and nucleic acid base component, except uracil for the selection of the pGFP-ATG8-URA3
97 plasmid. The carbon source is 2% glucose for SD-Ura or 2% glycerol for SG-Ura.
- 98 21. Yeast starvation medium (SD-N): 0.17% (w/v) YNB without amino acids and
99 ammonium sulfate supplemented with 2% (w/v) glucose.

100

101 **2.2 Affinity Chromatography**

102 1. Prepare lyophilised glutathione beads by adding 3 volumes of de-ionised water per 10
103 ml of lyophilised glutathione-agarose beads (*see Note 2*). Rotate beads 30 minutes at room
104 temperature then overnight at 4°C. Wash agarose beads with ten volumes of de-ionised
105 water or 1x PBS three times (*see Note 3*). Centrifuge at 300 x g for 5 minutes to separate
106 beads/buffer phases. Finally, resuspend the agarose beads in PBS to obtain 50% (v/v)
107 slurry and store at 4°C until use.

108 2. LC3 traps can be produced as indicated below or purchased (BMolecular).

109

110 **2.3 Dialysis**

111 1. Dialysis Buffer: 1x PBS, 0.8 M NaCl.

112 2. Dialysis tubing 12.4 kDa cut-off.

113

114

115 **3. Methods**

116 Carry out all steps at 4°C unless specified otherwise. All the procedure is illustrated in

117 **Figure 2.**

118

119 **3.1 Purification of recombinant LC3 traps**

120 1. Transform *Escherichia coli* BL21 strain with plasmids expressing GST control or
121 GST-LC3 traps containing 8 LIRs and plate onto LB-agar plates supplemented with 100
122 µg/ml of carbenicillin (*see Note 4*) (7).

123 2. Grow 1 single colony of transformed bacteria in precultures of 30 ml of LB

124 supplemented with 100 µg/ml of ampicillin, overnight at 28°C with shaking.

- 125 3. Grow the cells in 1 L of LB plus 100 µg/ml of ampicillin at 37°C with shaking. Start
126 with a 0.05-0.1 OD₆₀₀ culture that should reach 0.4-0.5 OD₆₀₀ in approximately 3-4 hours.
127 Put aside one aliquot (1 ml) of culture as control before induction with IPTG.
- 128 4. Induce expression of LC3 traps by addition of 1 mM of IPTG and shake culture
129 overnight at 20°C. Put aside an aliquot (1 ml) of culture after induction with IPTG.
- 130 5. Centrifuge saved 1 ml aliquots and wash bacterial pellets with 1x PBS. Discard the
131 supernatant and resuspend the pellet in 100 µg of EB buffer. Shake and boil samples at
132 95°C during 30 minutes.
- 133 6. Run both samples in SDS-PAGE. Stain gels with Coomassie blue to verify the
134 induction of the GST-LC3 traps with IPTG.
- 135 7. Centrifuge 1 L of bacterial culture at 6,600 x g during 30 minutes at 4°C. Wash pellets
136 twice with cold 1x PBS. Discard supernatant and store pellets at -20°C until use (*see Note*
137 **5**).
- 138 8. Resuspend frozen pellets in 20 ml of BL buffer.
- 139 9. Sonicate cell pellets at 30% Amp during 30 seconds. Repeat 6 times and between each
140 pulse incubate 1 minute on ice to avoid sample heating.
- 141 10. Supplement lysates with 2% (v/v) of Triton X-100, then rotate for 15 minutes at 4°C.
- 142 11. Clarify lysates by ultracentrifugation for 1 hour at 48,000 x g and 4°C. Save 100 µg of
143 clarified lysates (input) to be analysed together with all other fractions.
- 144 12. Equilibrate the glutathione-agarose beads (1 ml of beads per L of bacterial culture)
145 with BL buffer containing 2% (v/v) Triton X-100.
- 146 13. Incubate clarified lysates with equilibrate beads and supplemented with 2 mM DTT.
147 Incubate overnight rotating at 4°C (incubation time can be reduced at 4 hours) (*see Note*
148 **6**).

- 149 14. Load agarose beads into an empty plastic column. Collect flow through (FT) to verify
150 if most GST protein was captured in the column (*see Note 7*).
- 151 15. Wash 4 times with 10 column volumes of BL buffer supplemented with 1% Triton X-
152 100.
- 153 16. Elute LC3 traps with 6-12 fractions of 1 ml of 50 mM Tris-HCl pH 9.5 supplemented
154 with 0.8 M NaCl and 10 mM of reduced glutathione. Verify the protein elution peak by
155 using Bradford assay for each fraction (*see Note 8*).
- 156 17. Dialyse eluted fraction against PBS supplemented with 0.8 M NaCl. Load all samples
157 in one or two dialysis tubing with a 12.4 kDa cut-off. Perform dialysis at 4°C during 24
158 hours changing buffer every 2-3 hours of dialysis (*see Note 9*).
- 159 18. Concentrate LC3 traps up to 5 µg/µl by using ultracentrifuge filter with a 3 kDa
160 molecular weight cut-off (*see Note 10*). Protein concentration can be estimated by UV
161 absorbance.
- 162 19. Store purified LC3 traps at -80°C in PBS 0.8 M NaCl containing 10% glycerol in
163 small aliquots. Avoid refreezing samples (*see Note 11*).

164

165 **3.2 Pull down of recombinant ATG8 proteins**

- 166 1. Equilibrate glutathione-agarose beads with 1x PBS supplemented with 0.1% Igepal and
167 2 mM DTT.
- 168 2. Dilute the recombinant ATG8 proteins in 1x PBS supplemented with 0.1% Igepal and 2
169 mM DTT. The final volume should integrate the amount of LC3 traps required for the pull
170 down (*see Note 12*).
- 171 3. Save 1/10 of the sample to be used as input fraction. Dilute the sample in equal volume
172 of EB buffer to be analysed with other fractions.

- 173 4. Add to the 100 μ l of dried equilibrated glutathione-agarose beads, sufficient BL buffer
174 volume to complete the final volume of reaction of 500 μ l.
- 175 5. Incubate binding reaction by rotating at 4°C for 1 hour (*see Note 13*).
- 176 6. Centrifuge beads at 300 x g during 5 minutes at 4°C and collect supernatant (FT
177 fraction) for analysis.
- 178 7. Wash the beads three times with 1 ml of cold 1x PBS supplemented with BW buffer,
179 centrifuge beads at 300 x g during 5 minutes at 4°C. Discard supernatant and make sure no
180 liquid remains in the beads.
- 181 8. To elute bound fraction from beads using a shaker-incubator, add 100 μ l of EB buffer
182 and heat 5 minutes at 95°C with shaking at 1,000 rpm.
- 183 9. Analyse 10 μ l of eluted fractions in PAGE (15% polyacrylamide) according to the
184 molecular size of AT8 proteins of interest. Stain gels by Coomassie blue or detect proteins
185 by western blot (*see Note 14*).

186

187 **3.3 Capture and identification of ATG8 proteins from distinct biologic models**

188

189 3.3.1 Lysis of mammalian cells

- 190 1. Grow cells until the desired confluence. For example, 1-2 mg total proteins can be
191 extracted from 10-20 x 10⁶ HeLa cells (150 mm plate) after 48 hour growth. If any
192 treatment is used, set up conditions to select the best time of treatment to better accumulate
193 LC3/GABARAP proteins (e.g. bafilomycin A1) (*see Note 15*).
- 194 2. Before cell harvesting, wash them twice with 1x PBS to eliminate serum and treatments
195 used.
- 196 3. Centrifuge cells for 5 minutes at 500 x g at room temperature and remove supernatant.
- 197 4. Add 800 μ l to 1 ml of CLB on the pellet and slowly homogenise cells.

- 198 5. Incubate 15 minutes at 4°C (on ice).
- 199 6. Centrifuge lysed cells 15 minutes at 15,500 x g at 4°C and transfer supernatants.
- 200 7. Quantify protein in the extracts using Bradford assay, for example.
- 201 8. Incubate 10 minutes at 4°C cell extracts containing 0.5-1 mg of total proteins with 100
- 202 µg of LC3-trap to reach a final volume of 500 µl of CLB (*see Note 12 and Note 16*). Use
- 203 GST protein to control binding background.

204

205 3.3.2 Lysis of nematodes

- 206 1. The conditions for culturing nematode strains are very classical and will not be
- 207 detailed here. Synchronise worms and grow them on NGM, fed with *Escherichia coli*
- 208 strain OP50 (*see Note 17 and Note 18*).
- 209 2. Collect the worms at the 4th larval or adult stages by washing the plates with M9
- 210 buffer (*see Note 19*). Centrifuge 1 minute at 100 x g and remove the supernatant.
- 211 3. Wash the pellet in M9 buffer, centrifuge 1 minute at 100 x g and remove the
- 212 supernatant. Pellets of worms can be frozen at -80°C for several months.
- 213 4. Add a volume of CLB equal to the volume of the pellet and 100-200 µl of glass beads
- 214 of 600 µm (*see Note 20*).
- 215 5. Perform 2 cycles of grinding at 6,000 rpm for 1 minute in the pre-cooled shaking
- 216 machine (*see Note 21*). Total protein extracts can be frozen at -80°C at this stage.
- 217 6. Incubate for 10 minutes at 4°C cell pellets containing 0.5-1 mg of total proteins with
- 218 500 µl CLB supplemented with 100 µg of LC3-trap. Include GST as control.

219

220 3.3.3 Lysis of Yeast

- 221 1. Inoculate 1 single colony of wild type yeast cells (W303 or BY4741 for instance)
222 transformed with the pGFP-ATG8 plasmid (*see Note 22 and Note 23*) (8) into 2 ml of SD-
223 Ura and grow the cells overnight at 30°C with shaking.
- 224 2. In the morning, dilute the cells in 20 ml of SD-Ura to an OD₆₀₀ of 0.1 and grow the
225 cells at 30°C with shaking, until late exponential phase (OD₆₀₀: 1.5-2). At this step, the
226 level of GFP-ATG8 is very low.
- 227 3. Dilute the cells in 100 ml of SG-Ura to an OD₆₀₀ of 0.1. Grow the cells overnight at
228 30°C with shaking, to an OD₆₀₀ >1 (*see Note 24 and Note 25*). After 16-18 hours of
229 growth in glycerol containing media, the level of GFP-ATG8 is enhanced and some free
230 GFP resulting from GFP-ATG8 cleavage in the vacuole, a hallmark of autophagy, is
231 detected (7, 9).
- 232 4. Pellet cells with a 4,000 x g centrifugation during 5 minutes and wash pellets twice
233 with an equivalent volume of sterile water (*see Note 26*).
- 234 5. Incubate the cells at 30°C with shaking during 2 hours in an equivalent volume of
235 nitrogen depleted medium (SD-N) to induce mitophagy (7, 9) (*see Note 27*).
- 236 6. Measure the OD₆₀₀ of the yeast culture and harvest 100 OD₆₀₀ units by centrifugation
237 at 4,000 x g during 5 minutes. Resuspend the cells at a concentration of 1 mg/ml of yeast
238 cells in cold CLB.
- 239 7. Add an equal volume of 425-600 µm acid washed glass beads and break the cells by
240 vortexing at 4°C with 5 cycles of 30 seconds with 5-minute rests.
- 241 8. Transfer the yeast cell lysate and the beads on a mini spin chromatography column, to
242 get rid of glass beads without loosing cell lysate (optional), and centrifuge during 1 minute
243 at 3,500 x g and 4°C.

- 244 9. Centrifuge the elution from the column during 10 minutes at 3,500 x g and 4°C (*see*
245 **Note 28**). Collect supernatant and centrifuge again 10 minutes at 3,500 x g and 4°C (*see*
246 **Note 29**). Quantify protein content in the supernatant using a Bradford assay.
- 247 10. Mix 500 µg to 1 mg of total proteins with 500 µl CLB supplemented with 100 µg of
248 either LC3-trap or GST used as a control.
- 249 11. Incubate 10 minutes at 4°C.

250

251 3.3.4 Pull down and analysis of distinct ATG8 family members

- 252 1. Clarify cell lysate by centrifugation at 15,500 x g at 4°C for 30 minutes (*see Note 30*).
- 253 2. Take 1/10 of cell lysate volume to be used as input fraction. Dilute in an equal volume
254 of EB buffer to be analysed with other fractions.
- 255 3. Add 500 µl of the clarified lysate to 100 µl of dried glutathione-agarose beads to
256 complete 600 µl final volume of binding reaction.
- 257 4. Supplement with 1 mM of DTT to improve GST binding to the glutathione column.
- 258 5. Incubate reactions by rotating 4 to 6 hours at 4°C. Overnight binding can be considered
259 after verification that LC3 traps are not saturated (*see Note 31*).
- 260 6. Centrifuge beads at 300 x g during 5 minutes at 4°C and collect supernatant (FT
261 fraction) for analysis.
- 262 7. Wash three time with 1 ml of BW buffer, centrifuge beads at 300 x g during 5 minutes at
263 4°C. Discard supernatant until no liquid remains in the beads (*see Note 32*)
- 264 8. Elute bound fraction from the beads with 100 µl of EB buffer and heat 5 minutes at
265 95°C shaking-incubator 1,000 rpm (*see Note 33*).
- 266 9. Analyse eluted fractions (10 µl) on 8-15% PAGE according to the molecular size of the
267 protein of interest and detect by western blot. All other fractions can be analysed at this
268 point to control capture efficiency (*see Note 14*).

269 10. Typical results are illustrated in **Figure 3**.

270

271 **4. Notes**

272 **1.** Add protease inhibitors just before using the buffer.

273 **2.** Different commercially available glutathione beads have different sizes and are covered
274 with distinct densities of glutathione. Make sure to work with beads having at least the
275 capacity to capture 10 mg of protein per ml of glutathione dried beads.

276 **3.** Glutathione beads may come already in solution when purchased from distinct
277 distributors. In all cases, eliminate preservative reagents by exhaustively washing with 1x
278 PBS. Just before use, equilibrate beads in CLB.

279 **4.** Carbenicillin can be substituted to ampicillin and is preferred for LB-agar plate selection
280 since it is more resistant to light and temperature and can be stored for longer periods.

281 **5.** Long term storage of pellets can be done at -80°C. Thaw pellets on ice with direct
282 contact with the BL buffer.

283 **6.** GST binding to glutathione is a slow process, a minimum of 4 hour binding is
284 recommended to obtain >80% of protein binding to beads.

285 **7.** Store the FT at -20°C. If after gel verification this fraction contains a significant amount
286 of GST/LC3-trap protein, load it again into a new column to recover more protein.

287 **8.** Usually, protein peaks between fraction 2 and 7 but it depends on the quality of the
288 induction.

289 **9.** Most glutathione is eliminated during the first 2-3 hours of dialysis. However, to
290 optimise the recovery of the LC3-trap and captured ATG8 molecules from cell extracts, it
291 is highly recommended to extend the dialysis time.

292 **10.** LC3 traps are highly hydrophobic and the limit of solubility is around 3-5 mg/ml.

293 **11.** LC3 traps are highly hydrophobic and are thus prone to aggregation. It is advisable to
294 prepare small aliquots conserved at -20°C for short term storage and -80°C for long term
295 storage. Storage buffer contains 10% glycerol. LC3 traps should not be frozen and thawed
296 more than 2-3 times.

297 **12.** To avoid saturation of the LC3 traps, do not use too many proteins in the input
298 compared to the quantity of the traps added. The optimal amount of LC3 traps used
299 depends on the relative abundance of ATG8 proteins available in the samples. It is
300 recommended to set up conditions by analysing by western blot the different fractions from
301 the pull-down experiments to optimise the traps/target ratio. For example, for an optimised
302 pull down of recombinant GABARAP proteins using Fx8-LC3 traps, we recommend to
303 use at least 0.25 molar of traps per molar of target (i.e. traps/target molar ratios superior to
304 1:4).

305 **13.** Binding time could be decreased up to 30 minutes if the concentration of ATG8
306 proteins is high.

307 **14.** Ponceau, Coomassie and silver stainings provide useful information about size and
308 transfer of proteins, as well as efficiency and background of pull-down experiments
309 performed. These controls are strongly recommended while setting up a pull-down
310 experiment with LC3 traps.

311 **15.** This could be achieved by analysing total cell extracts by western blot or by
312 immunofluorescence.

313 **16.** The total protein concentration used depends on the cell type and relative abundance of
314 LC3/GABARAP proteins present in each cell type. Cell type depending settings are
315 recommended to be established before capture with LC3 traps. Alternative lysis buffer and
316 procedures can be considered as long as they do not alter LC3 traps conformation.
317 Compatibility of lysis buffer with an optimal LC3 trap capture must be performed.

318 **17.** Use nematode strains according to the needs of the experiments, for instance wild-type
319 strain (N2) versus transgenic strains of interest.

320 **18.** Worms can also be grown in liquid culture.

321 **19.** A minimum of 5,000 worms is needed but scaling up the culture allows to freeze
322 aliquots.

323 **20.** Grinding can be done using 1.4 mm and 2.8 mm ceramic beads (zirconium oxide) three
324 times for 20 seconds at 6,500 rpm separated by 30-second pauses.

325 **21.** It is essential to avoid heating of the samples. If a non-refrigerated grinder is used
326 samples must be kept on ice during 5 minutes between the 2 cycles.

327 **22.** The pGFP-ATG8 plasmid is optional since the traps interact with the LIR domain.
328 However, it is a precious tool to precisely follow induction of mitophagy by fluorescence
329 microscopy and western blot.

330 **23.** GFP-ATG8 is under the control of its natural promoter.

331 **24.** The doubling time for wild type yeast cells is different in glucose and glycerol
332 containing media. Depending on the genetic background, the doubling time can be 3 times
333 higher in glycerol (>180 minutes) *versus* glucose containing media (60-120 minutes).

334 **25.** Growth in glycerol induces respiration and enhances the mitochondrial mass.

335 **26.** These washing steps are essential to get rid of any trace of nitrogen.

336 **27.** In glucose containing SD-N media the cells undergo fermentation and the absence of
337 nitrogen induces autophagy. In such conditions, mitochondria are less essential. They can
338 be “sacrificed” and degraded by mitophagy.

339 **28.** During this step unbroken cells and cell debris are pelleted and discarded.

340 **29.** Repeat this step, if necessary, until all cell debris are pelleted.

341 **30.** These cell extracts are mainly cytoplasmic proteins. If total cell extracts are required,
342 cell lysis by sonication is recommended.

343 **31.** In case saturated signals of ATG8 proteins are observed in the FT fraction, binding
344 time can be increased up to overnight to optimise the capture of the target. If increasing the
345 binding time is not enough, consider increasing the amount of LC3 traps used.

346 **32.** To reduce background in the bound fraction, perform up to 6 washing steps with cold
347 BW buffer. Tween 20 concentration in 1x PBS can also be increased to 0.1%. Be aware
348 that increasing the stringency of the washes will reduce the binding of specific interacting
349 proteins.

350 **33.** pH of elution buffer must be between 8.5 and 9.5 for an optimal elution. A single
351 elution may not be sufficient. A second elution could be considered to elute proteins
352 retained to the beads. If a more concentrated eluted fraction is needed, the volume of EB
353 buffer can be reduced. Glutathione-agarose beads must be soaked in the EB buffer.

354

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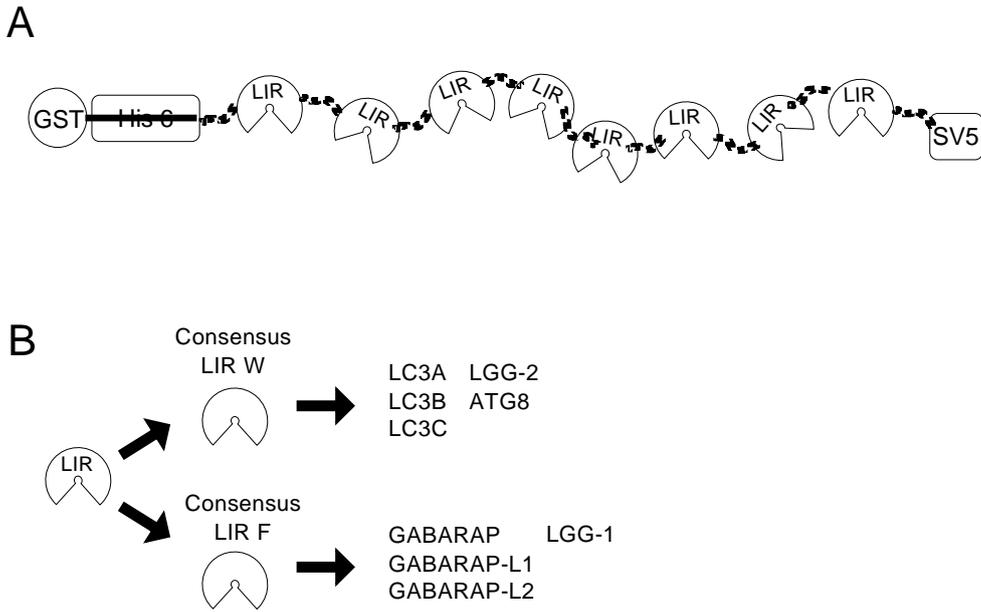
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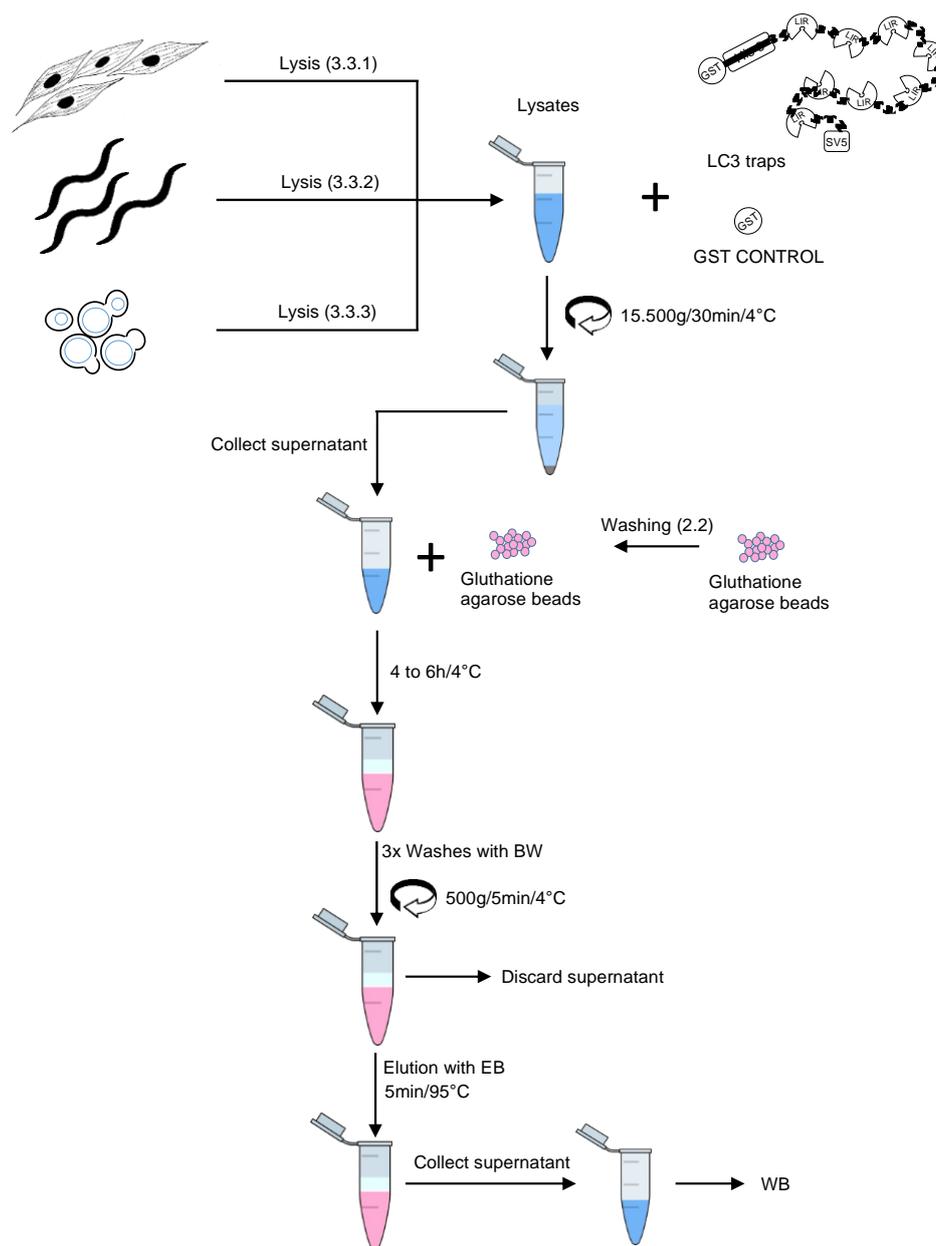
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394

395 **Figure 1. LC3 traps and molecules captured.** A) Cartoon representing LC3 traps
 396 containing 8 LIR motifs expressed in a GST vector tagged with an SV5 epitope at the C-
 397 terminus. B) Molecules that can be efficiently captured by the two LC3 traps based in a F
 398 and W LIR consensus.



399

400

Figure 2. Procedure for purification of ATG8 molecules using LC3 traps. The main

401

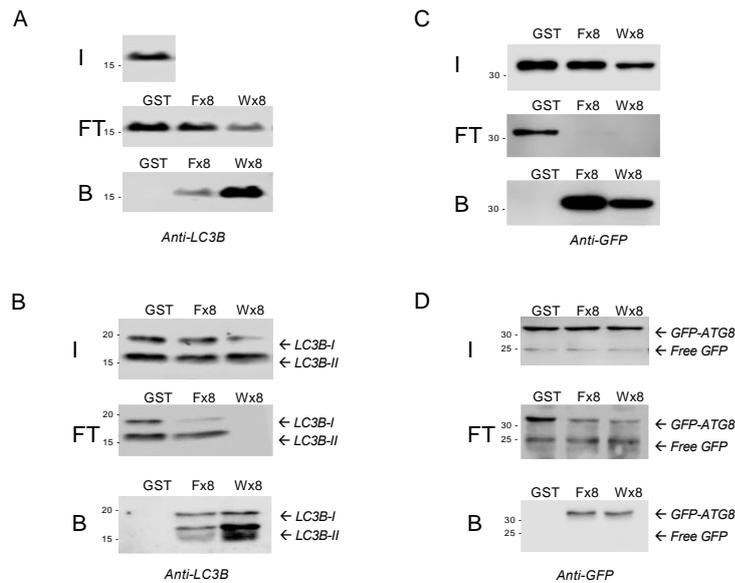
steps for the purification of ATG8 proteins from different species are illustrated. In

402

parenthesis, the sections with specific information are indicated. Washing Buffer (WB),

403

Elution Buffer (EB).



404

405

Figure 3: Examples of typical results obtained by pulling down ATG8 proteins in

406

several biological models using LC3 traps. A) Pull down of recombinant LC3B proteins.

407

1 μ g of recombinant LC3B proteins were incubated with 5 μ g of both LC3 traps for 20

408

minutes. Input, FT and bound fractions (I, FT, B, respectively) were blotted against LC3B.

409

B) Pull down of LC3B proteins from human cells. Cells were preincubated for 1 hour with

410

200 mM bafilomycin A1 to accumulate LC3-labelled autophagosomes. 1 mg of total

411

proteins from HeLa cells lysate were incubated for 6 hours with 100 μ g of LC3 traps. Input,

412

FT and bound fractions (I, FT, B) were blotted against LC3B. Unconjugated LC3B-I and

413

lipidated LC3B-II can be observed. **C) Pull down of LGG-1 proteins from *C. elegans***

414

strains stably expressing GFP-LGG-1. 500 μ g of total proteins from worm lysates were

415

incubated 6 hours with 100 μ g of LC3 traps. Input, FT and bound fractions (I, FT, B) were

416

blotted against GFP. **D) Pull down of ATG8 proteins from wild type yeast cells expressing**

417

GFP-ATG8. Yeast were grown for 18 hours before lysis in glycerol containing medium to

418

the stationary phase to accumulate ATG8 proteins. 500 μ g of total proteins from yeast

419

lysates were incubated 6 hours with 100 μ g of LC3 traps. Input, flow through and bound

420

fractions (I, FT, B) were blotted against GFP. GFP-ATG8 and free GFP can be observed.

421 GFP-ATG8 and free GFP can be observed. GFP is transiently resistant to vacuolar
422 proteases and the appearance of free GFP, a result of the degradation of GFP-ATG8 in the
423 vacuole, is indicative of an active autophagy.

424