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Studying protein ubiquitylation in yeast.

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Running head : Studying protein ubiquitylation in yeast.

1 **i. Summary/Abstract**

2 Ubiquitylation is a reversible post-translational modification that is critical for most, if not
3 all, cellular processes and essential for viability. Ubiquitin conjugates to substrate proteins
4 either as a single moiety (monoubiquitylation) or as polymers composed of ubiquitin
5 molecules linked to each other with various topologies and structures (polyubiquitylation).
6 This contributes to an elaborated ubiquitin code that is decrypted by specific ubiquitin-
7 binding proteins. Indeed, these different types of ubiquitylation have different functional
8 outcomes, notably affecting the stability of the substrate, its interactions, its activity or its
9 subcellular localization. In this chapter, we describe protocols to determine whether a
10 protein is ubiquitylated, to identify the site that is ubiquitylated, and provide direction to
11 study the topology of the ubiquitin modification, in the yeast *Saccharomyces cerevisiae*.

12

13 **ii. Key Words**

14 Yeast – ubiquitin – Histidine-tagged ubiquitin purification – immunoprecipitation in
15 denaturing conditions – ubiquitylation site mapping – ubiquitin chain topology

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22 **1. Introduction**

23 The conjugation of ubiquitin (hereafter referred to as “ubiquitylation”) is a complex
24 modification that can alter a protein’s ability to interact with other proteins, and as such
25 impacts on its stability, localization or function [1]. Ubiquitin is generally conjugated on the
26 ϵ -amino group of lysine residues of the target substrates, also called monoubiquitylation.
27 However, ubiquitin possesses itself 8 amino- groups (N-terminal + 7 lysines) that can be
28 used for ubiquitin conjugation, thereby generating a poly-ubiquitin chain
29 (polyubiquitylation). Hence, several factors will contribute to the signal generated by
30 ubiquitylation, such as the identity of the residue targeted by ubiquitin on the substrate, the
31 type of modification: mono- vs. polyubiquitylation, and in the latter case, the topology of the
32 chain (i.e. which lysine residue of ubiquitin is used for chain elongation) and its length [1].
33 Various ubiquitin-binding domains contribute to the decoding of this modification [2].
34 Consequently, different types of ubiquitylation create a wide range of molecular signals that
35 can lead to various functional outcomes, and altogether ubiquitylation contributes to many,
36 if not all, cellular pathways.

37 Ubiquitin is best known for its role as a signal for the recognition and subsequent
38 degradation of target proteins by the proteasome, which is mainly mediated by K48-linked
39 polyubiquitin chains. However, chains of all ubiquitin linkages have been identified and the
40 study of their structure and function is still undergoing [3]. K63-linked polyubiquitylation is
41 notably well known for its role in DNA repair [4-6], the regulation of signaling pathways [7]
42 or membrane trafficking [8,9]. Although K48-linked polyubiquitylation represents only a
43 fraction of the possible combinations of the ubiquitin code, it is the most abundant in all
44 cells studied to date [10-14]. This type of ubiquitylation is often a transient event in the cell,
45 occurring before the target protein is degraded, and can therefore be difficult to detect
46 experimentally.

47 Furthermore, ubiquitylation is a reversible modification. Several family of proteases act
48 as ubiquitin isopeptidases that cleave ubiquitin off substrates and process ubiquitin chains,
49 allowing a fine regulation of the ubiquitylation signal [15]. Therefore, another technical
50 problem when studying protein ubiquitylation is due to the activity of these enzymes when
51 preparing a protein extract in native conditions (eg. during an immunoprecipitation).

52 Here, we describe a protocol that allows the identification of ubiquitylated proteins in
53 denaturing conditions in yeast. This is based on a construct allowing the expression of
54 polyhistidine-tagged ubiquitin, which allows the purification of ubiquitylated proteins in
55 denaturing conditions by immobilized metal-ion affinity chromatography (IMAC).

56 Evidence that a protein is ubiquitylated can also be obtained by performing the reverse
57 experiment, in which a protein of interest is immunoprecipitated and the resulting sample is
58 blotted with anti-ubiquitin antibodies. In this type of experiment, the immunoprecipitation
59 should be performed on denatured samples, to ensure that the ubiquitylation signal detected
60 originates from the protein considered, and not from its potential interactants. Therefore, we
61 describe another protocol to achieve immunoprecipitation in denaturing conditions.

62 The identification of ubiquitylated sites on a protein of interest is often instrumental to
63 understand the functional contribution of this modification. We describe two possible
64 approaches that can lead to the identification of the ubiquitylation site on a protein. The first
65 approach is a tandem purification procedure to purify the protein of interest in denaturing
66 conditions. This may be helpful to identify ubiquitylation sites by mass spectrometry. We
67 also describe a general, truncation-based genetic method to identify the ubiquitylated sites
68 on a protein.

69 Finally, we will discuss on the possibilities offered to define the topology of ubiquitin
70 modification (linkage used within an ubiquitin chain on a substrate) and will provide an

71 example using a set of yeast strains carrying ubiquitin mutations, developed in the Finley lab
72 [5].

73

74

75 **2. Materials**

76 Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a
77 sensitivity of 18 MΩ cm at 25°C) and analytical grade reagents. Prepare and store all
78 reagents at room temperature (unless indicated otherwise).

79 **2.1 Components required for the visualization of ubiquitylation on crude extracts**

80 **(Section 3.1)**

- 81 1. Yeast culture medium (synthetic complete medium, SC): 1.7 g/L yeast nitrogen base ,
82 5 g/L ammonium sulfate , 20 g/L glucose). Autoclave. Add sterile-filtered amino
83 acid solution as required (e.g. dropout bases from Elis Solutions, Erpent, Belgium).
- 84 2. Plasmid encoding His-tagged ubiquitin (see section 3.1)
- 85 3. 100% TCA (Trichloroacetic acid) solution (w/v, i.e. 6.1 M). Store at 4°C.
- 86 4. 10% TCA (Trichloroacetic acid) solution (w/v, i.e. 0.61 M). Store at 4°C.
- 87 5. Vortex equipped with a microtube foam insert (placed in a cold room)
- 88 6. Glass beads: 0.4-0.6 mm (BBI-8541701, Sartorius Mechatronics)
- 89 7. Needles: 23G x 1" – 0.6 x 25 mm (Terumo Medical Corporation)
- 90 8. 5x Sample buffer (250 mM Tris-HCl pH 6.8, 500 mM dithiothreitol , 10% SDS ,
91 0.01% bromophenol blue ,50% glycerol). Store at -20°C.
- 92 9. 1M Tris-base: 1M Tris, non-buffered, in water.

- 93 10. 1x Sample buffer for TCA precipitates: 1 vol. of 5x Sample buffer , 1 vol. of Tris-
94 base, and 3 vol. water. Store at -20°C.
- 95 11. General equipment for SDS-PAGE and western blotting (gel casting, solutions, tanks
96 for migration/transfer, nitrocellulose membrane etc.)
- 97 12. Antibody directed against the protein of interest (or its tag if applicable)

98 **2.2 Components required for the purification of ubiquitin conjugates using poly-His-**
99 **tagged ubiquitin (Section 3.2)**

- 100 1. Yeast culture medium (synthetic complete medium, SC): 1.7 g/L yeast nitrogen base,
101 5 g/L ammonium sulfate, 20 g/L glucose). Autoclave. Add sterile-filtered amino acid
102 solution as required (e.g. dropout bases from Elis Solutions, Erpent, Belgium).
- 103 2. Plasmid encoding His-tagged ubiquitin (see section 3.1)
- 104 3. 100% TCA (Trichloroacetic acid) solution (w/v, i.e. 6.1 M). Store at 4°C.
- 105 4. 10% TCA (Trichloroacetic acid) solution (w/v, i.e. 0.61 M). Keep at 4°C.
- 106 5. Glass beads: 0.4-0.6 mm (BBI-8541701, Sartorius Mechatronics)
- 107 6. Vortex equipped with a microtube foam insert (placed in a cold room)
- 108 7. Needles: 23G x 1" – 0.6 x 25 mm (Terumo Medical Corporation)
- 109 8. 1M Tris-base: 1 M Tris, non-buffered, in water.
- 110 9. Buffer A: 6 M Guanidium-HCl, 20mM Tris pH8.0, 100 mM K₂HPO₄, 10 mM
111 imidazole, 100 mM NaCl, 0.1% Triton X-100 (v/v).
- 112 10. Ni-NTA superflow (Qiagen)
- 113 11. Micro Bio-Spin™ Chromatography Column (Bio-Rad)
- 114 12. Wash1 buffer: 20 mM Tris pH8.0, 100 mM K₂HPO₄, 20 mM imidazole, 100 mM
115 NaCl, 0.1% Triton X-100 (v/v).
- 116 13. Wash2 buffer: 20mM Tris pH8.0, 100 mM K₂HPO₄, 10 mM imidazole, 1 M NaCl,
117 0.1% Triton X-100 (v/v).

- 118 14. Elution buffer: 20 mM Tris pH8.0, 100 mM K₂HPO₄, 500 mM imidazole, 100 mM
119 NaCl. Store at 4°C for no longer than a month.
- 120 15. 5x Sample buffer: Tris-HCl 250 mM pH 6.8, dithiothreitol 500 mM, SDS 10%,
121 bromphenol blue 0.01%, glycerol 50%. Store at -20°C.
- 122 16. 100% acetone, ice-cold.
- 123 13. Ponceau S solution: 0.2% ponceau S (w/v), 3% TCA (w/v). Store at 4°C.
- 124 14. General equipment for SDS-PAGE and western blotting (gel casting, solutions, tanks
125 for migration/transfer, nitrocellulose membrane etc.)
- 126 15. Antibody directed against ubiquitin (e.g. mouse monoclonal P4D1 antibody: sc-
127 8017, Santa Cruz Biotech; use at 1/10000) and the protein of interest (or its tag if
128 applicable). Store at 4°C or -20°C.

129 **2.3 Components required for immunoprecipitation in denaturing conditions (Section**
130 **3.3)**

- 131 1. Yeast culture medium (synthetic complete medium, SC): yeast nitrogen base 1.7 g/L,
132 ammonium sulfate 5g/L, glucose 2% w/v). Autoclave. Add sterile-filtered amino
133 acid solution as required (e.g. dropout bases from Elis Solutions, Erpent, Belgium).
- 134 2. 10% TCA (Trichloroacetic acid) solution (w/v, i.e. 0.61 M). Keep at 4°C.
- 135 3. Vortex equipped with a microtube foam insert (placed in a cold room)
- 136 4. Glass beads: 0.4-0.6 mm (BBI-8541701, Sartorius Mechatronics)
- 137 5. Needles: 23G x 1" – 0.6 x 25 mm (Terumo Medical Corporation)
- 138 6. Protein-G-coupled Sepharose (e.g. GammaBind™ G sepharose™, GE Healthcare),
139 or if using an HA-tag, anti-HA affinity matrix (Roche)
- 140 7. IP dilution buffer: 50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl, 1.2 % Triton
141 X-100, 0.5 % Bovine Serum Albumin, yeast protease inhibitor cocktail (e.g. Sigma

- 142 P821520, diluted 1:100), 20 mM N-ethylmaleimide. Store at 4°C for no more than a
143 week.
- 144 8. Buffer B0: SDS 2% (w/v), bromophenol blue 0.05 % (w/v).
- 145 9. Buffer B1: 50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 % Triton X-100, 0.2
146 % SDS. Store at 4°C.
- 147 10. Buffer B2: 50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl, 0.1 % Triton X-100,
148 0.5 % SDS, 0.5 % Na-deoxycholate. Store at 4°C.
- 149 11. Buffer B3: 50 mM Tris pH 7.5, 2 mM EDTA, 500 mM NaCl, 0.1 % Triton X-100.
150 Store at 4°C.
- 151 12. Buffer B4: 50 mM Tris pH 7.5, 2 mM EDTA, 100 mM NaCl. Store at 4°C.
- 152 13. 5x Sample buffer: Tris-HCl 250 mM pH 6.8, dithiothreitol 500 mM, SDS 10%,
153 bromophenol blue 0.01%, glycerol 50%. Store at -20°C.
- 154 16. 100% acetone, ice-cold.
- 155 17. 1x Sample buffer: 1 vol. 5x Sample buffer, 4 vol. water. Store at -20°C.
- 156 18. General equipment for SDS-PAGE and western blotting (gel casting, solutions, tanks
157 for migration/transfer, nitrocellulose membrane etc.)
- 158 19. Antibody directed against the protein of interest (or its tag if applicable) and against
159 ubiquitin (e.g. mouse monoclonal P4D1 antibody: sc-8017, Santa Cruz Biotech; use
160 at 1/10000 ; or sc-8017 HRP, Santa Cruz Biotech; use at 1/2000). Store at 4°C or -
161 20°C.

162 **2.4. Components required for the tandem purification of a protein in denaturing**
163 **conditions for the identification of ubiquitin-conjugated sites (Section 3.4)**

- 164 1. Yeast culture medium: synthetic complete medium (SC): yeast nitrogen base 1.7 g/L,
165 ammonium sulfate 5g/L, glucose 2% w/v). Autoclave. Add required aminoacid as
166 sterile-filtered solutions (e.g. CSM-Ura dropout, Elis Solutions, Erpent, Belgium).

- 167 2. Yeast culture medium: synthetic complete raffinose medium: yeast nitrogen base 1.7
168 g/L, ammonium sulfate 5g/L, raffinose (2% w/v), 0.02% glucose (w/v) (*see Note 1*).
- 169 3. Galactose solution, 20% (w/v), sterile-filtered.
- 170 4. pBG1805-based plasmid containing the protein of interest (commercially available at
171 OpenBiosystems/GE Healthcare Dharmacon).
- 172 5. Protein-G-coupled Sepharose (e.g. GammaBind™ G sepharose™, GE Healthcare).
- 173 6. IP lysis buffer: 50 mM HEPES-KOH pH7.5, 0.25M NaCl, 10% glycerol, 1mM
174 EDTA. Filter sterilize and store at 4°C.
- 175 7. Protease inhibitors: yeast protease inhibitor cocktail (Sigma P821520; use at 1:100
176 dilution, store at -20°C), phenylmethanesulfonyl fluoride (PMSF) (100 mM stock in
177 EtOH; store at -80°C), N-ethylmaleimide (NEM) (1 M stock in EtOH; store at -
178 80°C), and MG-132 (Enzo Life Sciences; 100 mM stock in DMSO; store at -80°C).
- 179 8. Antibodies directed against the HA tag (e.g. mouse monoclonal F7 antibody: sc-
180 7392, Santa Cruz Biotech). Store at 4°C or -20°C.
- 181 9. Glass beads: 0.4-0.6 mm (BBI-8541701, Sartorius Mechatronics)
- 182 10. Vortex equipped with a microtube foam insert (placed in a cold room)
- 183 11. Needles: 23G x 1” – 0.6 x 25 mm (Terumo Medical Corporation)
- 184 12. Triton X-100 solution: 25% (v/v) in water.
- 185 13. Urea buffer: urea 8M, 10 mM Tris pH 6.3; 5 mM imidazole, 10 mM beta-
186 mercaptoethanol, 10 mM N-ethylmaleimide, 100 μM MG-132.
- 187 14. Ni-NTA superflow (Qiagene)
- 188 15. 5x Sample buffer: 250 mM Tris-HCl pH 6.8, 500 mM dithiothreitol, 10% SDS,
189 0.01% bromphenol blue, 50% glycerol. Store at -20°C.
- 190 16. Urea elution buffer: 8M urea , 10 mM Tris pH 6.3, 600 mM imidazole. Store at 4°C.
- 191 17. General equipment for SDS-PAGE and western blotting (gel casting, solutions, tanks

192 for migration/transfer, nitrocellulose membrane etc.)
193 18. Antibodies directed against ubiquitin (e.g. mouse monoclonal P4D1 antibody: sc-
194 8017, Santa Cruz Biotech; use at 1/10000 ; or sc-8017 HRP, Santa Cruz Biotech; use
195 at 1/2000). Store at 4°C or -20°C.

196

197 **3. Methods**

198 **3.1. Obtaining evidence of ubiquitylation on crude extracts.**

199 When detecting the protein of interest by western blot, higher molecular weight species are
200 sometimes observed, which may be caused by post-translational modifications such as
201 ubiquitylation. The expression of a tagged (i.e. heavier) ubiquitin causes a shift in all
202 ubiquitin-conjugated species, which can sometimes be sufficient to obtain a first indication
203 that a given protein is ubiquitylated. Several plasmids available for this application have
204 been described in the literature, either driving the expression of His-tagged ubiquitin under
205 the control of a copper inducible promoter (pJD421, pYEp96– 6His-Ub)[16,17] or the
206 strong *ADHI* promoter (m886) [11]. Note that His-tagged ubiquitin was previously shown to
207 be functionally comparable to non-tagged ubiquitin [18]. For expression of His-tagged
208 ubiquitin driven by the *CUPI* promoter, we noticed that the traces of copper in the synthetic
209 medium (DO) are sufficient to drive the expression of His-Ub to a level comparable as
210 endogenous Ub and therefore we recommend not to add extra copper in the medium to avoid
211 potential artifacts that may be due to a massive ubiquitin overexpression.

212 Therefore, this protocol describes a classical method to prepare crude extracts from cells
213 expressing tagged ubiquitin or not, that may allow to determine whether a shift in molecular
214 weight is due to ubiquitylation. The example of a result is illustrated in **Figure 1**.

- 215 1. **DAY 1.** In the morning, inoculate freshly streaked yeast cells in synthetic medium
216 (SC) medium supplemented with the required amino-acids (preculture). Use a strain
217 expressing your protein of interest together with His-tagged ubiquitin, and as a
218 control, use the same strain but that does not contain the His-tagged ubiquitin
219 plasmid.
- 220 2. In the evening, measure the OD₆₀₀ of the cultures and inoculate yeast cells in 5 mL
221 SC medium, at OD₆₀₀= 0.001. Grow overnight under agitation (200 rpm) at 30°C
- 222 3. **DAY 2.** Measure the OD₆₀₀ of the cultures. The cultures should have reached an
223 OD₆₀₀ of ~0.3-0.5. (*see Note 2*).
- 224 4. Take 1 mL of culture and place in a 1.5 mL tube.
- 225 5. Add 100 μL of a 100% (w/v) TCA solution
- 226 6. Incubate on ice for 10 min.
- 227 7. Spin down the cells by centrifugation for 1 min at 13,000 g at room temperature
- 228 8. Discard the supernatant.
- 229 9. Resuspend yeast cells in 100 μL of a 10% TCA solution
- 230 10. Add glass beads up to 1-2 mm below the meniscus
- 231 11. Lyse cells on a vortex equipped with a microtube foam insert for 10 min at 4°C.
- 232 12. After lysis, open the tube, poke a small hole at the bottom of the tube using a needle
233 (Terumo 23 G x 1" - 0.6 x 25 mm), place into a clean 1.5-mL tube to collect the
234 lysate, and close the cap
- 235 13. Centrifuge for a few seconds on a mini-centrifuge to collect the lysate and leave the
236 beads in the upper tube. Discard the tube with the beads (they should be dry).
- 237 14. Centrifuge the lysate for 1 min at 13 000 g at RT.
- 238 15. Remove as much supernatant as possible.

- 239 16. Resuspend the pellet in 1x Laemmli buffer for TCA precipitates, considering that 1
240 OD₆₀₀ unit should be resuspended in 50 μL
- 241 17. Denature at the desired temperature (37°C-95°C) for 5-10 min.
- 242 18. Use 7 μL for SDS-PAGE and transfer onto nitrocellulose membrane.
- 243 19. Blot with antibodies directed against the protein of interest.

244

245 **3.2. Purification of ubiquitin conjugates using polyHis-tagged ubiquitin**

246 In this protocol, total ubiquitin conjugates from cells will be purified by immobilized metal-
247 ion affinity chromatography. The presence of the protein of interest in this fraction is
248 addressed using a specific antibody (against the protein of interest or a tag). The advantage
249 of this purification is that it does not involve antibodies during the purification procedure,
250 which may interfere when revealing the presence of the protein of interest by western
251 blotting. It is also amenable to proteome-wide, mass spectrometry-based studies either alone
252 [11,19] or in combination to additional purification procedures [20-22].

253 This protocol (based on [11], and slightly modified in [23]) requires the prior transformation
254 of the yeast strain with a plasmid expressing His-tagged ubiquitin, described in the previous
255 section. Other protocols using slightly different procedures have been described in the past
256 [24,25]. Please note that this technique is not suitable to study the ubiquitylation of proteins
257 that contains an endogenous His-stretch, as their purification on the Ni-NTA column will
258 not depend on their ubiquitylation. A list of the *S. cerevisiae* proteins containing endogenous
259 poly-histidine (≥5) stretches is displayed in **table 1**. Of note, **Figure 2** shows the purification
260 of one of these proteins, Snf1, on Ni-NTA beads even in the absence of His-tagged ubiquitin.

- 261 1. **DAY 1.** Preculture: inoculate freshly streaked yeast cells in synthetic medium (DO)
262 medium supplemented with the required amino-acids in the morning. Use a strain
263 expressing your protein of interest together with His-tagged ubiquitin, and as a
264 control, use the same strain but that does not contain the His-tagged ubiquitin
265 plasmid.
- 266 2. In the evening, measure the OD₆₀₀ of the cultures and inoculate yeast cells in 100 mL
267 DO medium, at OD₆₀₀= 0.001. Grow overnight under agitation (200 rpm) at 30°C.
- 268 3. **DAY 2.** Measure the OD₆₀₀ of the cultures. The cultures should have reached an
269 OD₆₀₀ of ~0.3-0.5. (*see Note 2*). Spin down the cells in 2x50 mL tubes (3000 g, 3
270 min), remove the supernatant, place on ice.
- 271 4. Resuspend each pellet in 500 µL 10% TCA (w/v; i.e. 0.61 N), pool into a 1.5 mL
272 tube.
- 273 5. Leave on ice for at least 10 min (up to a few days).
- 274 6. Pellet cells: 1 min, 13 000 g at room temperature.
- 275 7. Resuspend the precipitated cells with 100 µL of 10% TCA by pipetting.
- 276 8. Add glass beads up to 1-2 mm below the meniscus.
- 277 9. Lyse cells on a vortex equipped with a microtube foam insert for 20 min at 4°C.
- 278 10. After lysis, open the cap, poke a small hole at the bottom of the tube using a needle,
279 place into a clean 1.5-mL tube to collect the lysate, and close the cap.
- 280 11. Centrifuge for a few seconds on a mini-centrifuge to collect the lysate and leave the
281 beads in the upper tube. Discard the tube with the beads (they should be dry). For
282 complete recovery, the glass beads can be washed with 200 µL of cold 10% TCA if
283 necessary, in this case, repeat the spin and pool with the first lysate.
- 284 12. Centrifuge the lysate for 10 min at 13 000 g at 4°C.
- 285 13. Remove as much supernatant as possible.

- 286 14. Wash the pellet with 100% ice-cold acetone, and centrifuge again for 10 min at 13
287 000 g at 4°C.
- 288 15. Neutralize the residual TCA present in the pellet by adding 30 µL of 1M Tris (non-
289 buffered) to the pellet. (*see Note 4*).
- 290 16. Add 200 µl of buffer A.
- 291 17. Resuspend the pellet. Add a few glass beads that will help to break the pellet, and
292 vortex. (*see Note 5*).
- 293 18. Add 800 µL buffer A to the resuspended pellet.
- 294 19. Let solubilize further by rotating for 1h at room temperature. Resuspension should be
295 complete.
- 296 20. Transfer the lysate to a new tube and centrifuge for 10 min at 13 000 g, at room
297 temperature. (*see Note 6*).
- 298 21. During the centrifugation, prepare Ni-NTA beads (Qiagene): transfer 200µL slurry to
299 2-mL tubes, wash the beads twice with 1.8 mL buffer A. Centrifuge for 1 min at 1
300 000 g between each wash. (*see Note 7*).
- 301 22. From the centrifugation (step 20), keep the supernatant, which corresponds to the
302 solubilized lysate, and transfer to a new tube. Keep 25 µL aside, at room
303 temperature, for TCA precipitation (“input fraction”) (see step 35).
- 304 23. Add the remaining sample to the tube containing the washed beads (see step 21), and
305 rotate for 2h at RT.
- 306 24. Centrifuge for 30 sec at 500 g to spin down the beads. Collect 25 µL of unbound
307 fraction and keep at room temperature for TCA precipitation (“Unbound fraction”)
308 (see step 35). Discard remaining supernatant.
- 309 25. Resuspend the beads in 1.8 mL buffer A

- 310 26. Transfer to a micro chromatography column placed in a tube holder (eg. floating
311 foam rack placed on top of a beaker), and let the wash solution drop by gravity.
- 312 27. Wash again with 1.8 mL buffer A, twice
- 313 28. Wash with 1.8 mL Wash1 buffer, three times
- 314 29. Wash with 1.8 mL Wash2 buffer, three times
- 315 30. Place the micro chromatography column in a 1.5 mL tube, centrifuge for 10 sec at
316 1000 g.
- 317 31. Place the micro chromatography column in a clean 1.5 mL tube, and add 100 μ L
318 elution buffer to the beads.
- 319 32. Incubate for 5 min at RT.
- 320 33. Spin down eluate for 1 min at 1 000 g, at room temperature.
- 321 34. Add 25 μ L of 5x SDS sample buffer and denature at the desired temperature (37°C-
322 95°C) for 5-10 min.
- 323 35. To prepare the “input” (see step 22) and “unbound” (see step 24) fraction, proceed as
324 follows. Dilute the 25 μ L-samples with 1.8 mL water, add 200 μ L of 100% TCA and
325 keep on ice for 10 min.
- 326 36. Spin down for 10 min at 13 000 g, at 4°C.
- 327 37. Discard supernatant. Wash the pellet with 1 mL ice-cold acetone (100%).
- 328 38. Spin down for 1 min at 13 000 g, at 4°C.
- 329 39. Discard supernatant. Let at room temperature with cap open until acetone has
330 evaporated completely.
- 331 40. Resuspend in 25 μ L of sample buffer for TCA precipitates and denature at the
332 desired temperature (37°C-95°C) for 5-10 min.

- 333 41. For SDS-PAGE, use 4 μ L of input / unbound fractions as prepared above, and 3-7
334 μ L of the eluate (depending on the expression level of the protein and its rate of
335 ubiquitylation). (*see Notes 8 and 9*).
- 336 42. Transfer on nitrocellulose membrane. After transfer, performing a staining of the
337 transferred proteins with a Ponceau S solution should allow to see His-Ub and a
338 ladder of ubiquitylated proteins (**Figure 3A**).
- 339 43. Blot with the antibody used to detect the protein of interest and with anti-Ubiquitin
340 antibodies (e.g. mouse monoclonal P4D1 antibody: sc-8017, Santa Cruz Biotech;
341 1/10000) to reveal Ub conjugates.

342

343

344 **3.3. Assessment of protein ubiquitylation after immunoprecipitation in denaturing** 345 **conditions**

346 The previous method involves the expression of tagged ubiquitin and the affinity
347 purification of ubiquitin conjugates, prior to detecting the presence of the protein of interest
348 within this fraction. Performing the opposite experiment, i.e. by immunoprecipitating the
349 protein of interest and studying the presence of ubiquitin in the immunoprecipitate, can also
350 provide evidence of the ubiquitylation of a given protein. A main caveat of this approach is
351 the fact that the ubiquitin signal detected in the immunoprecipitated sample may either be
352 due to the protein that was immunoprecipitated, or to potential interactants that were co-
353 immunoprecipitated. Therefore, we provide a protocol that allows the immunoprecipitation
354 of proteins in denaturing conditions, that was successfully used in the past [26,27]. These
355 immunoprecipitates can be used to reveal the ubiquitylation of the protein of interest.

356 An advantage of this technique is that it does not involve the use of overexpressed tagged-
357 ubiquitin, and as such may be more physiologically relevant. However, it depends on the use
358 of anti-ubiquitin antibodies, which also have their limits since some are specific of the
359 topology of the ubiquitin modification. We routinely use the P4D1 monoclonal antibody,
360 which is a good compromise for the detection of both mono- and poly-ubiquitin
361 modifications, although the monoubiquitylation signal is sometimes weak (see for instance
362 [28]).

- 363 1. **DAY 1.** Preculture: inoculate freshly streaked yeast cells in synthetic medium (DO)
364 supplemented with the required amino-acids in the morning. As a control, use a
365 strain which does not express the tagged protein, to confirm that the ubiquitin signal
366 observed after the immunoprecipitation is specific for the presence of the tagged
367 protein.
- 368 2. In the evening, measure the OD₆₀₀ of the cultures and inoculate the yeast cells in 100
369 mL DO medium, at OD₆₀₀= 0.001. Grow overnight under agitation (200 rpm) at
370 30°C.
- 371 3. **DAY 2.** Measure the OD₆₀₀ of the cultures. The cultures should have reached an
372 OD₆₀₀ of ~0.3-0.5. (*see Note 2*). Spin down the cells in 2x50 mL tubes (3000 g, 3
373 min), remove the supernatant, place on ice
- 374 4. Resuspend each pellet in 500 µL 10% TCA (w/v; i.e. 0.61 N), pool into a 1.5 mL
375 tube
- 376 5. Leave on ice for at least 10 min (up to a few days)
- 377 6. Prepare antibody-coupled beads for the immunoprecipitation. Take 50 µL of Protein-
378 G-coupled Sepharose slurry (*see Note 10*), wash in 1 mL IP dilution buffer,
379 resuspend in 1 mL IP dilution buffer and use the appropriate amount of antibodies
380 required for immunoprecipitation. Incubate for ≥1h at 4°C on a rotating wheel.

- 381 7. Pool the precipitated cells into one single 1.5 mL tube
- 382 8. Pellet the precipitated cells: 1 min, 13 000 g at room temperature.
- 383 9. Resuspend the precipitated cells with 100 μ L of 10% TCA by pipetting up and down
- 384 10. Add glass beads up to 1-2 mm below the meniscus
- 385 11. Lyse cells on a vortex equipped with a microtube foam insert for 20 min at 4°C.
- 386 12. After lysis, open the cap, poke a small hole at the bottom of the tube using a needle,
- 387 place into a clean 1.5-mL tube to collect the lysate, and close the cap
- 388 13. Centrifuge for a few seconds on a mini-centrifuge to collect the lysate and leave the
- 389 beads in the upper tube. Discard the tube with the beads (they should be dry). The
- 390 glass beads can be washed with 200 μ L of cold 10% TCA if necessary, in this case,
- 391 repeat the spin and pool with the first lysate.
- 392 14. Centrifuge the lysate for 1 min at 13 000 g at RT.
- 393 15. Remove as much supernatant as possible.
- 394 16. Wash the pellet with 1 mL ice-cold acetone (100%) (*see Note 11*).
- 395 17. Spin down for 1 min at 13 000 g, at 4°C.
- 396 18. Discard supernatant. Let at room temperature with cap open until acetone has
- 397 evaporated completely.
- 398 19. Resuspend in 100 μ L buffer B0. Make sure that the sample is blue (i.e. sample is not
- 399 acidic). If the sample is yellow, add increments of 1 μ L Tris-HCl 1M pH 7.5 until
- 400 the pH is neutralized.
- 401 20. Denature the sample at the desired temperature (37°C-95°C) for 5-10 min in a
- 402 thermomixer.
- 403 21. Spin down insolubilized material by centrifuging for 5 min at 16,000 g, at room
- 404 temperature
- 405 22. Transfer the supernatant to a clean tube.

- 406 23. Add 400 μ L of IP dilution buffer.
- 407 24. Keep 50 μ L aside for the “input” fraction ; keep on ice
- 408 25. Spin down the antibody-coated beads (see step 6) for 1 min at 300 g (4°C) and
- 409 remove the supernatant.
- 410 26. Add the protein sample prepared in step 23 to the antibody-coated beads
- 411 27. Incubate on a rotating wheel at 4° C for 2h.
- 412 28. Spin down the beads for 1 min at 300 g (4°C).
- 413 29. Take 50 μ L of the supernatant and keep 50 μ L aside for the “unbound” fraction;
- 414 keep on ice.
- 415 30. Carefully remove and discard remaining supernatant.
- 416 31. 1st wash: Resuspend the beads in 1 mL buffer B1, and spin down the beads for 1 min
- 417 at 300 g (4°C).
- 418 32. Repeat the 1st wash.
- 419 33. 2nd wash: Resuspend the beads in 1 mL buffer B2 and spin down the beads for 1 min
- 420 at 300 g (4°C).
- 421 34. Repeat the 2nd wash.
- 422 35. 3rd wash: Resuspend the beads in 1 mL buffer B3 and spin down the beads for 1 min
- 423 at 300 g (4°C).
- 424 36. Repeat the 3rd wash.
- 425 37. Last wash: Resuspend the beads in 1 mL buffer B4, and spin down the beads for 1
- 426 min at 300 g (4°C).
- 427 38. Repeat the last wash, and remove as much supernatant as possible.
- 428 39. Add 50 μ L of 1X sample buffer to the beads.
- 429 40. To the “input” and “unbound” fractions, add 12.5 μ L of 5X Laemmli sample buffer
- 430 44. Denature at the desired temperature (37°C-95°C) for 5-10 min.

- 431 41. Load 7 μ L of input / unbound fractions as prepared above, and 3-7 μ L of the eluate
432 (depending on the expression level of the protein and its rate of ubiquitylation).
433 42. Blot with the antibody against the protein of interest or its epitope, to evaluate the
434 efficiency of the immunoprecipitation, and with anti-Ubiquitin antibodies (1/10000
435 mouse monoclonal P4D1 antibody: sc-8017, Santa Cruz Biotech) to reveal Ub
436 conjugates. (*see Note 12*).

437

438 **3.4. Tandem purification of a protein in denaturing conditions for the identification of** 439 **ubiquitin-conjugated sites**

440 Once ubiquitylation of a protein has been documented, a traditional approach to understand
441 the consequences of this modification resides in identifying the sites targeted for
442 ubiquitylation, followed by their mutation to evaluate the outcome of the lack of
443 ubiquitylation on the protein's stability or function.

444 This protocol is based on a two-step purification. It involves the use of pBG1805-derived
445 constructs (*URA3*-based) [29], in which all yeast genes have been cloned and that are
446 commercially available. This allows the galactose-driven expression of the protein of
447 interest tagged at its C-terminus with multiple epitopes: 6xHis, HA, and a ProtA (ZZ
448 domains) preceded by a Protease 3C cleavage site (the size of the tag is 19 kDa). The first
449 step is a regular immunoprecipitation in native conditions using anti-HA antibodies,
450 followed by a Ni-NTA-based purification of the His-tag in denaturing conditions. It allows
451 to purify the protein (both ubiquitylated and non-ubiquitylated, see **Figure 4**) for further,
452 mass-spectrometry based, identification of the ubiquitylation sites (*See note 13*). Note that
453 Protein A itself can be ubiquitylated in yeast cells (S. Léon, unpublished results). It is
454 therefore necessary to identify the ubiquitylated sites by mass spectrometry to make sure
455 that ubiquitin is carried on the substrate, and not the tag. Consequently, this protocol should

456 merely be used to identify the ubiquitylation sites, and not to prove that a protein is
457 ubiquitylated.

458 **DAY 1.** Preculture: in the morning, inoculate freshly streaked yeast cells in 10 mL synthetic
459 glucose medium lacking uracil.

460 1. In the evening, measure the OD₆₀₀ of the cultures and inoculate the yeast cells in 180
461 mL synthetic raffinose medium, at OD₆₀₀= 0.004. Grow overnight under agitation
462 (200 rpm) at 30°C.

463 2. **DAY 2.** Measure the OD₆₀₀ of the cultures. The cultures should have reached an
464 OD₆₀₀ of ~0.3-0.5.

465 3. Add galactose (2% final) in the medium for the desired amount of time (*see Note 14*)

466 4. Measure the OD of the culture (*see Note 2*).

467 5. Spin down the equivalent of 150 OD units of cells at 3000 g, 3 min at room
468 temperature

469 6. Remove the supernatant, resuspend the pellet in 10 mL cold water

470 7. Spin down for 30 sec at 13,000 g, 4°C.

471 8. Discard supernatant and keep the pellet on ice.

472 9. Prepare antibody-coupled beads for the immunoprecipitation (see step 23). Take 200
473 µL of Protein-G-coupled Sepharose slurry, wash in 1 mL IP lysis buffer, resuspend
474 in 1 mL IP lysis buffer and use the appropriate amount of antibodies required for
475 immunoprecipitation (e.g. 10 µL of mouse monoclonal F7 antibody: sc-7392 from
476 Santa Cruz Biotech). Incubate for ≥1h at 4°C on a rotating wheel.

477 10. Resuspend the pellet in 2.5 mL IP lysis buffer containing protease inhibitors (yeast
478 protease inhibitor cocktail, 1 mM PMSF, 10 mM N-ethylmaleimide, 100 µM MG-
479 132).

480 11. Dispatch the resuspended pellet in 5 x 600 µL fractions, in 1.5 mL tubes

- 481 12. Add glass beads (0.4-0.6 mm) up to 1-2 mm below the meniscus.
- 482 13. Lyse cells on a vortex equipped with a microtube foam insert for 4 x 30 sec at 4°C
- 483 (cold room), with 1 min incubation on ice between each pulse.
- 484 14. After lysis, in the cold room, open the tubes, poke a small hole at the bottom using a
- 485 needle, place into a clean 1.5-mL tube to collect the lysate, and close the cap
- 486 15. In the cold room, centrifuge for a few seconds on a mini-centrifuge to collect the
- 487 lysate and leave the beads in the upper tube. Discard the tube with the beads (they
- 488 should be dry).
- 489 16. Pool lysates in a clean, cold centrifugation tube
- 490 17. To the lysate, add 100 µL of a 25% solution of Triton-X 100, and invert to mix. (*see*
- 491 *Note 15*).
- 492 18. Let solubilize on ice for 10 min.
- 493 19. Centrifuge the lysate for 5 min at 3 000 g at 4°C.
- 494 20. Transfer supernatant to a clean, cold 15-mL tube and keep on ice.
- 495 21. Keep 100 µL aside for the “input 1” fraction, keep on ice.
- 496 22. Spin down the beads coupled to antibodies (step 10) for 30 sec at 1000 g, 4°C.
- 497 23. Discard the beads’ supernatant.
- 498 24. Add the beads to the lysate.
- 499 25. Incubate on a rotating wheel at 4° C for 3h.
- 500 26. Spin down the beads for 30 sec at 1000 g, 4°C.
- 501 27. Keep 100 µL of the supernatant aside for the “unbound 1” fraction, keep on ice.
- 502 28. Discard remaining supernatant.
- 503 29. Add 1 mL of IP lysis buffer to the beads
- 504 30. Transfer to a cold, 1.5 mL tube
- 505 31. Incubate on a rotating wheel at 4° C for 10 min.

- 506 32. Spin down the beads for 30 sec at 1000 g, 4°C. Discard supernatant.
- 507 33. Repeat the wash twice and carefully remove supernatant.
- 508 34. Add 1 mL of urea buffer
- 509 35. Incubate for 5 min at room temperature under agitation (e.g. in a thermomixer, 1000
- 510 rpm)
- 511 36. Open the tube, poke a small hole at the bottom of the tube using a needle, place into
- 512 a clean 1.5-mL tube to collect the sample, and close the cap
- 513 37. Centrifuge for a few seconds on a mini-centrifuge to collect the eluate and leave the
- 514 beads in the upper tube. Discard the tube with the beads (they should be dry).
- 515 38. Keep 30 µL of the eluate aside for the “input 2” fraction, keep on ice.
- 516 39. To the remaining sample, add 500 µL Ni-NTA beads prewashed in urea buffer
- 517 40. Incubate overnight on a rotating wheel at 4°C.
- 518 41. To the “Input 1”, “Unbound 1” and “Input 2” fractions (steps 21, 27 and 38), add 1/5
- 519 volume of 5x sample buffer, denature all samples at 55°C for 10 min and store at -
- 520 20°C.
- 521 42. **DAY 3.** Spin down the beads (see step 40) for 30 sec at 1000 g, 4°C.
- 522 43. Keep 30 µL aside for the “unbound 2” fraction, keep on ice.
- 523 44. Discard remaining supernatant.
- 524 45. Add 2.5 mL of Urea buffer to the beads, incubate on a rotating wheel at 4° C for 10
- 525 min.
- 526 46. Spin down the beads for 30 sec at 1000 g, 4°C. Discard supernatant.
- 527 47. Repeat steps 45-46 and carefully remove supernatant.
- 528 48. Add 1 mL of Urea buffer to the beads, and transfer to a 1.5 mL tube
- 529 49. Incubate on a rotating wheel at 4° C for 10 min.

- 530 50. Spin down the beads for 30 sec at 1000 g, 4°C. Discard as much supernatant as
531 possible.
- 532 51. Add 100 µL of Urea elution buffer.
- 533 52. Incubate for 5 min at room temperature under agitation (e.g. in a thermomixer, 1000
534 rpm)
- 535 53. Open the tube, poke a small hole at the bottom of the tube using a needle, place into
536 a clean 1.5-mL tube to collect the eluate, and close the cap
- 537 54. Centrifuge for a few seconds on a mini-centrifuge to collect the sample and leave the
538 beads in the upper tube. Discard the tube with the beads (they should be dry).
- 539 55. The total volume of the eluate (incl. the bed volume of the beads) should be
540 approximately 175 µL.
- 541 56. To the “unbound 2” and the “eluate” samples (steps 43 and 55), add 1/5 volume of
542 5x sample buffer.
- 543 57. Denature all samples (input 1, output 1, input 2, output 2, eluate) at 55°C for 10 min.
- 544 58. Load 5 µL of input 1, output 1, input 2, output 2 and 10 µL of the final elution onto
545 an SDS-PAGE gel.
- 546 59. Blot with the antibody against your protein of interest or its epitope, to evaluate the
547 purification procedure, and with anti-Ubiquitin antibodies (1/10000 mouse
548 monoclonal P4D1 antibody: sc-8017, Santa Cruz Biotech) to reveal Ub conjugates.

549

550 **3.5 Identification of ubiquitylated sites within a protein of interest by a genetic**

551 **approach**

552 Ubiquitin conjugation can occur at defined sites within a protein, and the identification of
553 those sites (e.g. using the approach described in the above section) can help to generate non-
554 ubiquitylatable mutants after its/their mutation. However, the mutation of the target lysine is

555 sometimes compensated by the ubiquitylation of yet another site, because ubiquitylation can
556 show little specificity towards the sites targeted [30]. Therefore, we describe a mutant-based
557 approach that should lead to a construct that is not ubiquitylatable, regardless of the prior
558 identification of the ubiquitylation sites, that we successfully used in the past [23]. This is
559 based on a synthetic version of the ORFs encoding your protein of interest, in which all
560 lysine residues have been substituted to arginine (K0 construct) (**Figure 5A**). Then,
561 endogenous restriction sites can be used to construct chimeras. If no restriction sites are
562 available, silent restriction sites should be introduced at various places within the sequence
563 when designing the construct for gene synthesis, in which case a synthetic WT ORF must
564 also be ordered, with the same restriction sites. The ubiquitylation of the resulting chimeras
565 can be evaluated individually, to narrow down to the region and/or sites that are targeted by
566 ubiquitylation (**Figure 5B**).

567 Although it involves several cloning steps, this approach is still fairly efficient when dealing
568 with proteins carrying many lysines. Note that this approach requires that the ubiquitylated
569 site(s) lie(s) within a defined region of the primary structure of a protein. Below, we provide
570 the general directions to properly map the ubiquitylated site(s) using this approach.

571 1. Order synthetic gene versions encoding the protein of interest: WT and K0. Consider
572 adding silent restriction sites within the sequence that are not present on the plasmid in
573 which these synthetic genes will be cloned.

574 2. Clone the synthetic genes in the plasmid of interest. It is advisable to use a plasmid
575 allowing the expression of epitope-tagged protein, even if antibodies directed against the
576 protein are available, because the KR mutations may affect the antigenicity of the protein.

577 3. Assay for the ubiquitylation of the WT construct and the K0 mutant. The K0 should have
578 lost all traces of ubiquitylation, if ubiquitin is conjugated on a lysine.

- 579 4. Construct chimeras (Nt-WT/Ct-K0 and Nt-K0/Ct-WT) and assay again the ubiquitylation
580 of the chimeric proteins. The disappearance of ubiquitylated species for one of these
581 constructs indicates that the target lysine(s) are contained within those that have been
582 mutated. (*see Note 16*).
- 583 5. Prepare additional chimeras to narrow down to the minimal region.
- 584 6. If the density of lysines within the initial sequence is such that the final construct still
585 bears several lysine residues, then site-directed mutagenesis can be used to further
586 discriminate between each of those.

587 **3.6. Studying the topology of ubiquitin modification**

588 When dealing with a polyubiquitylated substrate, it may be interesting to determine the
589 topology of the ubiquitin chain, because the various structures obtained through various
590 ubiquitin linkages will lead to various functional outcomes.

591 Various tools have been developed to do so, such as antibodies directed against the most
592 abundant polyubiquitin chain linkages, namely K48- and K63-linked polyubiquitin, whose
593 use has already been described [31]. The development of tandem-ubiquitin binding entities
594 (TUBEs) [32,33] and other sensor proteins[34], consisting of multiple ubiquitin-binding
595 domains, also allow the purification of substrates that are polyubiquitylated with various
596 topologies and are commercially available.

597 An alternative approach consists in using ubiquitin mutants in which either of the ubiquitin
598 lysine residues have been mutated into an arginine, to hamper the ability to make a chain.
599 These chain-terminating mutants can be individually overexpressed in the context of a wild-
600 type strain in which these mutants will act as dominant-negatives, as previously described
601 [24]. Instead, here, we provide an example of the use of a set of yeast strains (“SUB”

602 strains) engineered by the Finley lab, in which all endogenous ubiquitin genes have been
603 disrupted and complemented with a plasmid encoding either WT or lysine point-mutants of
604 ubiquitin [5]. Below are guidelines on how to use these strains to determine ubiquitin
605 linkage. Note that this approach is limited to the study of non-K48-based polyubiquitin
606 chain substrates, because the expression of a K48R mutant in yeast as a sole source of
607 ubiquitin is not viable [35]. For this specific case, the overexpression of Ub-K48R mutant in
608 the context of a wild-type strain is therefore preferable [24]. Also, because the ubiquitin is
609 not tagged in these strains, it cannot be purified by Ni-NTA based purification. Therefore,
610 the analysis relies on the ability to see protein ubiquitylation on a crude extract (section 3.1)
611 (**Figure 6**). Alternatively, the protein may be purified from each of these strains by
612 denaturing IP, followed by a western blot using anti-ubiquitin antibodies as described in
613 section 3.3.

- 614 1. Transform the SUB strains with an *URA3*-based plasmid expressing a tagged version
615 of the protein of interest using standard yeast transformation procedure. These
616 includes SUB280 (WT ubiquitin), SUB515 (K6R Ub), SUB516 (K11R), SUB517
617 (K27R), SUB518 (K29R), SUB519 (K33R), and SUB413 (K63 Ub) [5] (*see Note*
618 *17*).
- 619 2. Grow cells as described in section 3.1 (for crude extracts) or in section 3.3 (for
620 denaturing IPs), in synthetic medium lacking uracil, starting at OD=0.010 for
621 SUB280 and OD=0.020 for the mutants.
- 622 3. For each strain, perform either a crude extract as described in section 3.1, or a
623 denaturing IP as described in section 3.3
- 624 4. Load samples on SDS-PAGE.
- 625 5. Blot with the antibodies used to detect the protein of interest.

626

627 **4. Notes**

- 628 1. The presence of 0.02% glucose allows a faster initiation of growth in raffinose
629 medium.
- 630 2. Cells will not lyse efficiently if they are not in exponential phase. We advise never to
631 reach an OD>1.0.
- 632 3. For proteins suspected to be modified by a K48-linked polyubiquitin chain, and/or to
633 be proteasomal substrates, the treatment of cells with a proteasome inhibitor (eg. MG132,
634 100 μ M final) prior harvest can lead to their stabilization, sometimes in its ubiquitylated
635 form (although this is usually counteracted by the presence of endogenous ubiquitin
636 isopeptidases). However, the use of MG132 in yeast cultures at this concentration is only
637 efficient in strains in which either *PDR5* or *ERG6* are disrupted, leading to an increased
638 permeability to various drugs [36,37]. An alternative method to promote the efficiency of
639 MG-132 in WT yeast strains has been described and is based on the use of a different culture
640 medium [38].
- 641 4. This step is essential, because His-tagged proteins will not bind to Ni-NTA beads in
642 acidic conditions.
- 643 5. A pipette tip can be used too, but we advise not to pipette up and down because the
644 pellet is sticky.
- 645 6. The guanidine-HCl in buffer A may precipitate upon sample cooling.
- 646 7. Do not incubate the beads with buffer A for too long, because they will titrate the
647 imidazole in buffer A, which may hinder their ability to bind to His-tagged proteins later on.
- 648 8. Preferentially use precast gradient gels (eg. NuPAGE® 4-12%, Bis-Tris, Life
649 Technologies).
- 650 9. Make sure that samples do not run out of the gel, because His-tagged ubiquitin is
651 about 10 kDa and will be used as a purification control.

652 10. It is advised to use Protein G- rather than Protein A-coupled beads except when the
653 source of the antibody is guinea pig. Neither Protein A nor Protein G bind chicken IgY.
654 Alternatively, when using HA-tagged proteins, use the ready-to-use anti-HA affinity matrix
655 (Roche): use 30 μ L, resuspend in 1 mL IP dilution buffer and keep on ice.

656 11. The use of a water-bath sonicator to resuspend the pellet helps for the final
657 solubilization of the sample.

658 12. Because of the presence of antibodies in the immunoprecipitate, we advise to use
659 antibodies to ubiquitin that are directly coupled to HRP to avoid the use of a secondary
660 antibody (e.g. mouse monoclonal P4D1 antibody: sc-8017 HRP, Santa Cruz Biotech; use at
661 1/2000).

662 13. Identifying post-translational modification sites by mass spectrometry can be
663 hindered by the spacing of Arg/Lys residues, at which the cleavage with trypsin occurs
664 within the protein of interest. Tryptic fragments that are either too long or too short will not
665 be analyzed, leading to a poor coverage and eventually, the inability to detect the post-
666 translational modification. The coverage of a given protein by mass spectrometry can be
667 visualized in repository databases, such as the Global Proteome Machine Database
668 (<http://gpmdb.thegpm.org>). In this case, other proteases than trypsin should be considered
669 prior to MS analysis.

670 14. The plasmid used is a 2 μ -based (high-copy) plasmid, and the expression of the
671 protein is driven by the strong galactose-inducible promoter. This can lead to a massive
672 overexpression of the protein, and the ubiquitylation detected may not be physiologically
673 relevant. To circumvent this problem, the expression level of the protein at various times
674 after galactose induction can be checked and compared with endogenous protein level.
675 Protein expression can usually be observed within 15 min of galactose induction.

676 15. Steps 17 and 18 are dispensable if dealing with a soluble protein. Adding TX-100

677 may lead to smears when visualizing the protein of interest by western blot. Note that 1%
678 TX-100 solubilizes many membrane proteins, but not all, so this should be checked prior to
679 the experiment.

680 16. If both chimeric proteins appear ubiquitylated, then it is likely that the protein is
681 ubiquitylated on multiple sites throughout the primary structure of the protein, or that
682 ubiquitylation can occur on any of those sites. In both cases, this approach should be
683 discontinued, and we advise to initiate a mapping of the ubiquitylated lysines using
684 biochemical purification and mass spectrometry (see section 3.4).

685 17. Note that only the *URA3* gene can be used for selection of transformants in the SUB
686 strains genetic backgrounds.

687

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693

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831 **Figure captions.**

832 **Figure 1. Obtaining evidence of ubiquitylation on crude extracts.** Protein extracts from
833 wild-type (BY4741) cells expressing the protein Ear1 tagged with mCherry (pSL22)
834 [39] together with either pCUP1:Ub (pRS426-based plasmid: pRHT79; left) or
835 pCUP1-His-Ub (pJD421; right) [16] were prepared as described in the text,
836 electrophoresed on SDS-PAGE and blotted with monoclonal anti-DsRed antibodies
837 (Clontech). The upper band observed on top of Ear1-mCh (gray arrowhead) is shifted
838 up in apparent size when expressing His-tagged ubiquitin (black arrowhead).

839 **Figure 2. Purification of the endogenously, polyhistidine-tagged protein Snf1 on Ni-**
840 **NTA column in the absence of His-tagged ubiquitin.** Proteins from a WT strain
841 (BY4741) expressing Snf1-HA from a plasmid (pSL204) were prepared as described
842 and purified on Ni-NTA resin in the absence of His-tagged ubiquitin. Equal amounts
843 of extract (input, 1st lane) and unbound fraction (2nd lane) were loaded, showing the
844 total depletion of Snf1, which is recovered in the eluate (3rd lane). Phosphoglycerate
845 kinase (PGK) is used as a control and does not bind to the beads. Other proteins that
846 may show a similar behavior are displayed in **Table 1**.

847 **Figure 3. Purification of ubiquitin conjugates using polyHis-tagged ubiquitin.** Protein
848 extracts from WT cells (BY4741) expressing either Ub or His-tagged Ub (see Figure
849 1) were prepared and treated as described for the purification of His-tagged ubiquitin.
850 **A.** Ponceau S staining of the nitrocellulose membrane after transfer of the indicated
851 sample, showing the purification of His-Ub in the eluate fraction (El) (arrow). **B.**
852 Western blot using anti-ubiquitin antibodies on a similar experiment. The gel shows
853 ubiquitin dimers (Ub₂) in both cell types, and His-Ub+Ub dimers in cells expressing
854 His-Ub. **C.** Example of a purification of His-tagged ubiquitin from yeast cells grown

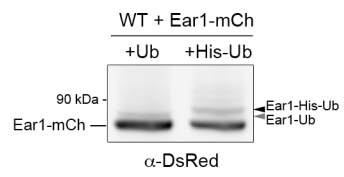
855 either in lactate or glucose medium and expressing the arrestin-related protein Rod1
856 tagged with GFP. Displayed are the “input “ (Inp) and eluate fractions (El.). Rod1 is
857 phosphorylated in lactate medium (asterisk) and becomes ubiquitylated rapidly after
858 glucose addition (black circle) (See also [23]).

859 **Figure 4. Tandem purification of a protein in denaturing conditions for the**
860 **identification of ubiquitin-conjugated sites. A.** WT cells (BY4741) expressing the
861 galactose-inducible Ear1-His₆-HA-ProtA construct were subjected to a double
862 purification as described. First, an immunoprecipitation using anti-HA antibodies was
863 performed on a crude extract in native conditions (input 1 and unbound 1). The eluate
864 of this immunoprecipitation was used as the input fraction (input 2) for the second,
865 Ni-NTA based purification of the protein in denaturing conditions (unbound 2 and
866 final eluate). A western blot using anti-HA antibodies allows to evaluate the
867 purification procedure. **B.** From the same fraction, 1 μ L or 25 μ L of eluate were
868 loaded on the same gel. The gel was cut, and the left part was subjected to a western
869 blot using anti-HA antibodies, whereas the right part was used for colloidal blue
870 staining, showing the purity of the protein obtained.

871 **Figure 5. Identification of ubiquitylated sites within a protein of interest by a genetic**
872 **approach. A.** Example of the method: identification of the ubiquitylated region
873 within the arrestin-related protein, Rod1, using a synthetic construct in which all
874 lysines have been mutated (K0) and its derived chimeric constructs [23]. **B.** Western
875 blot showing the ubiquitylation status of several of the chimeric constructs depicted in
876 **A.** The asterisk indicates phosphorylated Rod1, which is only observed when yeast
877 cells are grown in the absence of glucose, such as in lactate medium (Lac) [23]. The
878 black circles indicate Rod1 ubiquitylation, which occurs only after glucose exposure

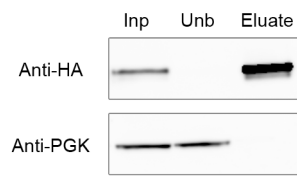
879 and for constructs in which the ubiquitylation site is still intact. PGK is used as a
880 loading control.

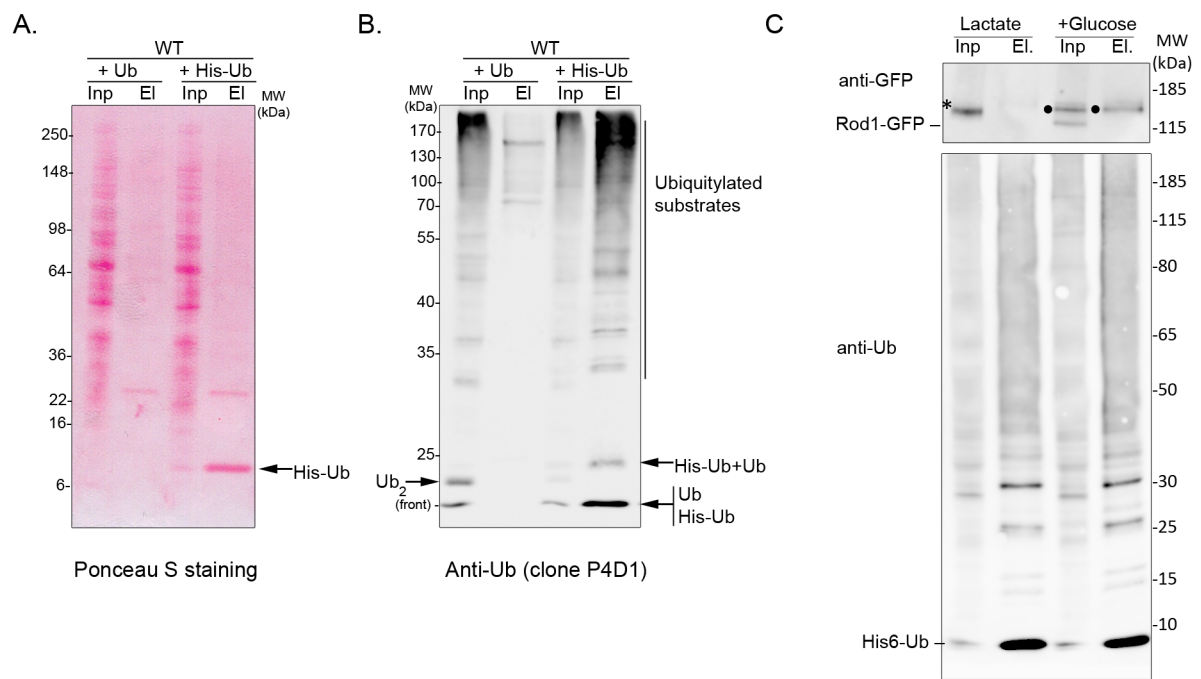
881 **Figure 6. Studying the topology of ubiquitin modification.** An example of the use of the
882 SUB strains (which express K-to-R ubiquitin mutants) to gain information on the topology
883 of the ubiquitin modification on a substrate (here, the endosomal Rsp5 adaptor protein,
884 Ear1) . Black arrowheads: Ear1 ubiquitylated adducts. White arrow: the ubiquitin
885 modification appears as a single band in the SUB413 strain, suggesting that Ear1 is
886 polyubiquitylated with a short K63 chain (for more details, see also [40]). PGK is used as a
887 loading control.



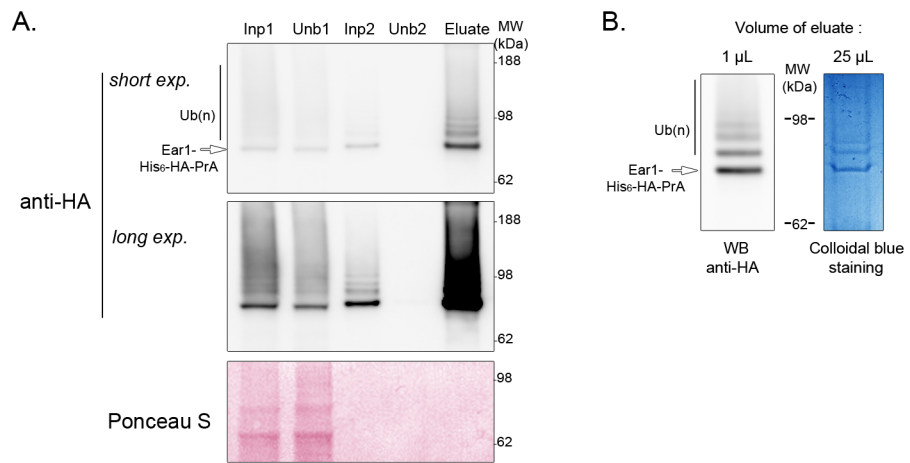
Hovsepian et al. Fig. 1

Snf1-HA strain

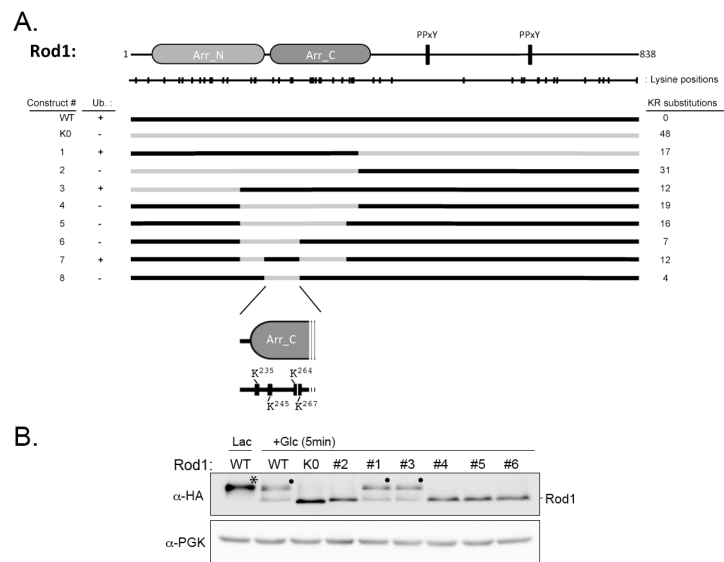




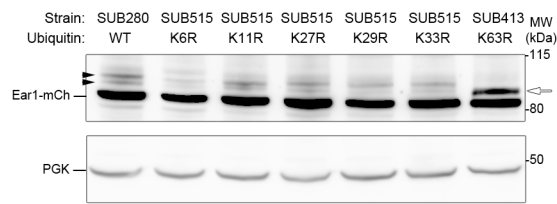
Hovsepian et al., Figure 3



Hovsepian et al. Figure 4



Hovsepian et al. Figure 5



Hovsepian et al., Figure 6