Unmasking functional redundancy of deubiquitylases enables specificity profiling and discovery of novel proteasome substrates

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Abstract

Deubiquitylating enzymes (DUBs) counteract ubiquitylation to control the stability or activity of their substrates. Identifying DUB substrates is challenging and genetic approaches can be thwarted by redundant action of DUBs. Here, we circumvented redundancy by broadly inhibiting DUBs in *Xenopus* egg extract and used quantitative mass spectrometry to identify over thirty proteins that undergo proteasomal degradation, the majority of which have not been reported as DUB substrates. These results were confirmed with recombinant human proteins, demonstrating the conservation of their DUB-dependent stability. We used these substrates to profile the ability of a panel of DUBs to rescue degradation. This approach revealed that USP7, uniquely among the 14 DUBs tested, has a broad ability to rescue degradation. USP21, which is used widely to nonspecifically deubiquitylate proteins in vitro, was unable to rescue degradation, highlighting the importance of profiling enzyme activity in a physiological system. Together, we identify new DUB substrates and present a system to characterize physiological DUB specificity, overcoming the challenges posed by DUB redundancy.

15 **Introduction**

16 The covalent addition of ubiquitin to proteins is a widespread, highly regulated post-
17 translational modification. The Ubiquitin-Proteasome System (UPS) is best known for its ability
18 to target specific proteins for degradation, but ubiquitylation also regulates protein localization,
19 activity and function independently of degradation[1,2,3]. Ubiquitin contains seven lysine residues,
20 each of which can be conjugated further with additional ubiquitin moieties to form polyubiquitin
21 chains of different topologies that may result in different fates[4]. For example, lysine-63 linked
22 chains promote non-degradative functions such as DNA repair or endocytosis[5,6], while lysine-48
23 linked chains and branched chains such as K11/K48 or K29/K48 promote degradation by the
24 proteasome[7], a vital process that contributes to cellular homeostasis.

25 Ubiquitin is covalently attached to substrates via a cascade of E1-E2-E3 enzymes[8]. In
26 contrast, deubiquitylating enzymes (DUBs) catalyze ubiquitin removal[9]. As such, a substrate's
27 ubiquitylation status is a product of a carefully tuned balance between ubiquitylation and
28 deubiquitylation activities. By counteracting substrate ubiquitylation, DUBs can rescue proteins
29 targeted to proteasomal destruction. The human genome encodes approximately 100 DUBs,
30 divided into two families: the zinc metalloprotease class (10 DUBs), and the cysteine-protease
31 class that contains most other DUBs (~90)[10,11]. A small number of DUBs have been well-studied,
32 with many characterized substrates, but most DUBs still do not have any known substrates,
33 limiting our understanding of how DUB substrate specificity is achieved. DUB specificity is
34 complex, as it can arise through binding specificity mediated by unique protein-protein
35 interaction domains[12], or through recognition of specific topologies of ubiquitin chains[13,14,15].
36 Most DUB substrates have been discovered by first identifying proteins that interact with

37 specific DUBs. This approach has been used globally[16], but it may miss substrates that interact
38 weakly with DUBs or identify regulators or scaffold proteins rather than true substrates.

39 Identifying physiological UPS substrates, such as DUB targets, is challenging due to the
40 prominence of protein-quality control pathways that ubiquitylate newly synthesized proteins that
41 do not fold properly in growing cells[17]. Therefore, it can be difficult to discern which
42 ubiquitylation or degradation events are physiologically relevant, and which are simply a result
43 of misfolding during protein biogenesis. In addition, because DUBs may function
44 redundantly[18,19], inactivating a single DUB may not destabilize its substrates or affect a particular
45 biological process. Consistent with the presence of functional redundancy between DUBs, only
46 the metallo-protease RPN11 is essential for viability in yeast[20]. In the case of proteome stability,
47 it is not known which DUBs have the broadest impact in rescuing proteins from proteasome
48 degradation or to what extent DUBs act redundantly in this process. Identifying the DUBs that
49 have a major role in counteracting proteasomal degradation is of therapeutic interest, because the
50 UPS targets for destruction misfolded, damaged proteins and proteins involved in a wide range
51 of diseases[21]. Inhibiting DUBs that target these proteins may accelerate their elimination to
52 provide a therapeutic benefit.

53 Here, we first took an unbiased quantitative proteomic approach and discovered new
54 substrates protected from proteasomal degradation by DUBs, using the *Xenopus* egg extract
55 model system. By broadly inhibiting cysteine-protease DUBs, we circumvented the possible
56 effects of their redundancy on proteome stability. Because transcription and translation are not
57 active in this system, protein instability caused by DUB inhibition is not a consequence of
58 protein quality control ubiquitination activity. We recapitulated protein destabilization with
59 expression of recombinant human orthologs in extract, demonstrating that DUB-dependent

60 protein stability is evolutionarily conserved. Next, we took advantage of these newly identified
61 DUB substrates and tested the ability of a set of DUBs to rescue their degradation, uncovering a
62 broad ability of USP7 to rescue protein instability in extract. However, specific inhibition of
63 USP7 with a small molecule inhibitor was not sufficient to promote degradation of most of the
64 substrates we identified, suggesting that USP7 functions redundantly with other DUBs in this
65 system. Our work highlights the impact of DUB redundancy on proteome stability and reveals
66 the specificity and activity of DUBs whose function would otherwise be masked by redundancy.
67

68 **Results**

69 **UbVS treatment induces rapid depletion of available ubiquitin and labels a broad set of**
70 **cysteine-protease DUBs in *Xenopus* extract**

71 Our laboratory previously showed that the broad cysteine-protease DUB inhibitor UbVS
72 induces depletion of available ubiquitin in *Xenopus* extract[22]. In particular, UbVS-treatment
73 inhibited proteasome degradation of cyclin B, which could be efficiently rescued by addition of
74 50 μM exogenous ubiquitin[22]. We confirmed that 10 μM UbVS was sufficient to rapidly deplete
75 free ubiquitin, (Fig. 1a, 1b) which was associated with discharge of the Ube2C thioester (E2)
76 within 5 minutes (Fig. 1a, bottom). Addition of exogenous ubiquitin to UbVS-treated extract
77 fully recharged the Ube2C thioester, confirming that the E2's discharge was due to the depletion
78 of free ubiquitin (Fig.1a, bottom). To determine the spectrum of cysteine-containing DUBs
79 targeted by this concentration of UbVS, extract was treated with 10 μM HA-tagged UbVS (HA-
80 UbVS) and sensitive DUBs were visualized as discrete bands by anti-HA Western blotting
81 analysis (Fig. 1b, right). To identify these DUBs, we performed mass spectrometry analysis of
82 immunopurified HA-UbVS from extract and identified 88 proteins (Table S2), 35 of which

83 were cysteine-protease DUBs (Table S1, S2) that are likely direct targets of UbVS. All known
84 classes of cysteine-protease DUBs were found (Fig. 1c, Table S1), whereas metallo-protease
85 DUBs (JAMM) were not identified, as expected[23]. Prior proteomic analysis of *Xenopus* extract
86 detected 54 DUBs[24] (Table S1), 51 of which were cysteine-proteases. Thus, 10 μM HA-UbVS
87 labeled 35 of 51 (69%) of cysteine-protease DUBs present in extract, consistent with broad
88 specificity of UbVS for this class of DUBs[23]. The remaining DUBs were likely not either
89 because they were present in low abundance or because they do not react rapidly with UbVS.
90 Our HA-UbVS pull-down also isolated proteins that are likely to associate very tightly with
91 DUBs (Table S2), due to us carrying out the pull-down in presence of high salt (500 mM). A
92 large number of these proteins (31) are components of the proteasome, consistent with the
93 known interaction of UbVS-sensitive DUBs UCHL5 and USP14[25] with the proteasome. Other
94 known interactors of DUBs that we isolated included GBP1/GBP2, which binds USP10[26], and
95 the SPATA proteins (SPATA2/SPATA2L), which have been identified as CYLD interactors[16] in
96 human cells (Table S2), suggesting evolutionary conservation of these interactions.
97

98 **UbVS treatment induces degradation of a limited set of proteins in *Xenopus* extract when**
99 **available ubiquitin is restored**

100 We first wanted to investigate how inhibition of multiple DUBs influenced stability of
101 the proteome in *Xenopus* extract. We hypothesized that simultaneous inhibition of 35 DUBs with
102 UbVS might lead to destabilization of proteins that require ongoing deubiquitylation to maintain
103 their stability. By simultaneously inhibiting a large number of DUBs, we predicted that we might
104 be able to identify new substrates of DUBs, including substrates whose stability is conferred by
105 the action of redundant DUBs. We used a multiplexed Tandem Mass Tag (TMT)-based

106 quantitative proteomics approach[27] (Fig. 1d) and we measured protein abundance over time in
107 untreated extract, as well as extract to which ubiquitin, UbVS or the combination
108 (UbVS/ubiquitin) were added (Fig. 1e). Proteins decreasing specifically in the presence of UbVS
109 and/or UbVS/ubiquitin may normally be protected from degradation by UbVS-sensitive DUBs.
110 We performed 2 independent experiments using *Xenopus* extract prepared from 2 sets of eggs
111 (collected from different animals and processed separately), to ensure reproducibility. In each
112 experiment we quantified around 8000 proteins, with an overlap of 94.8% between the 2
113 experiments.

114 First, we observed that in the absence of any perturbation, the proteome was stable in
115 *Xenopus* extract over the course of 60 minutes (Fig. S1a). Few proteins changed in abundance
116 over time and none reproducibly in both the experiments. This degree of proteome stability is
117 consistent with the idea that bulk rates of translation are relatively quiescent in the *Xenopus*
118 system[28] and that the extract was treated with cycloheximide to prevent protein synthesis and
119 mitotic entry. Proteome stability in untreated extract was not a consequence of limiting ubiquitin
120 availability because addition of exogenous ubiquitin alone did not stimulate protein degradation
121 (Fig. S1a). With the exception of ubiquitin, which was added to the extract, we did not observe
122 any proteins that increased in abundance (Fig. S1a) in both the experiments. Addition of UbVS
123 alone also had no measurable effect on protein stability, possibly due to depletion of available
124 ubiquitin (Fig. S1a). However, addition of UbVS together with ubiquitin (UbVS/ubiquitin)
125 induced degradation of 34 proteins, as defined by a reduction in their abundance by at least 1.5-
126 fold in both experiments (Fig. 1f, g). Choosing a more relaxed threshold based on the 5% of
127 proteins whose abundance decreased most in UbVS/ubiquitin (FDR 2%) revealed additional
128 proteins that decreased in both experiments (Fig. S3a). Because transcription and translation

129 were inactive in extract, the decrease in protein abundance of this specific set of proteins was
130 likely a consequence of protein degradation that occurred as a direct consequence of DUB
131 inhibition. Thus, these proteins represent putative DUB substrates that are protected from
132 degradation by UbVS-sensitive DUBs.

133 Because only a limited number of proteins were destabilized by the addition of 10 μM
134 UbVS/ubiquitin, we tested whether stronger inhibition of UbVS-sensitive DUBs, by addition of a
135 higher concentration of UbVS, led to destabilization of a larger number of proteins. We
136 compared protein stability in untreated extract and in extract co-treated with ubiquitin and 10 μM
137 (as in the previous experiments) or 30 μM UbVS (Fig. S2a) using TMT-based quantitative
138 proteomics as before. We confirmed the degradation of the proteins observed previously using
139 10 μM UbVS, but we did not observe an increase in the number of proteins degraded in the
140 presence of 30 μM UbVS (Fig. S2b). On the contrary, the proteins decreasing in 10 μM UbVS
141 were more stable at the higher UbVS concentration (Fig. S2b, S2c). This finding suggested that
142 addition of a higher concentration of UbVS might lead to faster ubiquitin depletion, thereby
143 hampering the ability of UbVS to stimulate protein degradation. To test this hypothesis, we
144 monitored the discharge rate of the E2-Ub thioester after ubiquitin addition to extract pre-treated
145 with increasing concentrations of UbVS. Whereas addition of 50 μM ubiquitin was sufficient to
146 completely sustain Ube2C charging for 30 minutes in extract treated with 10 μM UbVS, this
147 concentration of ubiquitin was insufficient to maintain the E2 charged in extract treated with
148 higher concentration of UbVS (Fig. S2d). Thus, we cannot drive broader protein instability by
149 stronger DUB inhibition because ubiquitin becomes depleted too rapidly.

150 **Confirmation of UbVS-dependent proteasomal degradation of the newly identified**
151 **substrates with human orthologs**

152 Most of the 34 substrates that we identified have reported physiological functions, but
153 only 9 are known UPS substrates (Fig. 2a, Fig. S3a, Table S3). Furthermore, for only four of
154 them have specific DUBs been identified that control their stability. Two of these proteins are
155 ubiquitin ligases, MARCH7 and BIRC3, which are known to be protected from degradation by
156 cysteine-protease DUBs in human cells (by USP7 or USP9[29] and USP19 respectively[30]). In
157 addition, we identified Stam and NFX1, which are known to be deubiquitylated by the cysteine-
158 protease DUBs USP8[31] and USP9 respectively[32]. The ability of our screen to identify known
159 DUB substrates suggests that our approach is capable of identifying physiologically relevant
160 proteins whose stability requires DUB activity.

161 For the majority of the proteins we identified, DUBs have not been implicated in
162 regulation of their stability. We identified four additional ubiquitin ligases (MKRN1, RNF138,
163 ZNF598, UBOX5), whose stability is not known to be dependent on DUB activity. This
164 enrichment for ubiquitin ligases is again consistent with the fact that DUBs are known to protect
165 them from auto-ubiquitination and consequent degradation[17,33]. Other functional classes of
166 proteins that we identified included transcriptional regulators (GTSF1, SOX3, SOX15, TGIF2),
167 signaling proteins (GYGIf1, PLK3, WEE2, CSNK1E, BORA, PIM3), cytoskeletal regulators
168 (CRIPT, WASL, KIAA1430, HN1, ARHGEF19), proteins involved in RNA processing
169 (FAM32, RNASEH1, RBM18, SYF2, ZMAT22, ZFP36L11, EIF1), DNA damage components
170 (ETAA1, SPRTN), a lipid storage protein (PLIN2), and an uncharacterized protein (C6ORF132).
171 Together these findings suggest that ongoing deubiquitylation in the *Xenopus* system is
172 important to maintain the stability of proteins that regulate a wide variety of biological processes.

173 We looked for possible characteristics sequence characteristics of these substrates (Table
174 S3) that could contribute to their recognition by the UPS. Consistent with the PEST sequence[34]

175 being a signal that promotes ubiquitin and proteasome-dependent degradation, we found one or
176 multiple PEST sequences in 13 of the 34 hits (Table S3). Because efficient protein degradation
177 by the proteasome requires unstructured regions in its substrates[35], we analyzed their sequences
178 using ProViz[36]. We found that 8 substrates were predicted to be completely or highly disordered
179 whereas 25 had significantly disordered regions consistent with the possibility that they are
180 proteasome substrates (Table S3).

181 To directly test whether degradation of the identified DUB substrates was proteasome-
182 dependent, we quantified protein stability in extract treated with UbVS/ubiquitin in the presence
183 of the proteasome inhibitor MG262 or DMSO (as a control) with a TMT-based quantitative
184 proteomics experiment (Fig. S4a, S4b). We found that 19 proteins became unstable after
185 UbVS/ubiquitin addition (Fig. S4b, S4c), 14 of which were among the 34 substrates identified
186 (Fig. 1g). All of these proteins were stabilized by proteasome inhibition (Fig. S4b, c). Thus,
187 UbVS-sensitive DUBs antagonize proteasome-mediated degradation of these proteins.

188 To verify the findings of the proteomic experiments and directly test whether these
189 proteins become destabilized by DUB inhibition, we expressed 13 candidate substrates in rabbit
190 reticulocyte lysate and labeled them with ^{35}S-methionine. We chose ten proteins from our list of
191 34 candidates, as well as three proteins that were destabilized in at least one of the two
192 experiments of Fig.1f. We translated *in vitro* human orthologs, with the exception of PIM3,
193 where we tested the *Xenopus* protein. Subsequently, we added the translated proteins to untreated
194 extract or to extract pre-treated with ubiquitin, UbVS or UbVS/ubiquitin and monitored their
195 stability over time. We found that 12/13 proteins were degraded in the presence of
196 UbVS/ubiquitin but were stable in the other conditions (Fig. 2b, 2c and Fig. S3b, 3c). STAM was
197 the only protein that was not destabilized in the presence of UbVS/ubiquitin, but instead showed

198 strong poly-ubiquitination (Fig. 2d). As a control, two proteins that were stable in the proteomic
199 experiments (KPNA2 and HSPA8), were also tested and found to be stable when assayed by this
200 approach (Fig. 2b, 2c) Thus, because we could recapitulate the behaviour of the endogenous
201 *Xenopus* proteins using human orthologs, the DUB-dependent regulation of these proteins is
202 likely conserved, as is known already for MARCH7, BIRC3, NFX1 and STAM.

203 **Identification of DUBs sufficient to rescue proteins from degradation in UbVS/ubiquitin-**
204 **treated extract**

205 Next, we used these substrates to investigate the role of cysteine-protease DUBs in
206 counteracting their proteasomal degradation. If the proteins degraded in UbVS/ubiquitin-treated
207 extract were true DUB substrates, addition of recombinant DUBs to the extract should be able to
208 rescue their degradation. Furthermore, by testing the sufficiency of each DUB to rescue
209 degradation of these substrates, we can evaluate the activity and specificity of each DUB, even if
210 they normally function redundantly. We reasoned that the DUBs efficiently targeted by UbVS
211 were most likely to rescue the stability of the proteins degraded. Thus, we measured the fraction
212 of each DUB labeled by UbVS. After we treated the extract with 10 μM HA-UbVS, we
213 subsequently depleted all the HA-UbVS (and associated proteins) from the extract by
214 immunodepletion with anti-HA antibodies coupled to beads. Using TMT-based quantitative
215 proteomics, we compared the proteins remaining in extract after immunodepletion of HA-UbVS
216 to the proteins present in extract after incubation with empty beads. As expected, the proteins
217 depleted were mostly DUBs (Fig. S5a). We detected 32 cysteine-protease DUBs in undepleted
218 extract and found that 25 of them were depleted by HA-UbVS (Fig. 3a), with 19 DUBs being
219 depleted by at least 30%. There was no clear correlation between the reported abundance[24] of the
220 DUBs in extract (Table S1) and their fractional depletion by HA-UbVS (Fig. 3a). We selected 12

221 DUBs that were depleted at least 30% to test if they could rescue substrate degradation in
222 UbVS/ubiquitin treated extract (Fig.3b, in bold). In addition, we tested USP14 because it can
223 rescue several substrates from proteasomal degradation[37], as well as USP21 because it is a highly
224 active and non-specific DUB widely used in in vitro assays.

225 First, we confirmed the activity of each recombinant DUB with a UbVS-reactivity assay
226 (Fig. S5b, c). All DUBs tested showed almost complete labelling with UbVS, with the exception
227 of USP14 that requires activation by the proteasome[23,37], which was absent from these in vitro
228 assays. We then proceeded to add each recombinant DUB to extract pre-treated with
229 UbVS/ubiquitin and monitored the stability of ^{35}S-methionine-labeled ING2, CSNK1E and
230 CRIPT (Fig.3b). We chose these proteins as model substrates because they have not been
231 reported to be regulated by DUBs and they have unrelated functions. Extract was treated with
232 UbVS for 30 minutes, instead of 10 minutes, to ensure that the UbVS would react completely
233 with the endogenous DUBs and not inhibit the exogenous DUBs added to the extract (data not
234 shown). We began by assessing all DUBs at the same concentration (800 nM), which is
235 substantially greater than the endogenous concentration for ten of the fourteen DUBs tested (Fig.
236 S6a). We compared the ability of each DUB to rescue degradation of the selected substrates,
237 creating a "DUB profile" that reports the percentage of substrate rescued by each DUB (Fig. 3b).
238 We observed that only USP7, USP15, USP25 and USP28 could rescue degradation of these
239 substrates (Fig. 3b, Fig. S6b). Whereas USP28 and USP15 are present at low concentration in
240 extract (4 and 20 nM respectively), USP25 was not detected in the previous study indicating that
241 its concentration is very low, so its activity was not further evaluated. USP7 is much more
242 abundant in extract (150 nM), suggesting that it might be able to rescue degradation of these
243 substrates at its physiological concentration. In fact, when tested at physiological concentrations,

244 USP7 remained capable of strongly rescuing CRIPT stability, with partial rescue of ING2
245 stability (Fig.3c, S6c). In contrast, USP15 and USP28 did not rescue substrate degradation when
246 used at lower concentrations, even though these exceeded their endogenous concentrations by 5-
247 to 15-fold, respectively (Fig. 3c, S6c). Therefore, among all the DUBs screened, USP7 seemed
248 unique in its ability to rescue degradation of the substrates when tested at its physiological
249 concentration. In contrast, USP4, USP9X, OTUD3, USP8, USP10 and USP21 did not rescue
250 degradation of the three substrates, even though they were tested well above their endogenous
251 concentrations. We were puzzled by the fact that the two known proteasome associated DUBs,
252 UCHL5 and USP14, did not affect degradation of the substrates tested. Because they are very
253 abundant in extract, we retested them together with UCHL3 (another very abundant DUB in
254 extract) at 5 times their endogenous concentration and we compared them to USP7. None of
255 these DUBs had an effect comparable to USP7 in this assay (Fig.3d).

256 Because USP7, USP15, USP25 and USP28 was each sufficient to stabilize all three
257 substrates when used at 800 nM concentration, we wondered whether these DUBs were also
258 capable of restoring ubiquitin availability in UbVS-treated extracts. We treated extract with 10
259 μM UbVS, added each of the recombinant DUBs and monitored the charging status of Ube2C-
260 Ub thioester as a readout of ubiquitin availability. We included USP21 as a positive control, as it
261 broadly deubiquitylates substrates in vitro, as well as USP5, which can regenerate free ubiquitin
262 by acting on unanchored ubiquitin chains[38]. As expected, addition of exogenous ubiquitin fully
263 restored charging of the Ube2C-Ub thioester (Fig.3e), as did addition of USP21, consistent with
264 its known broad substrate specificity. USP5 did not have any effect, suggesting that free
265 unanchored ubiquitin chains are not generated at high levels in UbVS-treated extract. Of the 4
266 DUBs able to rescue substrate degradation, only USP15 was able to fully restore charging of

267 Ube2C. This finding suggests that USP7, USP25 and USP28 have a restricted substrate
268 specificity that is capable of rescuing substrate degradation without impacting ubiquitin
269 availability in the extract, whereas USP15 seems to have a broader specificity that allows it to
270 rescue degradation and also restore ubiquitin recycling. On the other hand, despite the ability of
271 USP21 to regenerate free ubiquitin, it is not able to rescue the degradation of any of the tested
272 substrates.

273 **USP7 broadly rescues protein degradation in UbVS/ubiquitin treated extract**

274 As USP7 seemed to be the most efficient DUB in rescuing degradation of the substrates
275 tested, we decided to investigate how broad an effect USP7 had in rescuing proteins from
276 degradation in extract treated with UbVS/ubiquitin. We performed a TMT-based quantitative
277 proteomics experiment comparing protein stability in extract treated with UbVS/ubiquitin, and in
278 extract in which USP7, USP9X or USP4 were added at 800 nM each (Fig. 4a). We tested USP9X
279 and USP4 as they were good targets of HA-UbVS (Fig. 3a) yet were not sufficient to rescue the
280 degradation of the panel of substrates that we tested (Fig 3c). We found that USP7 could rescue
281 16 of the 20 proteins degraded in UbVS/ubiquitin (Fig. 4c). However, we did not find any
282 protein stabilized by addition of USP4 or USP9X (Fig.4b, 4c), consistent with the results of our
283 earlier screen using the panel of ^{35}S-labeled substrates. The fact that human recombinant USP7
284 can stabilize endogenous *Xenopus* proteins suggests again that DUB-dependent regulation of the
285 stability of these proteins is likely evolutionarily conserved.

286 Because USP7 broadly rescued substrate degradation in UbVS/ubiquitin-treated extract
287 (Fig. 4b, c) and has been reported to be associated with the proteasome[39,40], USP7 might inhibit
288 the proteasome independent of its catalytic activity, as is known for USP14[37]. Thus, we tested if

289 the catalytic activity of USP7 was required for substrate rescue. Pre-incubation of USP7 with
290 UbVS inhibited rescue of substrate degradation (Fig.4d), confirming that deubiquitinating
291 activity of USP7 is required for the rescue. Second, we investigated if USP7 could inhibit
292 degradation of other known proteasome substrates. We tested if USP7 can rescue the APC
293 substrates cyclin B1 and securin from proteasome degradation. Surprisingly, we found that USP7
294 was not able to rescue them from degradation (Fig. 4e), indicating that USP7 does not generally
295 inhibit the degradation of all substrates of the UPS.
296
297 **Inhibition of Usp7 is not sufficient to induce protein instability suggesting that DUBs**
298 **function redundantly to control protein stability in extract**
299 Because USP7 could stabilize most of the proteins degraded in extracts treated with
300 UbVS/ubiquitin, we tested if inactivation of USP7 using the specific inhibitor XL-188[41] was
301 sufficient to induce degradation of the substrates that we identified. We also tested the effect of
302 USP14 inhibition on proteome stability using the specific inhibitor IU-47[42] alone or in
303 combination with XL-188, since USP14 inactivation promotes degradation of some proteasome
304 substrates in vitro and in human cells[43]. First, we verified these compounds were able to inhibit
305 the endogenous *Xenopus* DUBs. We pre-incubated extract with the active compounds (IU-47 and
306 XL-188) and the respective inactive derivatives (IU-C and XL-203) for 30 minutes, and then
307 added 10 μM HA-UbVS. As expected, only the active compounds were able to prevent HA-
308 UbVS labelling of the specific targeted DUB (Fig. S7a). After we verified that the inhibitors
309 were working in extract, we compared protein stability in extract pre-treated with 10 μM UbVS,
310 100 μM IU-47 (USP14i), 200 μM XL-188 (USP7i) or the combination of the latter inhibitors,
311 with TMT-based quantitative proteomics (Fig. S7b). In all conditions we added ubiquitin to

312 minimize any impact of DUB inhibition on ubiquitin recycling. In this experiment, we confirmed
313 again that 14 of the 34 candidates (Fig. S7b) were degraded in UbVS/ubiquitin treated extract.
314 Among these proteins, only HN1 was destabilized after specific USP7 inhibition. There were no
315 other proteins (detected with more than one peptide) that were destabilized following specific
316 USP7 inhibition. Inhibition of USP14 did not promote protein instability in *Xenopus* extract, as
317 none of the proteins degraded in UbVS/ubiquitin, or any other protein, was degraded in IU-47-
318 treated extract (Fig. S7c, d). Inhibition of both DUBs, as expected, caused the degradation of
319 Hn1 that was destabilized by the USP7 inhibitor. In addition, we observed degradation of three
320 other proteins: DBN1, KHLC1, and Supervillin (SVIL), a known USP7 substrate[44] (Fig.S7c,
321 bottom). Together these findings suggest that inhibition of USP7 alone or in combination with
322 USP14 is not sufficient to drive the broader pattern of protein instability that we observe in
323 extracts treated with UbVS/ubiquitin. Instead, our results support a model in which multiple
324 DUBs play redundant functions in maintaining stability of these substrates.

325 **Discussion**

326 In this study, we discovered novel DUB substrates that are protected from proteasome
327 degradation by cysteine-protease DUBs using a non-biased proteomic approach. One of the
328 challenges in identifying DUB substrates is that DUBs may function redundantly. Here, we
329 circumvented DUB redundancy by inactivating multiple DUBs simultaneously. We took
330 advantage of the *Xenopus* egg extract model system in which the quality control pathway is
331 silent, as protein synthesis is inhibited. Thus, proteins destabilized by DUB inhibition are more
332 likely to be direct DUB substrates as transcription and translation are suppressed. We
333 recapitulated DUB-dependent stability with recombinant human proteins, indicating that their
334 DUB-dependent regulation is evolutionary conserved. We showed how these novel physiological
335 DUB substrates can be a valuable tool for studying DUB activity and specificity. By testing the
336 ability of individual DUBs to rescue their degradation in a system in which DUBs are broadly
337 inhibited, we found that USP7 has a broad capacity to rescue substrate degradation. However, we
338 found that specific inhibition of USP7 is not sufficient to induce substrate instability, suggesting
339 that stability of the substrates that we identified is likely controlled by multiple DUBs. Together
340 our findings highlight the role of DUB redundancy and the importance of inhibiting multiple
341 DUBs to reveal new DUB substrates.

342 Our approach began with identifying proteins protected from proteasomal degradation by
343 cysteine-protease DUBs. Several of these proteins are known to be regulated by specific DUBs,
344 including MARCH7, BIRC3, STAM, NFX1,TRIP12, SVIL, and NEK2A[29,30,32,45,31,44,46].
345 Furthermore, PIM3 and WASL have not been connected previously to DUBs, but are similar in
346 sequence to well-established DUB substrates (PIM2[47] and WASH[48] respectively). Together,
347 these findings validate that our approach can identify true DUB substrates. However, the vast

348 majority of the proteins we identified have not been directly implicated as DUB substrates,
349 demonstrating the novelty of our approach. Some proteins that we identified have a proposed
350 physiological function, but have not been deeply studied otherwise, explaining why UPS-
351 dependent control of their stability has not yet been reported. As examples, we highlight CRIPT,
352 a neuronal cytoskeletal protein, as well as SYF2, ZMAT2 and RBM18 that are involved in RNA
353 processing. In contrast, a number of the other substrates we identified, including Inhibitor of
354 growth 2 (ING2), SPRTN, ETAA1, and the Casein Kinases ε and δ, have been extensively
355 studied, so it is surprising that DUB-dependent control of their stability has not yet been
356 reported. ING2 belongs to the family of ING proteins, which are tumor suppressors that regulate
357 DNA repair, cell proliferation, differentiation, senescence and apoptosis[49]. ING2 levels are low
358 in a variety of cancer cells and ING2 knockout mice develop a high incidence of cancer[49]. ING2
359 is targeted to the proteasome by the ubiquitin ligase Smurf1[50]. Here we confirmed that ING2 is a
360 UPS substrate and we discovered that cysteine-protease DUBs antagonize its degradation,
361 providing a novel mechanism that regulates its stability. SPRTN and ETAA1 are both involved
362 in DNA replication and DNA damage and are essential for maintaining genome stability[51,52]. A
363 specific mutation in SPRTN causes a syndrome that predisposes patients to cancer (the Ruijs-
364 Aalfs syndrome)[53] while ETAA1 (Ewing tumor-associated antigen 1) was discovered as a tumor-
365 specific antigen in Ewing's sarcoma[54]. Our results provide the first evidence that the UPS
366 controls the stability of ETAA1. SPRTN is a cell cycle-regulated protein that is known to be
367 ubiquitylated by APC/CDH1[55] and degraded upon exit from mitosis. Our data suggest that
368 UbVS-sensitive DUBs could ensure SPRTN stability during S-phase or during the DNA-damage
369 response when its activity is required. Casein Kinases ε and δ are versatile proteins that
370 participate in multiple processes such as cell cycle control, spindle organization and circadian

371 rhythm[56]. They auto-phosphorylate themselves inhibiting their catalytic activity[57]. Our study
372 suggests that DUBs could modulate their degradation by the UPS as another mechanism to
373 regulate their activity.

374 Our candidate substrates were enriched for ubiquitin ligases (n=12), extending our
375 understanding of the importance of DUBs in counteracting their auto-ubiquitination and
376 consequent degradation[17,33]. Makorin Ring Finger protein 1 (MKRN1) ubiquitylates p53 and p21
377 during cell growth, targeting them to proteasomal degradation[58]. Yet despite intensive study, a
378 role of DUBs in controlling MKRN1 stability has not been described. Our data suggest that
379 autoubiquitination of MKRN1 could be an important control mechanism, as it is for MDM2,
380 another p53 ubiquitin ligase[59]. Among the new substrates we identified, we found multiple
381 transcription factors (n=7) and proteins involved in RNA metabolism (n=12). These data further
382 support the idea that DUBs may regulate these processes[16,20] and suggest that changes in protein
383 levels observed in growing cells after DUB inhibition must be carefully interpreted as they could
384 be due to an indirect effect induced by changes in transcription/translation.

385 Having identified a set of novel physiological DUB substrates, we next tested the ability
386 of specific DUBs to antagonize their degradation under conditions in which the physiological
387 rates of substrate ubiquitination are maintained. The most interesting finding to emerge from this
388 analysis was the ability of USP7 to rescue a large number of substrates from degradation. USP7
389 has been highly studied and has attracted attention as a pharmacological target, as it regulates the
390 stability of the tumor suppressor p53 and its regulator MDM2[60]. Among the DUBs we tested,
391 USP7 has the greatest number of identified substrates, most of which are targeted to the
392 proteasome[61]. Among the substrates that we identified, a few such as MARCH7, SVIL, NEK2A
393 and TRIP12 are known targets of USP7[29,44,46,45] whereas two others, PIM3 and WASL, are likely

394 USP7 targets as USP7 is known to regulate related proteins (PIM2[47] and WASH[48] respectively)
395 in human cells.

396 What explains the broad ability of USP7 to antagonize proteasomal degradation? USP7
397 has been reported to associate with the proteasome[39,40], where it could intercept proteins that are
398 targeted for degradation. However, the physiological significance and mechanism of this
399 association has not been carefully studied. An intriguing possibility is that USP7 acts on
400 substrates that are insensitive to the other proteasome-associated DUBs, USP14 and UCHL5. For
401 example, USP14 has been shown to deubiquitylate proteins that carry more than one ubiquitin
402 chain[62]. As such, it follows that USP7 could deubiquitylate substrates bearing single ubiquitin
403 chain substrates that are insensitive to USP14. More studies are needed to fully understand the
404 substrate specificity of the proteasome-associated DUBs and the possible role of USP7 in this
405 context. USP7 may have a broad ability to rescue substrates from degradation because it directly
406 binds a wide range of proteins through recognition of common degenerate motifs (P/AxxS and
407 Kxxx/KxK) that are found in many proteins[61]. Another plausible explanation is that USP7 is
408 kinetically fast, and thus competent to counteract the rates of ubiquitination of ubiquitin ligases
409 that target substrates for degradation. This idea is supported by USP7 being the most active DUB
410 in Ub-AMC assays among a panel of 12 cysteine DUBs tested[63].

411 Despite USP7 being able to broadly rescue substrates from degradation, it was unable to
412 rescue the APC/C substrates Securin and CyclinB1. This result was not due to a lack of activity
413 of USP7 in mitosis because we verified that USP7 was still active in mitosis (data not shown).
414 The insensitivity of these proteins to USP7 could be due to the fact that USP7 is unable to
415 recognize K11-linked ubiquitin and mixed ubiquitin chain types that are synthetized by the

416 APC[22]. USP7 has been reported to deubiquitylate all types of ubiquitin linkages [63,64], but activity
417 towards mixed ubiquitin linkages (such as K48/K11) remains unclear.

418 Despite the broad ability of USP7 to rescue substrates from degradation, USP7 was
419 unable to rescue ubiquitin depletion in UbVS-treated extract. In contrast, Usp21 (a highly active
420 and nonspecific DUB) was able to rescue ubiquitin depletion, but was unable to rescue
421 degradation of the tested substrates. These finding suggest that even if USP7 broadly rescues
422 substrates from degradation, it must have a distinct and probably restricted substrate specificity
423 compared to USP21. For example, USP21 may deubiquitylate a limited number of highly
424 abundant proteins present in extract, whereas USP7 may target a distinct set of low abundance
425 proteins. Identifying non-degradative targets of USP7 and USP21 will be an important question
426 to address in future research to better understand the features underlying substrate specificity of
427 these enzymes.

428 Our study suggests that the large number of substrates protected from degradation by
429 USP7 reported in the literature is not simply due to USP7 having been deeply studied, but arises
430 from its broad ability to rescue substrates from degradation. Although many USP7 substrates
431 have been reported, our work suggests that USP7 may contribute to the stabilization of an even
432 larger number of substrates, as the identification of some substrates may have been masked by
433 DUB redundancy. In our system, despite USP7's ability to broadly rescue substrates from
434 degradation, we observed that specific inhibition of USP7 was not sufficient to induce
435 degradation of these substrates. Similar results were obtained for the inhibition of USP14, a
436 proteasome-associated DUB whose inhibition enhances proteasome activity[37]. Even
437 simultaneous inhibition of USP7 and USP14 destabilized very few proteins. Together these
438 findings support the notion that DUBs function redundantly to maintain the stability of the

439 proteins in this system. Because degradation is an irreversible step in the protein lifecycle, DUB
440 redundancy may help set a higher threshold for ubiquitination required for degradation of a
441 protein, beyond what is required for direct recognition by the proteasome.

442 **Materials and Methods**

443 **Gene nomenclature**

444 The human gene symbols have been used for consistency and simplicity in the manuscript.

445

446 **Reagents**

447 Commercial antibodies used for Western blotting analysis were as follow: anti-Ube2C
448 (A-650, Boston Biochem), anti-ubiquitin (P4D1; sc-8017; Santa Cruz Biotechnology), anti-HA-
449 peroxidase (3F10; Roche), anti-USP7 (A300-033A; Bethyl). Secondary antibodies used included
450 anti-rabbit IgG-HRP (NA934V) and anti-mouse IgG-HRP (NA931V) from GE Healthcare.
451 Chemicals used in this study included: Ubiquitin vinyl sulfone (U-202), MG262 (I-120) and
452 Ubiquitin vinyl sulfone tagged with HA (U-212), purchased from Boston Biochem. For
453 immunoprecipitation anti-HA-Agarose beads (A2095) from Sigma were used. Ubiquitin (U-
454 100H) and recombinant DUBs were provided by Boston Biochem (E-519, USP7; E-520, USP8;
455 E-320, USP5; E-325, UCHL3; E-325, UCHL5; E-546, USP25; E-552, USP9x; E-570, USP28;
456 E-574, OTUD3; E-592, USP10; E-594, USP15; E-596, USP4).

457

458 **Preparation of *Xenopus* egg extract**

459 Interphase extract was prepared as described[65] but using 2 μg/ml calcium ionophore
460 (A23187, Calbiochem) for egg activation. Entry into mitosis was induced by addition of 1 μM
461 non-degradable cyclin B (MBP-Δ90) as previously described[66].

462

463 **HA-UbVS immunoprecipitation**

464 Following treatment with 10 μM HA-UbVS (10 minutes at 24°C), *Xenopus* extract was
465 diluted three times with XB buffer (10 mM potassium HEPES pH 7.7, 500 mM KCl, 0.1 mM
466 CaCl2, 1 mM $MgCl_2$, 0.5% NP40) and incubated with anti-HA beads or empty beads at 4°C (1h
467 and 30 minutes). At the end of the incubation, beads were washed three times with XB buffer,
468 SDS sample buffer was added and samples were subjected to SDS gel electrophoresis. Samples
469 were processed according to the GeLC-MS/MS strategy[67].
470
471 **HA-UbVS immunodepletion**
472 *Xenopus* extract was pre-treated with 10 μM HA-UbVS (10 minutes at 24°C) diluted
473 three times with XB buffer and incubated with anti-HA beads or empty beads overnight at 4°C.
474 Supernatants from the beads were collected and treated for Mass Spectrometry analysis by SL-
475 TMT method (described below).
476
477 **Degradation of ^{35}S-methionine labeled substrates**
478 Extract was pre-treated with UbVS (10 μM) for 10 min at 24°C before addition of
479 ubiquitin (50 μM) and substrates. The preincubation time with UbVS was extended to 30
480 minutes when recombinant DUBs were added to the extract. Pre-treatment of extract with
481 MG262 (200 μM) or specific DUB inhibitors was done at 24°C for 30 minutes. Substrates were
482 expressed and labeled using ^{35}S-methionine (Perkin Elmer, NEG709A500UC) with the TNT kit
483 (Promega: L1770). Each substrate was amplified with primers by PCR to allow T7-dependent
484 transcription of the PCR product or transcribed directly if plasmids contained a T7 promoter. The
485 translation reaction mix was added to the *Xenopus* extract at 8% final volume. Samples of the
486 reactions were collected at the indicated time, quenched with sodium dodecyl sulfate (SDS)

487 sample buffer, and processed for SDS gel electrophoresis and phosphor imaging (Bio-Rad PMI).
488 Quantification was performed using Quantity One software (Bio-Rad).
489
490 **UbVS labeling of recombinant DUBs to verify their activity**
491 Each DUB (1μM) was incubated with saturating amount of UbVS or HA-UbVS as
492 indicated (1 hour at 30°C). Reactions were stopped with addition of sodium dodecyl sulfate
493 (SDS) sample buffer and run on SDS-PAGE. After SDS-PAGE, the gel was stained with
494 Coomassie Blue.
495
496 **Streamlined Tandem Mass Tag Protocol**
497 Peptide extraction and digestion
498 The TMT labeling protocol and mass spectrometry analysis were based on the SL-TMT
499 sample preparation strategy[27]. *Xenopus* extract was collected, resuspended in the appropriate
500 buffer (1% SDS, 5mM DTT, 50mM Tris pH 8.8 and Pierce protease inhibitor tablet) and flash
501 frozen in liquid nitrogen. Methanol–chloroform precipitation was performed. Four volumes of
502 methanol were added to each sample and briefly vortexed. One volume chloroform was added to
503 the samples and vortexed again. Lastly, three volumes water was added and vortexed. The
504 samples were centrifuged (5 minutes, 14000 RPM) and subsequently washed twice with cold
505 methanol. Samples were resuspended in 200 mM EPPS, pH 8.5 and digested overnight at 24°C
506 with Lys-C protease (Wako Chemicals). Later samples were incubated for 6 hours at 37°C for
507 digestion by trypsin protease (Pierce Biotechnology).
508
509 Isobaric labeling and fractionation

510 Tandem mass tag (TMT) isobaric reagents (Thermo Fisher Scientific) were resuspended
511 in anhydrous acetonitrile (final concentration of 20 μg/μL). 10 μL of the labeling reagents plus
512 30 μl of acetonitrile was added to the peptides obtained by the previous digestions (~100 μg).
513 After incubation at room temperature (90 minutes), the reaction was quenched using
514 hydroxylamine to a final concentration of 0.3% (v/v). The TMT-labeled samples were pooled at
515 a 1:1 ratio across all the samples. Fractions were fractionated off-line using basic pH reversed
516 phase chromatography (BPRP). Fractions were pooled into 6 or 12 super-fractions which were
517 acidified with formic acid to a final concentration of 1%. Each fraction was desalted using
518 homemade StageTip, dried with vacuum centrifugation, and reconstituted in 5% acetonitrile and
519 5% formic acid for LC-MS/MS processing.
520
521 Peptide detection, identification and quantification
522 All samples were analyzed on an Orbitrap Fusion mass spectrometer (Thermo Fisher
523 Scientific) coupled to a Proxeon EASY-nLC 1000 liquid chromatography (LC) pump (Thermo
524 Fisher Scientific). Peptides were separated on a column packed with 35 cm of Accucore C18
525 resin (2.6 μm, 150 Å, Thermo Fisher Scientific). The column had a 100 μm inner diameter
526 microcapillary. For each experiment, 2 μg of peptides were loaded onto this column. Peptides
527 were separated, using a flow rate of 450 nL/min., with a 150-minute gradient of 3 to 25%
528 acetonitrile in 0.125% formic acid. Each analysis used an MS3-based TMT method, which it is
529 known to reduce ion interference if compared to MS2 quantification. The scan sequence starts
530 with an MS1 spectrum (Orbitrap analysis, resolution 120,000, 400−1400 Th, automatic gain
531 control (AGC) target 5E5, maximum injection time 100 ms). For subsequent MS2/MS3 analysis,
532 only the top 10 precursors were selected. MS2 analysis included: collision-induced dissociation

533 (CID), quadrupole ion trap analysis, automatic gain control (AGC) 2E4, NCE (normalized
534 collision energy) 35, q-value 0.25, maximum injection time 120 ms), and isolation window at
535 0.7. After we acquire each MS2 spectrum, we collected an MS3 spectrum in which multiple MS2
536 fragment ions were captured in the MS3 precursor population with isolation waveforms using
537 multiple frequency notches. MS3 precursors were fragmented by HCD and analyzed using the
538 Orbitrap (NCE 65, AGC 1.5E5, maximum injection time 150 ms, resolution was 50,000 at 400
539 Th). For MS3 analysis, we used charge state-dependent isolation windows: For charge state z=2,
540 the isolation window was set at 1.3 Th, for z=3 at 1 Th, for z=4 at 0.8 Th, and for z=5 at 0.7 Th.
541 Collected Spectra were processed using a Sequest-based software pipeline. Spectra were
542 converted to mzXML using MS Convert [68]. Database searching included all the entries from the
543 PHROG databas. This database includes many lower abundant proteins and multiple splice
544 isoforms (not present in other databases). In the original study, around 11,000 proteins were
545 identified[24]. Thus, our study (with ~8000 proteins) represents an acceptable coverage of the
546 *Xenopus* proteome. This database was concatenated with one composed of the sequences in the
547 reversed order. Searches were performed using a 50 Th precursor ion tolerance and the product
548 ion tolerance was set to 0.9 Da. Oxidation of methionine residues (+15.995 Da) and, where
549 indicated. Peptide-spectrum matches (PSMs) were adjusted to a 1% false discovery rate (FDR).
550 PSM filtering was performed using a linear discriminant analysis, as described previously and
551 assembled to a final protein-level FDR of 1%. Proteins were quantified by summing reporter ion
552 counts across all matching PSMs[69]. Reporter ion intensities were adjusted to correct for the
553 isotopic impurities of the different TMT reagents according to manufacturer specifications. The
554 peptides signal-to-noise (S/N) measurements assigned to each protein were summed and
555 normalized so that the sum of the signal for all proteins in each channel was equivalent, thereby

556 accounting for equal protein loading. Lastly, each protein was scaled such that the summed
557 signal-to-noise for that protein across all channels was 100, thereby generating a relative
558 abundance (RA) measurement.
559
560 **TMT Mass-spectrometry analysis**
561 A two-sided Student's t-test was used as a measure of statistical confidence of the
562 observed $\log_2$ fold change. Selected candidates met both thresholds (Fc≤ -1.5 and *p*-value<0.05)
563 in the experiments. For Figure 1g, the candidates were included if at least one peptide was
564 identified in both of the experiments. For subsequent figures, where a single experiment was
565 performed, candidates were included only if they were detected with at least 2 different peptides.
566 When multiple isoforms of the same protein decreased in UbVS/ubiquitin, only the isoform with
567 more peptides was selected (for simplicity isoforms are not indicated in the figures).

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727 **Author contributions**

V.R. and R.W.K designed the experiments

V.R. performed all the experiments

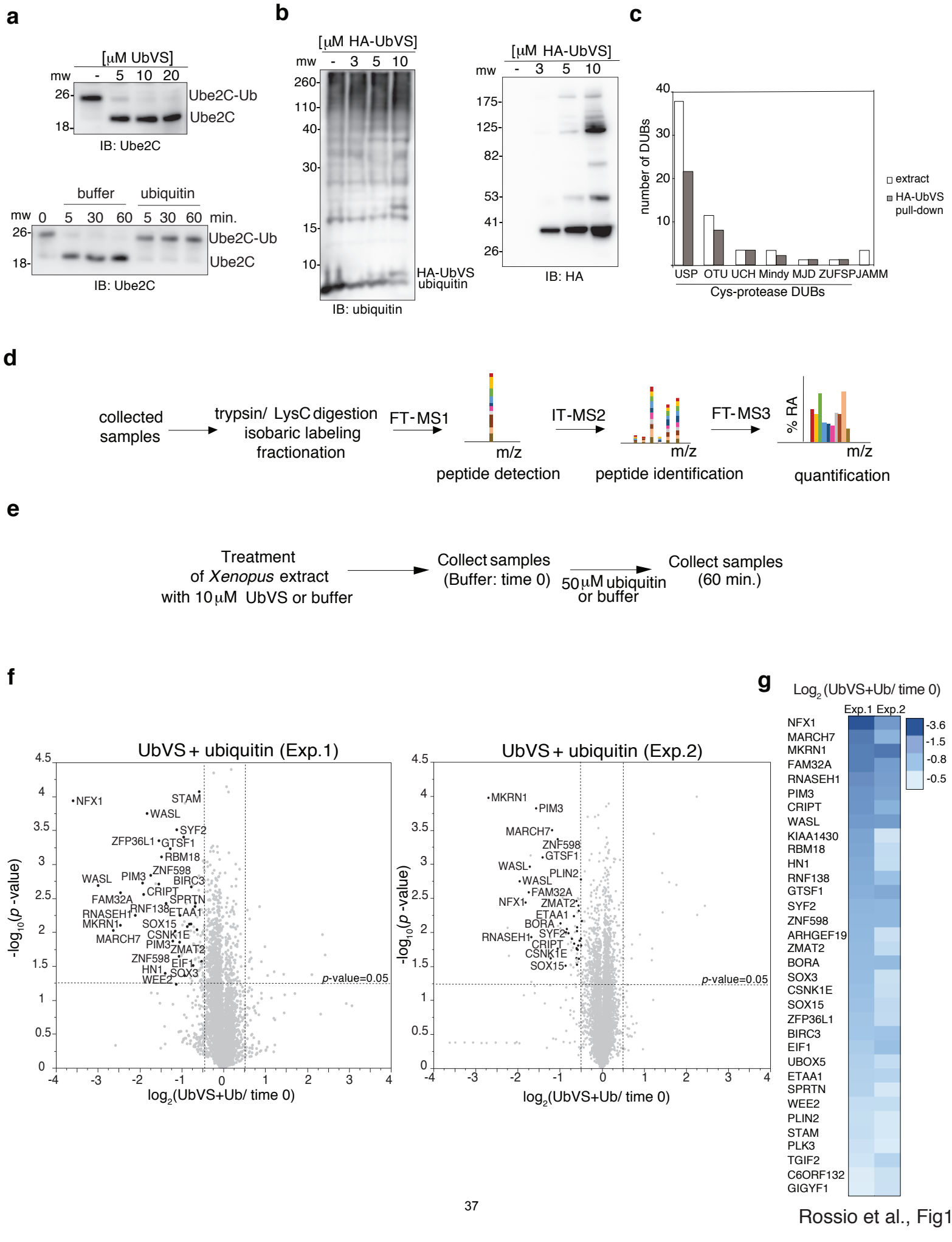
B.B. provided advice on DUB selection and DUB assays

J.A.P., J.C. and S.P.G. performed mass spectrometric analysis

R.W.K and V.R. wrote the manuscript with input from all the authors

Competing interests

The authors declare no competing interests



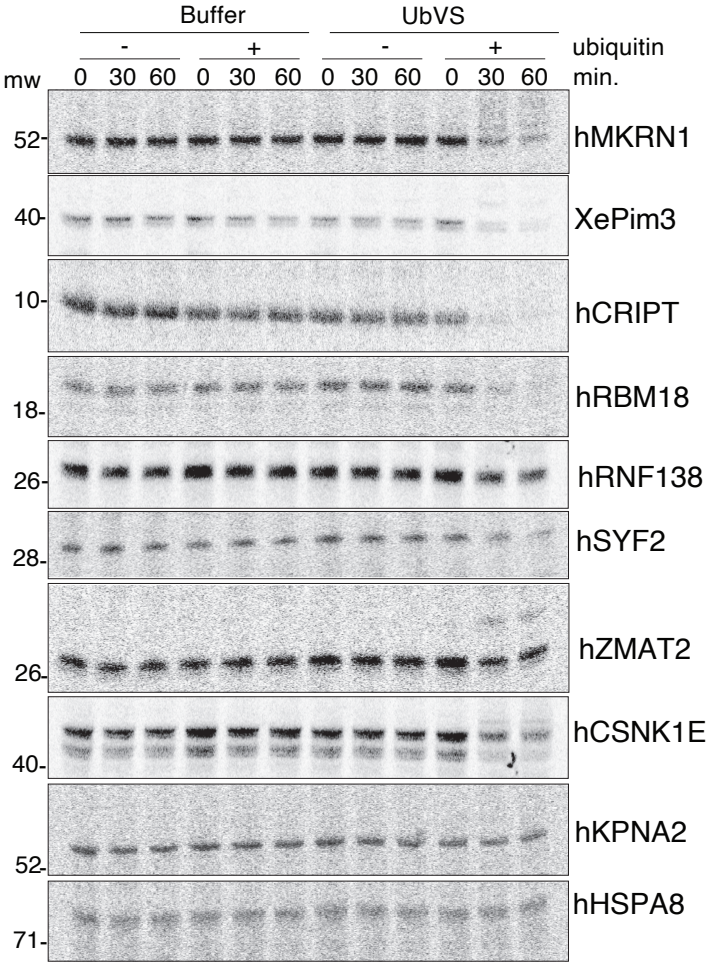
Rossio et al., Fig1

735 **Fig.1 Inhibition of cysteine-protease DUBs by UbVS causes rapid ubiquitin depletion and**
736 **degradation of a specific set of proteins. (a)** Interphase *Xenopus* extract was treated with the
737 indicated concentrations of UbVS or **(b)** HA-UbVS. After 10 minutes (time 0), 50 μM ubiquitin
738 or buffer were added (A, bottom). Aliquots were quenched with **(a)** non-reducing or **(b)** reducing
739 sample buffer and subjected to immunoblotting (IB) for the indicated proteins. **(c)** Extract was
740 incubated with 10 μM HA-UbVS (10 minutes) and subjected to anti-HA pull-down followed by
741 analysis of eluates by tandem mass spectrometry. The number of DUBs identified with the anti-
742 HA pull-down (gray) is compared to the number of DUBs detected previously in extract[24]
743 (white). **(d)** Mass spectrometry analysis by Streamlined-Tandem Mass Tag (SL-TMT) method is
744 summarized. **(e)** Ubiquitin or buffer were added to the extract (time 0) pre-incubated with buffer
745 or 10 μM UbVS for 10 minutes. Samples were collected in duplicate at time 0 and 60 minutes
746 and processed as described in (d). **(f)** Volcano plots of TMT analysis comparing the proteins
747 detected in UbVS/ubiquitin at 60 minutes with the proteins detected at time 0 (2 independent
748 experiments). Statistical significance ($-\log_{10} p$-value) is plotted against ratio (average $\log_2$).
749 Proteins significantly decreasing in both the experiments are showed in black. **(g)** Log_2 ratio heat
750 map of the proteins significantly downregulated in UbVS/ubiquitin in both the experiments in
751 (f).

a

Proteins	Function
MKRN1 UBOX5 (putative) **MARCH7 RNF138** BIRC3 NFX1 (putative) ZNF598	UPS (E3s)
RBM18 SYF2 EIF1 FAM32A **ZMAT2** RNASEH1 ZFP36L1	RNA metabolism
SOX3 SOX15 TGIF2 GTSF1	Transcription factors
CRIPT ARHGEF19 KIAA1430 WASL	Cytoskeleton
BORA WEE2 **PIM3** GIGYF1 HN1 PLK3	Proliferation/cell death
SPRTN ETAA1	DNA damage
STAM (trafficking) **CSNK1E** (circadian period) PLIN2 (lipid storage) C6ORF132 (?)	Others

b



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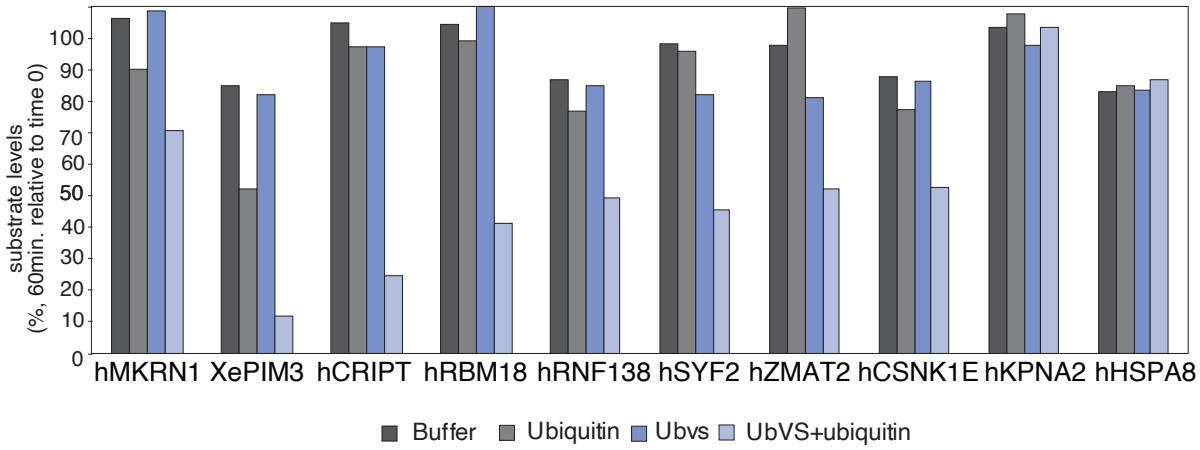

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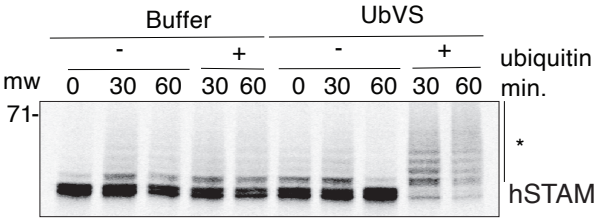
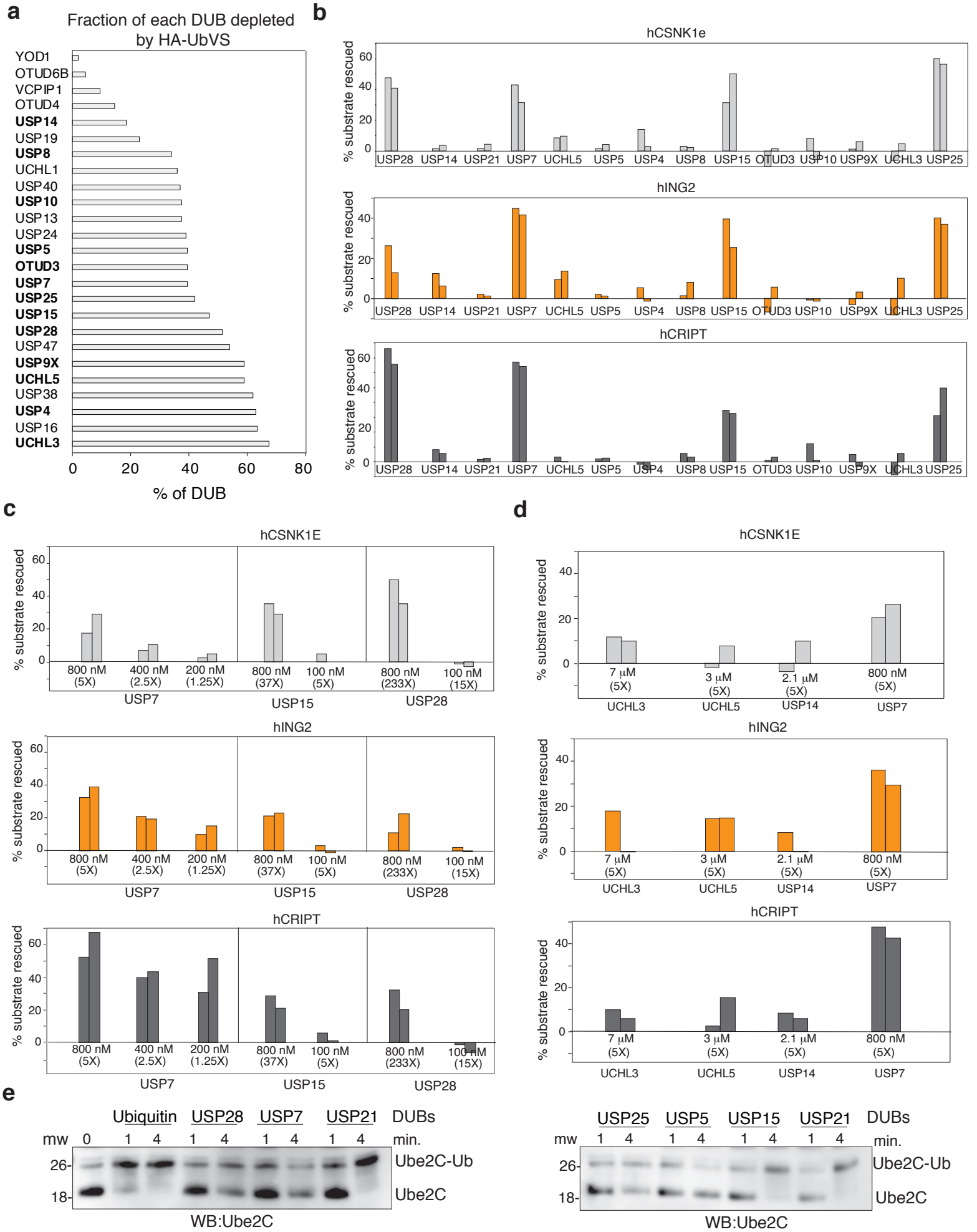


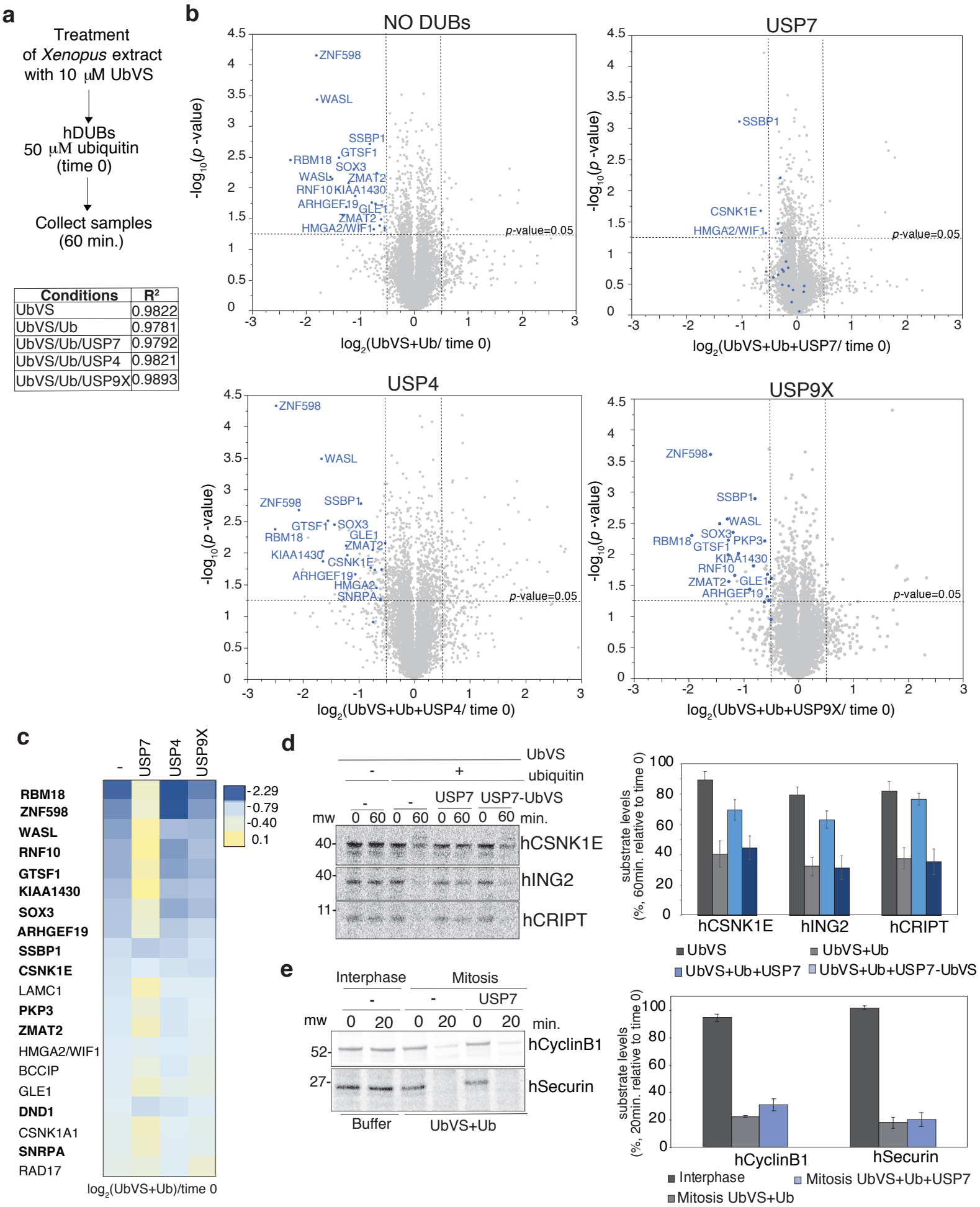
Rossio et al.,Fig2

752 **Fig. 2 Independent validation of the proteins protected from degradation by UbVS-**
753 **sensitive DUBs (a)** Functions of the proteins down-regulated in UbVS/ubiquitin-treated extract
754 (Fig.1f, g). Proteins independently validated in are shown in bold. (**b)** Human orthologs (prefix
755 h) of the indicated proteins, with the exception of PIM3, where the *Xenopus* protein was used,
756 were expressed in rabbit reticulocyte lysate, labeled with ^{35}S-methionine and added to the
757 extract, treated as described for the TMT proteomic experiments (Fig.1e). Aliquots were
758 collected at the indicated times, quenched with SDS sample buffer and processed for SDS-PAGE
759 and phosphorimaging. UbVS/ubiquitin-dependent degradation of these proteins was confirmed
760 in 2 independent experiments. **(c)** Quantification of one experiment. Quantification of the second
761 experiment is shown in Fig. S3d **(d)** Performed as in (b) *: ubiquitinated species.



Rossio et al., Fig3

762 **Fig 3. Identification of DUBs sufficient to rescue degradation of the selected substrates in**
763 **UbVS/ubiquitin-treated extract (a)** Extract was incubated with 10 μM HA-UbVS (10 minutes)
764 overnight at 4°C with anti-HA or empty beads. Supernatants were collected in triplicate for mass
765 spectrometry analysis by the SL-TMT method. Percentage of each DUB depleted by 10 μM HA-
766 UbVS. Bold: DUBs tested in subsequent experiments. **(b)** Extract was pre-incubated with 10 μM
767 UbVS (30 minutes), after which recombinant DUBs (800 nM), ^{35}S-methionine labeled substrates
768 and buffer or ubiquitin were added. After 60 minutes, aliquots were quenched with sample buffer
769 and processed for SDS-PAGE and phosphorimaging**.** Quantitative profiles for the 3 model
770 substrates tested were created. The percentage of each substrate rescued by the addition of the
771 indicated DUBs is reported for two independent experiments. Gels for one of the experiments are
772 shown in Fig. S6 **(c)** and **(d).** Dose response analysis, performed as in (**b**) but at varying DUB
773 concentrations. The tested concentration is shown; the value in parentheses indicates the fold-
774 increase of the tested concentration relative to the endogenous concentration. **(e)** Performed as in
775 **(b)** with the exception that ubiquitin was not added to the extracts with the DUBs. Samples were
776 collected at the indicated times (time 0), quenched with non-reducing sample buffer and
777 subjected to immunoblotting.



Conditions	R^2
UbVS	0.9822
UbVS/Ub	0.9781
UbVS/Ub/USP7	0.9792
UbVS/Ub/USP4	0.9821
UbVS/Ub/USP9X	0.9893

Rossio et al., Fig4

778 **Fig.4 USP7 broadly rescues substrates from degradation in UbVS/ubiquitin-treated extract**

779 **(a)** Top: Extract was incubated with 10 μM UbVS (30 minutes) followed by addition of DUBs
780 (800 nM) and ubiquitin. Samples were collected in duplicate and processed for mass
781 spectrometry analysis by SL-TMT method. Bottom: R^2 of the technical replicates for each
782 condition. **(b)** Volcano plot of quantitative proteomics analysis comparing proteins detected in
783 the different conditions. Statistical significance ($-\log_{10}$ *p*-value) is plotted against ratio (average
784 $\log_2$ ratio). Proteins significantly decreasing in UbVS/ubiquitin are indicated by blue dots in all
785 the conditions tested. **(c)** Log_2 ratio heat map of the proteins significantly downregulated in
786 UbVS/ubiquitin after addition of the indicated DUB. Bold: Proteins decreasing in both
787 experiments in Fig. 1f. **(d)** Extract was incubated with 10 μM UbVS (30 minutes) followed by
788 addition of USP7 (800 nM), ubiquitin and ^{35}S-methionine labeled substrates (time 0). USP7 was
789 added to extract after pre-incubation (30 minutes) with buffer or UbVS (USP7-UbVS). Right:
790 Quantification of the experiment. Error bars: SEM of three independent experiments. One
791 representative experiment is shown. **(e)** ^{35}S-methionine labeled substrates were added to
792 interphase extract or to mitotically arrested extract pre-incubated with UbVS (30 minutes).
793 Concomitantly, USP7 and ubiquitin were added (time 0). Aliquots were collected (20 minutes),
794 quenched as usual and processed for SDS-PAGE and phosphorimaging. Right: Quantification of
795 the substrate present at 20 minutes in the experiment on the left. Error bars: SEM of three
796 independent experiments. One representative experiment is shown.

797