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1 **UFMylation: a ubiquitin-like modification**

2

3 Xingchen Zhou^{1,2}, Sayyed J Mahdizadeh³, Matthieu Le Gallo^{1,2}, Leif A Eriksson³, Eric

4 Chevet^{1,2*}, Elodie Lafont^{1,2*}

5

6 ¹Inserm U1242, University of Rennes, Rennes, France.

7 ²Centre de Lutte Contre le Cancer Eugène Marquis, Rennes, France.

8 ³Department of Chemistry and Molecular Biology, University of Gothenburg, Sweden

9

10 *Corresponding authors: eric.chevet@inserm.fr or elodie.lafont@inserm.fr

11

12 ORCIDs: Xingchen Zhou 0000-0003-2291-8181, Sayyed J Mahdizadeh 0000-0002-4844-

13 6234, Matthieu Le Gallo 0000-0001-9085-8155, Leif A Eriksson 0000-0001-5654-3109, Eric

14 Chevet 0000-0001-5855-4522, Elodie Lafont 0000-0003-1978-7491

15

16 X handles: Inserm U1242 laboratory: @CSignaling; Eric Chevet: @Eric_Chevet; Elodie

17 Lafont: @lafont_elodie

18

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21 **Abstract**

22 Post-translational modifications (PTM) add a major degree of complexity to the proteome
23 and are essential controllers of protein homeostasis. Amongst the hundreds of PTMs
24 identified, ubiquitin and ubiquitin-like (UBL) modifications are recognised as key regulators
25 of cellular processes through their ability to affect protein-protein interactions, protein
26 stability, and thus the functions of their protein targets. Here, we focus on the most recently
27 identified UBL, Ubiquitin-Fold Modifier 1 (UFM1), and the machinery responsible for its
28 transfer to substrates (UFMylation) or its removal (deUFMylation). We first highlight the
29 biochemical peculiarities of these processes, then we develop on how UFMylation and its
30 machinery control various intertwined cellular processes and we highlight some of the
31 outstanding research questions in this emerging field.

32 **UFM1: an emerging type I ubiquitin like (UBL) protein**

33 Ubiquitin and ubiquitin-like (UBL) modifications regulate a broad variety of cellular
34 processes. UBL proteins, classified as type I or II, are characterised by the conserved
35 presence of a “ubiquitin-fold” [1]. Further, type I UBLs, such as SUMO, ISG-15 and UFM1,
36 are covalently conjugated to substrates, while type II UBLs are domains within multi-
37 domain proteins whose functions are mostly related to the transfer or the removal of
38 ubiquitin or type I UBL. **UFM1**, (for **Ubiquitin-Fold Modifier 1**, see **Glossary**), is the most
39 recently identified type I UBL [1, 2]. **UFMylation**, the covalent attachment of UFM1 on target
40 proteins, displays a peculiar biochemistry and has recently emerged as a critical regulator
41 of multiple cellular processes. Herein, we describe the machinery involved in the addition
42 and removal of UFM1 from target proteins and the known cellular roles of UFMylation.

43

44 **Components of the UFMylation/deUFMylation machinery**

45 *The UFMylation/deUFMylation machinery: discovery and localisation*

46 Similar to ubiquitin, UFM1 is covalently transferred to the primary amine of a lysine residue
47 in target proteins via the coordinated action of three enzymes: a UFM1-activating E1, a
48 UFM1-conjugating E2, and a UFM1-ligase E3, and several components of this UFMylation
49 machinery were identified in 2004 [2]. Specifically, Komatsu *et al* discovered the UFM1-
50 activating E1 enzyme, **UBA5**, in a yeast two-hybrid screen while searching for interactants
51 of another UBL, GABARAPL2. UBA5 was not found to act as an E1 for GABARAPL2.
52 Hence, the authors searched for UBA5 interacting partners, ultimately identifying UFM1 and
53 also the UFM1-conjugating E2 enzyme 1, **UFC1**. They further demonstrated that UBA5 and
54 UFC1 respectively function *in vitro* as E1 and E2 enzymes for UFM1 [2] (**Figure 1A, B**).
55 Building on their previous work, the Komatsu laboratory identified **UFL1** as an E3 ligase for
56 UFM1, and **DDRKG1** (also named UFBP1) as a substrate of UFL1 [3]. They also highlighted
57 that DDRKG1 is UFMylated at K267, a modification which impacts UFMylation of some, but
58 not all, substrates of UFL1. In addition, DDRKG1 regulates UFL1 localisation, favouring its
59 anchoring to the Endoplasmic Reticulum (ER) [4] (**Box 2**), and is crucial for UFL1 activity
60 [5]. Kwon *et al.* and Wu *et al.* identified a second partner of UFL1, **CDK5RAP3**, by tandem
61 affinity purification followed by mass spectrometry (MS) sequencing [4] [6]. CDK5RAP3 acts
62 as an accessory protein in UFMylation, controlling the activity of the UFM1-UFC1-UFL1-
63 DDRKG1 complex towards specific substrates [5, 7]. In line with the involvement of
64 UFMylation in several specialized but key cellular processes, UFL1, DDRKG1 and
65 CDK5RAP3, which regulate each other's stability in multiple human cell types (e.g., [4, 6,

66 7]) appear to be ubiquitously expressed [4]. Their genetic deletion in mice results in
67 embryonic lethality [8-10] characterized by haematopoiesis defects similarly to the deletion
68 of UBA5 (60). Beyond their role as core components of the E3 ligase for UFMylation, UFL1
69 and DDRGK1 have also co-evolved and are expressed in some species which do not
70 express UFM1 and the rest of the UFMylation machinery, implying that they might possess
71 UFMylation-independent roles [11].

72 As with ubiquitin and several other UBL proteins [1], UFM1 is expressed as a pro-
73 form which is matured through cleavage at its C-terminus [2] (**Figure 1B**). This is mediated
74 by UFM1-Specific Proteases (UFSP), which are also responsible for **deUFMylation** of
75 substrates [2] (**Box 1**). Maturation of pro-UFM1 exposes the conserved Gly83 residue which
76 is essential for the covalent attachment of UFM1 via an isopeptide bond to lysines within
77 targets [2]. Whether additional amino acids (e.g., Ser, Thr, Cys), N-terminal of proteins or
78 non-proteinaceous substrates are also UFMylated, as described for ubiquitin, still remains
79 unknown. The number of UFMylation substrates validated in a cellular context is currently
80 limited and includes the UFL1 partner DDRGK1 [3], the ribosomal proteins RPL26 [12-15],
81 RPS3 [13, 14, 16], RPS20 [13, 14], RPL10 [13, 14] along with the translation initiation factors
82 eIF6 [13], eIF4G1, eIF4H, eIF4E, eIF4A1, eIF4B, eIF3A and eIF2alpha [16], as well as the
83 proteasomal protein PSMB5 [17], the oligosaccharyltransferase subunit RPN1 [18], the
84 transcription coactivator ASC1 [19], histone H4 [20], the nuclease MRE11 [21, 22], the
85 NADH-cytochrome B5 reductase 3 CYB5R3 [17, 23], and the cellular trafficking-related
86 proteins RAB1A/B, RAB5C, ARF4, and clathrin [18], suggesting that UFMylation plays roles
87 in various cellular processes, and most prominently **ER proteostasis**. UFM1 presents less
88 than 21% sequence identity with ubiquitin in human but displays a UBL fold comprising 4 β -
89 strands, one central α -helix and a small α -helix [2], and, along with the UFMylation
90 machinery, is evolutionary conserved in most eukaryotes [11, 24] (**Box 2**). UFM1 possess
91 6 lysine residues and can itself be the substrate of UFMylation, forming poly-UFM1 chains.
92 In cells, only poly-UFMylation linked through Lys69 of UFM1 has been reported [19],
93 however a small proportion of K7- and K9-linked chains is also observed in cell-free
94 reconstitution assays [5].

95

96 *Peculiarities of UFMylation biochemistry*

97 As described above, UFMylation requires a three-step enzymatic reaction [25]. Contrary to
98 the ubiquitination process, which can be catalysed by hundreds of enzymes in human cells
99 [26], UFMylation solely relies on one E1, one E2 and one E3 enzyme. UFMylation is

100 reversible, through deUFMylation (**Box 2 and Figure I**). Thereafter, we focus on the most
101 notable aspects of these steps (**Figure 1A-C**).

102

103 Activation of UFM1

104 E1 enzymes, classified as canonical or non-canonical, catalyse the activation of the Ub/UBL
105 terminal carboxylate via consecutive adenylation and thio-esterification reactions. Canonical
106 E1s display two **adenylation domains** (AD): an inactive and an active AD (IAD, AAD,
107 respectively) and a separate, flexible relative to the AD, catalytic Cys domain [27, 28]. Unlike
108 canonical E1s, the catalytic Cys of non-canonical E1s is close to, or embedded within their
109 AD [27, 28].

110 UBA5 displays an AD (aa 57-329) comprising the ATP-binding pocket and the
111 catalytic Cys250 [2], and is therefore considered as a non-canonical E1 [29, 30]. However,
112 with only 404 aa, UBA5 is a particularly small E1, and the way it activates UFM1 is a field of
113 active research. The activation of UFM1 by UBA5 requires two steps, with the use of one
114 ATP, generating first the adenylated UFM1 intermediate, which is non-covalently associated
115 to UBA5, and second the UBA5-S~UFM1 thioester formed through nucleophilic attack of
116 the adenylated UFM1 intermediate by UBA5 Cys250 [31]. Consistent with the requirement
117 of UFM1 C-terminal tail (aa 79-83) for its charging to UBA5 [32], the pathogenic mutation
118 Arg81Cys of UFM1 limits UFMylation [33] (**Table 1, Figure 1D-E**).

119 C-terminal to the AD, the **UFM1-interacting sequence (UIS)** (aa 334-346) of
120 UBA5 is critical for UFM1 activation [34, 35]. Indeed, as for other non-canonical E1s [28],
121 UBA5 forms homodimers via its AD [29], and this dimerization is key to the UFM1-activation
122 step (**Figure 1C, (1)**). Specifically, one UBA5 non-covalently interacts with one UFM1 via
123 the UIS, thereby placing UFM1 towards the AD, and therefore the catalytic Cys, of the
124 opposing UBA5 of the dimer [32]. This trans-binding mechanism of UFM1 to UBA5, unique
125 to UBA5 amongst E1 enzymes [32, 36], might have been favoured as it also contributes to
126 stabilising the UBA5 dimer and to enhancing ATP binding [36-38]. In addition to the existing
127 UBA5 inhibitors which target the ATP-binding pocket [39] or Cys 250 [40], this peculiar trans-
128 binding mechanism might be pharmacologically targeted to regulate UBA5 activity. Of note,
129 the C-terminal part of UBA5 is also able to interact with GABARAP proteins, and even
130 though UBA5 does not activate these UBLs, such a binding contributes to UBA5 recruitment
131 to the ER [41-43].

132 The N-terminal part of UBA5, and in particular aa 37-56, is not essential for
133 UFMylation but augments UBA5 ATP-binding affinity and thus UFM1 activation velocity [38].

134 Accordingly, pathogenic mutations are described in UBA5 N-terminus (**Table 1**), *e.g.*,
135 Arg55His, which reduce its activity [44]. Similar contributions to ATP-binding of N-terminal
136 residues are also described in other E1s, such as Arg15 of APPB, part of the APPB1/UBA3
137 E1 complex for the UBL NEDD8 [45]. Since UBA5 can be found in two splicing isoforms in
138 humans, differing by the 56 aa at the N-terminus [46], future studies may decipher whether
139 alternative splicing of UBA5 is triggered in particular biological contexts to control
140 UFMylation.

141

142 Conjugation of UFM1

143 Following activation by the E1, the Ub/UBL is transferred from the catalytic Cys of the E1
144 activating enzyme to the catalytic Cys of the E2 conjugating enzyme through a trans-thio-
145 esterification reaction [1]. Recruitment of the corresponding E2 to a canonical E1 is mainly
146 mediated by the ubiquitin fold domain (UFD) at the C-terminal end of the AAD [1]. However,
147 as a non-canonical E1, UBA5 does not possess such a UFD and instead interacts with UFC1
148 via a C-terminal UFC1-binding site (UBS) [29, 30, 41, 47]. UBA5 transfers UFM1 to UFC1
149 through a trans mode of transfer requiring UBA5 homodimerization [32]. Indeed, the Cys116
150 [48] of one UFC1 bound to one UBA5 subunit, accepts the UFM1 from the other UBA5
151 subunit [32] (**Figure 1C, (2)**). This mechanism is similar to the transfer of the UBL ATG8
152 from the homodimeric E1 enzyme ATG7 to the E2 enzyme ATG3 [49]. The linker region
153 between the UIS and the UBS of UBA5 is also important for UFM1 conjugation [47]. In
154 particular, the close proximity of the linker of UBA5 to Cys116 of UFC1 is required to
155 enhance the nucleophilic activity of the catalytic exposed Cys116, a peculiar
156 complementation feature unique to this E2/E3 pair [47]. Correspondingly, the pathogenic
157 compound mutation (*i.e.*, combined with a second UBA5 mutation) of UBA5 Ala371Thr, in
158 the linker region, impairs the transfer of UFM1 from UBA5 to UFC1 [44] (**Table 1**). In
159 addition, the N-terminal part of UBA5 present in the long isoform also promotes UFM1
160 transfer from UBA5 to UFC1 in the presence of ATP [38].

161 On the UFC1 side, a hydrophobic pocket, comprising residues from α -helix 1 and
162 β -strand 1 and structurally opposite of the Cys116 interacts with the UBS [47]. This brings
163 both UBA5 and UFC1 active cysteines in proximity, supporting therefore the trans-thio-
164 esterification reaction and the transfer of UFM1 [47]. As this same binding pocket of UFC1
165 is suggested to mediate binding to the N-terminal UBS of UFL1 [7, 50], UFL1 likely
166 outcompetes discharged UBA5 to bind to UFC1, a phenomenon also described for other E2
167 enzymes [51]. Canonical E2s display a His-Pro-Asn motif [52] in which the Asn is required

168 for the transfer of the Ub/UBL1 to the Lys of the substrate [53] and His stabilizes the HPN
169 motif; yet this motif is lacking in UFC1, another peculiarity of this E2. Instead, UFC1 presents
170 a Thr-Ala-Lys motif (aa 106-108) which is required for aminolysis [5] as exemplified by the
171 destabilizing effect on the TAK motif of the pathogenic mutation Thr106Ile and impaired
172 UFM1 conjugation activity [33] (**Figure 1F, Table 1**).

173
174 Ligation of UFM1 to its targets
175 After Ub/UBL conjugation, the role of the E3 ligase enzyme is to cooperate with the E2
176 enzyme to transfer the Ub/UBL from the E2 enzyme to a lysine residue on the substrate [54]
177 (**Figure 1B**).

178 It was long suggested that UFL1, which does not contain a catalytic Cys [5], is a
179 **scaffold E3 ligase** [10, 19]. As demonstrated recently [5, 50], UFL1 forms an active complex
180 with DDRGK1 and UFC1 [7, 50] (**Figure 1C, (3)**), interacting with these two proteins via its
181 N-terminal part [3, 7, 19, 50]. This complex UFMylates ASC1 and RPL26 *in vitro* and
182 catalyses mono- and K69-linked poly-UFMylation [5]. Importantly, the UFL1/DDRGK1
183 complex is required for UFC1 to discharge UFM1 on Lys residues through aminolysis *in*
184 *vitro*, and also promotes UFC1 ability to form free K69-linked poly-UFM1 chains [5].

185 Even though the structure of the full UFL1/UFC1/DDRGK1 complex has not been
186 resolved experimentally, recent predictions using Alphafold highlight that the UFL1 N-
187 terminal region may comprise five winged helix (WH) domains, a sixth partial WH (pWH)
188 domain preceded by an N-terminal helix whilst DDRGK1 completes UFL1 pWH by a
189 complementary C-terminal pWH and a full WH (WH1) [5, 7]. This structural complementarity
190 explains both the ability of DDRGK1 to stabilize UFL1 and to promote its E3 ligase activity
191 [5, 7]. In agreement with these discoveries, UFMylation of several other substrates relies on
192 DDRGK1 too (*e.g.*, RAB1A/B, RAB5C, ARF4, clathrin [16, 18]). The minimal domains
193 required to promote UFM1 discharge from UFC1 *in vitro* were mapped to region 207-314 of
194 DDRGK1 (*i.e.*, its pWH and WH1), and 1-116 of UFL1 (*i.e.*, its pWH and WH3) [5]. However,
195 depending on the substrate, additional parts of UFL1/DDRGK1 are required for proper
196 substrate modification as demonstrated for RPL26, MRE11 and H4 [5]. As discussed in [5],
197 WH domains are also known to bind nucleic acid [55], and might also thereby contribute to
198 the UFMylation of ribosomal substrates or of substrates primarily localized in the nucleus
199 like H4, ASC, or MRE11.

200 Importantly, the addition of CDK5RAP3 to the DDRGK1/UFL1 complex modifies
201 its substrate specificity [5, 7]. *In vitro*, CDK5RAP3 limits UFL1/DDRGK1 ability to promote

202 the discharge of UFM1 from UFC1 [5]. Furthermore, CDK5RAP3 allows mono-UFMylation
203 of RPL26 whilst it represses H4, MRE11 and ASC1 UFMylation [5]. At the ER, binding of
204 CDK5RAP3 to UFL1, DDRGK1 and UFC1 is able to divert UFL1 from UFMylation of DDRGK1
205 at K267, and on the contrary promotes RPL26 UFMylation [7]. Even though a
206 UFL1/DDRGK1 purified from bacteria (and thus devoid of such PTM) is able to promote
207 UFC1 discharge [5], mutation of K267 residue in cells limits ER-phagy [23]. The amount of
208 CDK5RAP3-associated to UFL1/UFC1/UFM1/DDRGK1 complexes might therefore
209 contribute to coordinate ER-bound ribosome quality control and ER-phagy. Overall,
210 CDK5RAP3 might therefore restrict/determine substrate specificity of UFMylation in cells [5,
211 7, 10, 56], and the exact mechanisms involved for its impact on substrates UFMylation will
212 need further exploration.

213
214 **Cellular roles of the UFMylation machinery**
215 UFMylation is involved in various inter-connected cellular processes (**Figure 2A**).
216 Noteworthy, many of these pathways are linked to ER protein homeostasis (a.k.a. ER
217 proteostasis) [57].

218
219 *UFMylation in the maintenance of genome integrity and gene expression*
220 UFMylation plays a role in genome integrity maintenance (**Box 3 and Figure II**) and controls
221 transcription through various mechanisms. UFMylation has also been shown to control the
222 activity of transcription factors. For instance, ER α (Estrogen receptor- α) when activated (by
223 17 β -estradiol), translocates to the nucleus where it recruits coactivators such as SRC1, p300
224 and ASC1 to activate target gene transcription (e.g., cyclin D and c-Myc). ASC1 UFMylation
225 [19] enhances the recruitment of p300, SRC1 and ASC1 to the promoters of ER α -target
226 genes [19]. However, given that the ER-anchored DDRGK1 is required for ASC1 UFMylation
227 [19], the mechanism by which the complex is formed (e.g., cytoplasmic of ASC1 or formation
228 of ER invaginations in the nucleoplasm) need to be documented. Contrary to its role in
229 promoting ER α -target gene expression, UFMylation rather represses NF- κ B-dependent
230 gene expression [4, 6, 58]. Knockdown of CDK5RAP3 in U2OS or HeLa cells promotes
231 basal and TNF α or IL-1 α -induced NF- κ B-dependent expression of IL-8, MMP-9, MCP-1,
232 Cox-2 and I κ B α [58]. Conversely, CDK5RAP3 overexpression represses TNF α -induced NF-
233 κ B activity and sensitises cells to TNF α -induced loss of viability [59]. Mechanistically,
234 CDK5RAP3 interacts with the p65 NF- κ B subunit and limits its phosphorylation at S536 [58].

235 In addition, other studies did corroborate a role for UFL1 as a regulator of NF- κ B-regulated
236 gene expression [4, 6].

237

238 *Roles of UFMylation in ER proteostasis*

239 Conditional ablation of the components of the UFMylation machinery in various mouse
240 tissues, including hematopoietic cells, pancreatic beta-cells, hepatocytes, intestinal cells, is
241 accompanied by an increased expression of ER stress markers and of cell death, e.g., [8-
242 10]. As such UFMylation controls ER proteostasis by several means.

243

244 Control of protein synthesis, ER protein translocation, quality control (QC) and ER-phagy

245 Translation in eukaryotes includes multiple factors, promoting mRNA recruitment and
246 scanning, initiator tRNA selection and the association with ribosomal subunits. UFMylation
247 of ribosomal proteins was reported by several teams [12-16]. For instance, UFL1 was
248 identified in the ribo-interactome using MS-based proteomics [13] and UFMylation occurs
249 on the ribosomal proteins RPS3, RPS20, RPL10 and the translation initiation factor eIF6
250 [12-16]. The UFMylation and deUFMylation machineries were also identified in a
251 CRISPR/Cas9 screen as factors promoting SYVN1-dependent ER-associated protein
252 degradation (ERAD), yet their impact on this process turned out to be quite modest and cell-
253 type specific [12]. However, UFMylation can control ER proteostasis independently of ERAD
254 as well [12]. Indeed, UFMylation contributes to the elimination of translocon-engaged
255 arrested nascent protein chains [12, 15, 60, 61] (**Figure 2B**). Specifically, RPL26 is
256 UFMylated on K132 and K134, two residues nearby the interface between the nascent
257 peptide's exit tunnel and the SEC61 translocon [12, 15]. This modification, which is induced
258 upon ribosome stalling at the ER promotes the targeting of translocon-engaged arrested
259 peptides to the lysosomes for degradation through a SAYSD1-dependent sensing
260 mechanism [15, 61]. Recently, Scavone *et al.* reported that UFMylation promotes the
261 handling of the translocon-engaged arrested proteins by the canonical ribosome-associated
262 QC [60]. They described that the nascent stalled proteins could be CATylated (C-terminal
263 addition of alanine and threonine) by NEMF, ubiquitinated by LTN1 before being extracted
264 from the translocon by the AAA+ ATPase p97/VCP and degraded by the proteasome.
265 UFMylation, potentially by weakening the interaction of the stalled ribosome with the SEC61
266 translocon could promote LTN1 access to the arrested peptide [60] (**Figure 2B**).

267 UFMylation is also involved in ER-phagy. First, a CRISPRi screen identified the
268 UFMylation machinery as regulating starvation-induced ER-phagy [18]. In this context,

269 interaction of UFL1 with DDRGK1 and the anchoring of DDRGK1 to the ER were both
270 required for their ER-phagy-promoting role. Interestingly, UFMylated proteins included
271 Ribophorin1 (RPN1), a subunit of the oligosaccharyl transferase (OST) complex in proximity
272 to the translocon, and the ribosomal protein RPL26 and several studies have further
273 implicated UFMylation in linking ER-phagy to resolving ribosome stalling [11, 56]. In this
274 context, CDK5RAP3 binds to ATG8 (GABARAP isoform), allowing its recruitment to
275 autophagosomes to mediate ER-phagy, and also likely redirects the activity of the UFL1 E3
276 ligase complex towards RPL26 as mentioned earlier [11, 56] (**Figure 2B**).

277

278 Roles in the Unfolded Protein Response

279 The Unfolded Protein Response is an adaptive pathway triggered to restore ER
280 proteostasis. It is transduced by three transmembrane ER-resident sensors, namely IRE1 α
281 (hereafter referred to as IRE1), PERK and ATF6 α [62]. Blunting UFMylation is accompanied
282 by UPR activation in multiple cell types [8, 9, 63-65]. All the UFMylation machinery
283 components are also targets of the IRE1/XBP1s arm of the UPR [57]. In addition, one study
284 suggested that DDRGK1 promotes IRE1-XBP1s signalling by stabilizing IRE1 [66].
285 However, this is at odds with multiple other studies [8, 18, 63, 64] and the underlying
286 mechanism remains unclear. Functional links between UFMylation, the UPR and ER-phagy
287 were also described and could be activated in response to ER stress [4, 63]. In fact,
288 depletion of UFL1, UFM1 or DDRGK1 leads to increased IRE1 and PERK expression as
289 well as increased expression of CLIMP63, a marker of ER sheets [18]. At last, IRE1
290 depletion partially rescues the ER-phagy defect associated with DDRGK1 depletion [18].
291 However, the role of UFMylation versus individual components of its machinery in autophagy
292 remains unclear [8, 9, 67].

293

294 *Roles of UFMylation in other cellular processes*

295 Cell cycle progression

296 Cell cycle progression is controlled by checkpoints ensuring the coordination of key events
297 (e.g., spindle assembly) before the next stage of the cell cycle. These checkpoints involve
298 cyclins and cyclin-dependent kinases (CDKs) and are modulated by UFMylation. Indeed,
299 CDK5RAP3 regulates G2/M by controlling CDK1 phosphorylation [68, 69]. In addition,
300 UFMylation-depleted RPE-1 cells present a delay in G1 as they fail to pass the restriction
301 point [16]. Mechanistically, UFMylation promotes both transcription and translation of cyclin
302 D1 which together with CDK4/6 is responsible for Rb phosphorylation preceding the one

303 mediated by CDK2/cyclin E/A that drives restriction point. UFMylation might also either
304 promote [70] or impair [9] the stability of p53, which might impact on cell cycle control [71]
305 (**Figure 2C**).

306

307 Cell death and differentiation

308 UFMylation can also control cell death and differentiation during normal development and in
309 cancer. For example, UFSP2, UFL1, UBA5 and UFC1 were identified amongst the top hits
310 in a genome-wide CRISPR screen aiming at identifying regulators of glioblastoma stem cells
311 (GSC) maintenance [72]. Individual KO of these genes, or inhibition of UBA5 with the
312 pharmacological drug DKM 2-93 which covalently binds UBA5 Cys250 [40], lead to reduced
313 GSC fitness [72]. Interestingly, the same screen identified the ERAD factors SEL1L and
314 HRD1 as crucial for GSC fitness [72], and both PERK and IRE1 have been suggested to
315 control cell differentiation in glioblastoma [73, 74]. Thus, it is tempting to speculate that part
316 of the effect of disrupting UFMylation on GSC fitness is linked to its roles in ER proteostasis.
317 UBA5 KO mice die *in utero* between E12.5 and E13.5 days, from anaemia due to a mild
318 defect of primitive and a pronounced defect of definitive erythropoiesis [75]. Moreover, a
319 defect in the differentiation of common myeloid progenitors to megakaryocyte/erythroid
320 progenitors and massive cell death of hepatic erythroid progenitors is observed in UBA5 KO
321 embryos. In line with their essential roles in UFMylation, UFL1 and DDRGK1 knockout mice
322 also display embryonic lethality and defective erythropoiesis [8, 9]. Yet, the bases for the
323 roles of UFMylation in erythropoiesis and cell differentiation in general are however still
324 elusive. Of note, disruption of ribosome homeostasis particularly impacts erythropoiesis [76,
325 77] and erythroid differentiation increases RPL26 UFMylation [15]. It is thus tempting to
326 speculate that part of the phenotypes described above could be linked to the role of
327 UFMylation in resolving ribosome stalling.

328

329 Cellular response to viral infection and immune receptor signalling

330 Several virus types can also hijack and/or be controlled by the host UFMylation machinery.
331 Indeed, UFL1 promotes hepatitis A virus (HAV) HM175/18f replication by enhancing viral
332 RNA translation in a RPL26-dependent manner [65], and the Epstein Barr Virus protein BILF
333 promotes MAVS UFMylation and lysosomal degradation to limit inflammasome activation
334 and cell death [78]. In contrast, UFL1 can promote the anti-viral cellular response, with
335 several potential mechanisms proposed, such as the stabilization of the proton channel
336 STING in macrophages by UFL1 [79], or RIG-I/MAVS signalling [80, 81], which both result

337 in enhanced type I interferon production. Beyond the response to viral infection, control of
338 immune receptor signalling by UFMylation could also affect cancer cell cross-talk with their
339 micro-environment. In line with this, a recent study suggests that UFL1 limits the stability of
340 PD-L1 in various cancer cell lines [82]. Whether and how UFMylation might functionally link
341 immune receptor signalling and control of proteostasis will also most likely be an exciting
342 research direction.

343

344 **Concluding remarks**

345 Mutations or modulation of the expression of UFMylation components influence disease
346 development in animal models and in a variety of human diseases, most prominently Central
347 Nervous System-related diseases [33, 44, 83-86]. The biochemical mechanisms underlying
348 UFMylation and deUFMylation are currently being uncovered, just as the cellular processes
349 they control. Yet, many questions remain (see **outstanding questions**), pertaining to the
350 fundamental peculiarities of UFMylation and deUFMylation but also to the impact of these
351 processes on the interconnections of the cellular functions they control. In addition, several
352 promising efforts are ongoing to identify modulators of the UFMylation and deUFMylation
353 processes, for example [39, 40, 82]. Understanding how UFMylation impacts the cross-talk
354 between different cell types/compartments will be key for deciphering the physiological
355 importance of this PTM and for evaluating the potential of its pharmacological targeting.

356 **Authors contributions**

357 Conceptualization: EL and EC. Writing of original draft: EL and XZ. Revising the draft: EL,
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373

374 **Conflict of interest**

375 EC is a founder of Thabor therapeutics (www.thabor-tx.com). LAE and SJM are co-founders
376 of ANYO Labs (www.anyolabs.com). LAE is co-founder of Cell Stress Discoveries Ltd
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378

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652
653

654 **BOX 1 - Principles and peculiarities of deUFMylation**

655 UFM1 can be removed from substrates through the action of UFM1-Specific Proteases
656 (UFSPs) through their isopeptidase activity [87, 88]. UFSP1 and UFSP2 were first identified
657 in mouse, encoded by two different genes [89]. Their activity, specific to UFM1, can be
658 inhibited by NEM (N-ethylmaleimide) or mutation of their catalytic Cys residues [90, 91].
659 Whether human UFSP1 is actually catalytically active has long been debated since, contrary
660 to human UFSP2 and to UFSP1 in other species, it displays a partial protease domain.
661 However, in certain human cell types, KO of UFSP2 leads to increased global UFMylation,
662 implying that at least another protease catalyses the maturation of pro-UFM1 [12, 65, 90].
663 This elusive enzyme was recently shown to be a long isoform of UFSP1 [90, 91], whose
664 translation is initiated at an upstream ²¹⁷CUG codon instead of the annotated ⁴⁴⁵AUG codon
665 and displays 38% protein sequence identity with UFSP2 [90, 91]. *In vitro*, both UFSP1 and
666 UFSP2 can reverse substrate UFMylation (*e.g.*, DDRGK1, ASC1) [3, 91] and catalyse the
667 maturation of pro-UFM1 to UFM1 [90, 91]. UFSP1 and UFSP2 double KO cells are devoid
668 of UFMylated proteins and lack maturation of pro-UFM to UFM1, strongly arguing for the
669 existence of only two UFSPs [90, 91], at least in the cell types tested so far. Some
670 differences can however be noted between these two UFSP. For instance, UFSP2 KO leads
671 to a drastic accumulation of UFMylated proteins, which is much more modest in UFSP1 KO
672 cells. UFSP2, but not UFSP1, efficiently removes UFMylation from RPL26, whereas UFSP1
673 is more efficient at maturing pro-UFM1 [90, 91]. Of note, UFSP1 is required to remove UFM1
674 from Lys122 of UFC1, a residue structurally close to UFC1 catalytic Cys116, and whose
675 UFMylation might inhibit UFC1 activity [90]. Whether UFSP1 serves to alleviate this potential
676 auto-inhibition is still unproven. Both UFSP present a conserved catalytic site comprising a
677 Cys-Asp-His triad along with a Tyr residue [25, 87] common to other cysteine-based Ub/UBL
678 proteases. However, these enzymes differ by several regulatory loops which, in addition to
679 their distinct sub-cellular localisation and expression levels (see **Box 2**), may influence their
680 substrate repertoire (see [25]). Of note, several pathogenic mutations residing in the catalytic
681 triad of UFSP2 have been reported, such as Y290H, H428R, D426A, and the V115E
682 mutation which is part of the ODR4-binding site (86) see **Figure I** and **Table 1** for structural
683 and cellular consequences.

684

685 **BOX 2 – Evolution, expression and localisation of the UFMylation/de-UFMylation**
686 **components**

687 UFM1 and the machinery of UFMylation/de-UFMylation is found in multiple eukaryotes,
688 including *Homo sapiens* and several model organisms like *Caenorhabditis elegans*, *Mus*
689 *musculus*, and *Arabidopsis thaliana*. Yet, its expression is not restricted to multicellular
690 organisms [24], as it is for example expressed by *Chlamydomonas reinhardtii* [11]. UFM1,
691 CDK5RAP3 and UFC1 seem to have largely co-evolved and are restricted to eukaryotes,
692 whilst the rest of the UFMylation machinery is thought to have ancestral mosaic origins from
693 both bacteria (for UBA5) and Asgard archaee (for UFL1, DDRGK1) [11]. Since the
694 UFMylation machinery has been completely or partially lost in fungi, in some algae and
695 alveolate parasites [11, 24], these organisms must display alternative pathways for ER
696 stress tolerance. Of note, the UFMylation machinery is encoded by the genome of some
697 parasites such as *Leishmania donovani* and contribute to this parasites' proliferation in
698 human macrophages as well as their virulence in mice [92].

699 In line with their roles in key cellular processes, all components of the UFMylation
700 and deUFMylation machinery seem to be ubiquitously expressed in murine and human
701 tissues, albeit with variable abundance [2-4, 46, 90, 93]. UFSP1 protein expression is
702 generally much lower than that of the other members of the whole UFMylation/de-
703 UFMylation machinery, and UFM1 is on the contrary the most abundant [90]. The
704 stoichiometry between the different components seems key in ensuring UFMylation. For
705 instance, it was shown that overexpression of UBA5 induced a back-transfer of UFM1 from
706 UFC1, which was reversed upon overexpression of UFM1 [94]. UFM1 is localised in the
707 nucleus and diffusely in the cytoplasm [2]. UBA5 and UFL1 mainly localise to the cytoplasm
708 [2, 46, 93], whereas UFC1 is predominantly nuclear with a small cytoplasmic fraction [2].
709 Sub-cellular localisation of CDK5RAP3 includes the nucleus, the nucleolus, the centrosome
710 and the cytoplasm [68, 95, 96] whilst DDRGK1 is anchored to the ER membrane [4]. Most
711 of the UFMylation substrates are ER-localized, but some are found in the nucleus, and it is
712 puzzling how the whole UFMylation machinery access them. UFSP1 is mainly cytosolic
713 whilst UFSP2 localizes to the ER membrane, due to its interaction with the ER-anchored
714 ODR4 [90, 97]. Whether or not ODR4 also impacts on UFSP2 activity in addition to
715 determining its localization and promoting its stability [90] is unknown.

716

717 **BOX 3 - UFMylation and the maintenance of genome integrity**

718 Genomic instability can result from altered DNA damage repair and contribute to cancer
719 development [98]. Among DNA damage, double-strand breaks (DSB) are the most
720 deleterious. When DSB happen the MRE11-RAD50-NBS1 (MRN) complex can be recruited
721 to the damage and triggers activation of the ATM kinase which orchestrates cell cycle
722 progression and DNA repair through homologous recombination [99-101] Many PTMs,
723 including ubiquitylation and UFMylation, regulate the DDR [20, 21, 102, 103]. Indeed, UFL1,
724 through a mechanism which remain elusive, is rapidly recruited to DNA damage sites upon
725 UV or X-ray treatment in U2OS cells and increases activation of ATM [101]. In
726 overexpression systems, MRE11 was shown to be UFMylated at K282 [21] which has also
727 been observed in several other studies (*e.g.*, [17, 22]). K282R MRE11 mutation limits MRN
728 complex formation and may impact genome stability [21]. Activation of ATM also depends
729 on the recruitment of the acetyltransferase Tip60 to Suv39h1-trimethylated histone H3Lys9
730 [104]. In turn, Tip60 acetylates ATM leading to its activation [105]. Irradiation-induced UFL1
731 nuclear localisation may prompt UFL1-mediated mono-UFMylation of histone H4 on K31 as
732 well as Suv39h1-dependent ATM activation and homologous recombination [20, 103].
733 Consistently, UFL1-depleted cells display an increased sensitivity to irradiation [20].
734 However, since both MRE11 K282 [106] and histone H4 K31 [107] are ubiquitinated, one
735 may wonder whether all the DDR-repressing effects associated with mutating these sites
736 are solely due to defective UFMylation. In response to genotoxic stress, contradicting effects
737 towards DSB repair and cell death were reported upon knockdown of UFMylation
738 components, highlighting unexplained cell type differences in the signaling triggered by
739 different DNA damage-inducers (*e.g.*, [20, 68, 71]) (**Figure II**). UFMylation could also
740 contribute to genome stability by promoting telomere maintenance. Indeed, UFM1- or UFL1-
741 deficient zebrafish present telomere instability and zebrafish expressing a MRE11 which
742 cannot be UFMylated phenocopies the loss of UFM1, with telomere shortening and
743 decreased erythrocytes number [22]. Multiple links are described between activation of the
744 UPR and the DDR. Thus, one may wonder whether the induction of expression of
745 UFMylation components by the UPR might provide another mean to safeguard genome
746 integrity [108].

747 **Table 1: Mutations of the UFMylation/deUFMylation machinery**

748

Protein	SNP and protein change	Biochemical/cellular impacts of the mutation	Pathology	Ref
UFM1	rs1033946108 ARG81CYS	Homozygous, hypomorphic: - <i>In vitro</i> : reduced thioester formation with UBA5; reduced transfer to UFC1 - In patients lymphoblasts: reduced UFM1-UBA5 and UFM1-UFC1 intermediates formation See also Figure 1D	Leukodystrophy, hypomyelinating,	[33]
	rs747359907 3-BP DEL - 273TCA (PROMOTER)	Homozygous Reduced reporter gene expression in SY-5Y neuroblastoma and U373 astrogloma cell lines		[85]
		Homozygous		[109]
UBA5	rs114925667 ALA371THR	Compound mutation with several variants, hypomorphic: - <i>In vitro</i> : no/very limited impact on thioester formation with UFM1; reduced transfer of UFM1 to UFC1 - In cells: reduced UFM1-conjugates formation	Developmental and epileptic encephalopathy	[44, 83, 110]
	rs774318611 ARG55HIS	Compound mutation with ALA371THR - <i>In vitro</i> : reduced thioester formation with UFM1; reduced transfer of UFM1 to UFC1; impaired binding to ATP of Arg55Ala mutation - In cells: dramatically reduced UFM1-conjugates formation; reduced mRNA and protein level See also Figure 1E		[38, 44]
	rs745968949 TYR285*	Compound mutation with ALA371THR, nonsense variant - In cells: reduced mRNA and protein level		[44]
	rs886039756 ARG61*	Compound mutation with ALA371THR		[44]
	rs374052333 ARG188*	Compound mutation with ALA371THR		[44]
	rs886039757 GLN302*	Compound mutation with ALA371THR - <i>In vitro</i> : no/very limited thioester formation with UFM1; no/very limited transfer of UFM1 to UFC1 - In patient fibroblasts: reduced UBA5-UFM1 levels		[83]
	rs886039758 LYS324ASNfs *14	Compound mutation with ALA371THR <i>In vitro</i> : dramatic reduction of thioester formation with UFM1; dramatic reduction of transfer of UFM1 to UFC1		[83]
	rs886039759 VAL260MET	Compound mutation with ASP389TYR, hypomorphic: - <i>In vitro</i> : reduced thioester formation with UFM1; reduced transfer of UFM1 to UFC1 - In cells, slight reduction of UFBP1 UFMylation		[83, 111]

	rs886039760 ASP389TYR	Compound mutation with VAL260MET - <i>In vitro</i> : nearly normal thioester formation with UFM1; nearly normal transfer of UFM1 to UFC1 - <i>In cells</i> : slightly reduced DDRGK1 UFMylation		[83, 111]
	rs532178791 MET57VAL	Compound mutation with GLY168GLU, hypomorphic: - <i>In vitro</i> : reduced thioester formation with UFM1; reduced transfer to UFC1 - <i>In cells</i> : slight reduction of DDRGK1 UFMylation See also Figure 1E		[83, 111]
	rs886039761 GLY168GLU	Compound mutation with MET57VAL - <i>In vitro</i> : no/very limited thioester formation with UFM1, no/very limited transfer to UFC1 - <i>In cells</i> : drastically impaired formation of UFM1 conjugates		[83, 111]
	rs540839115 ARG246*	Compound mutation with LYS310GLU <i>In cells</i> : reduced protein half-life, increased nuclear localisation, impaired UBA5-UFM1 interaction	Spinocerebellar ataxia, autosomal recessive (1 family)	[84]
	rs886039762 LYS310GLU	Compound mutation with ARG246* <i>In cells</i> : slightly reduced protein half-life; no/very limited impact on UBA5-UFM1 interaction		
UFC1	rs1553232770 THR106ILE	Homozygous, hypomorphic: - <i>In vitro</i> : No/very limited UFC1-UFM1 thioester formation - Patients lymphoblasts: reduced UFM1-UFC1 intermediate and UFM1 conjugates See also Figure 1F	Neurodevelopmental disorder with spasticity and poor growth	[33]
	rs1181612302 ARG23GLN	Homozygous, hypomorphic: - <i>In vitro</i> : No/very limited UFC1-UFM1 thioester formation - Patients lymphoblasts: reduced UFM1-UFC1 intermediate and UFM1 conjugates		
DDRGK 1	rs1325869434 IVS3DS,G-A, +1	Homozygous - Patients lymphoblasts: no protein expression	Spondyloepimetaphyseal dysplasia, Shohat type	[86]
UFSP2	rs796052130 TYR290HIS	Heterozygous <i>In vitro</i> : Impaired deUFMylation activity for the Tyr282His murine oxyanion Tyr mutation equivalent See also Figure II (BOX I)	Hip dysplasia, beukes type (1 family)	[88, 112]
	rs1554022725 ASP426ALA	Heterozygous <i>In vitro</i> : Reduced deUFMylation activity for the Asp418Ala murine catalytic Asp equivalent See also Figure II (BOX I)	Spondyloepimetaphyseal dysplasia, Di Rocco type	[88, 113]
	rs2095515802 HIS428ARG	Heterozygous <i>In vitro</i> : No deUFMylation activity for the His420Ala murine catalytic His mutation See also Figure II (BOX I)		[88, 114]

	rs142500730 VAL115GLU	Homozygous In patients' fibroblasts: reduced UFSP2 protein level, accumulation of UFMylated substrates (ASC1, DDRGK1, RPL26) See also Figure II (BOX I)	Developmental and epileptic encephalopathy	[115]
Note: coding single nucleotide polymorphisms (SNP) or short indels, annotated as pathogenic or likely pathogenic were downloaded from OMIM.				

749

750 **Glossary**

751 **Adenylation domain:** domain found in UBA5 which comprises in particular a catalytic
752 cysteine and an ATP-binding pocket allowing it to catalyze the activation of UFM1, which is
753 the first step of UFMylation (adenylation and thio-esterification of UFM1).

754 **CDK5RAP3:** CDK5 Regulatory Subunit Associated Protein 3, is a direct binding partner of
755 UFL1, which controls its E3 ligase activity.

756 **DDRKG1:** DDRGK Domain-Containing Protein 1, also called UFBP1 (UFM1-Binding and
757 PCI domain containing Protein 1), is a direct interacting partner of UFL1 which structurally
758 complements UFL1 and promotes its stability and E3 ligase activity.

759 **deUFMylation:** catalytic process by which UFM1 is cleaved from protein substrates,
760 performed by the UFM1-specific proteases (deUFMyases) UFSP1 and UFSP2 in human
761 cells.

762 **ER proteostasis:** Protein homeostasis ensured by various ER-associated quality-control
763 processes and by the unfolded protein response (UPR).

764 **ER stress:** condition resulting from disruption of ER proteostasis, usually characterized by
765 the accumulation of unfolded or misfolded proteins in the ER lumen and morphological
766 changes of the ER. ER stress leads to the activation of an adaptive cellular program called
767 the UPR.

768 **Scaffold E3 ligase:** E3 ligase enzyme that bring the E2 activating enzyme and the substrate
769 in close proximity for direct transfer of the Ub/UBL from the E2 enzyme to the substrate. In
770 opposition to Cys-dependent E3 ligase enzymes, where the Ub/UBL is transferred from the
771 Cys of the E3 enzyme to the substrate.

772 **UBA5:** Ubiquitin-fold modifier activating enzyme 5, also called UBE1DC1, is the E1-
773 activating enzyme which catalyses the first step of UFMylation (adenylation and thio-
774 esterification reactions).

775 **UFC1:** Ubiquitin fold-modifier conjugating enzyme 1, is the E2 conjugating enzyme which
776 catalyses the second step of the UFMylation process (trans-thiolation reaction).

777 **UFL1:** Ubiquitin fold-modifier specific ligase 1 is the E3 ligase enzyme catalysing the last
778 step of UFMylation, bringing in close proximity the UFM1-charged UFC1 to transfer UFM1
779 on the substrate (aminolysis reaction).

780 **UFMylation:** catalytic process, relying on the coordinated action of three enzymes (the E1
781 UFM1-activating UBA5, the E2 UFM1-conjugating UFC1 and the E3 UFM1-ligase UFL1),
782 by which the C-terminal glycine of UFM1 is covalently linked to the primary amine of lysine
783 residues on protein substrates, resulting in protein monoUFMylation or polyUFMylation.

784 **UFM1:** Ubiquitin-Fold Modifier 1 is a 85-amino acid protein (83 in its mature form),
785 structurally resembling ubiquitin, and therefore part of the ubiquitin-like (UBL) family of
786 proteins.

787 **UFM1-interacting sequence:** sequence in UBA5, outside its adenylation domain, and
788 which mediates its non-covalent binding to UFM1.

789 **UFSP (1 or 2):** UFM1 specific proteases, or deUFMyases, are cysteine proteases which
790 catalyse the maturation of pro-UFM1 (peptidase activity) as well as the removal of UFM1
791 from substrates (isopeptidase activity).

792

793 **Figure legends**

794

795 **Figure 1. Overview of the UFMylation/deUFMylation machinery and processes and**
796 **impact of selected pathogenic mutations. A.** Key domains and residues of UFM1, UBA5,
797 UFC1 and UFL1. **B.** Overview of the UFMylation and deUFMylation processes. UFMylation
798 includes three steps: **the first step** corresponds to the activation of UFM1 (**1**); **the second**
799 **step** corresponds to the conjugation of UFM1 (**2**) and **the final step** involves the ligation of
800 UFM1 to its targets (**3**). UFM1 conjugation to the substrate is reversible through the action
801 of the UFM1-specific cysteine proteases, UFSP1 and UFSP2. **C.** Schematic details of the
802 three steps of UFMylation. **D.** Structure of human UBA5 (dimer) in complex with two UFM1
803 (from PDB ID: 5IAA). R81C UFM1 mutant impairs four hydrogen bonds and one salt bridge
804 with the indicated residues of UBA5 (**Table 1**). **E.** Structure of human UBA5 (dimer) in
805 complex with two UFM1 (from PDB ID: 6H77). R55H mutant of UBA5 impairs 2 hydrogen
806 bonds and one salt bridge with ATP. Of note, the proximity of the M57 residue, which is
807 pathogenic when mutated to valine (**Table 1**). **F.** Structure of human UFC1 (from PDB ID:
808 2Z6P). T106I mutant impairs two hydrogen bonds, one with the side chain of E149 and one
809 with the backbone of A107 (**Table 1**). See also Figure I legend for molecular modelling
810 methods.

811

812 **Figure 2. Overview of the cellular functions controlled by UFMylation. A.** Cellular
813 functions controlled by UFMylation. **B.** Impact of UFMylation on ER ribosome-associated
814 quality control and ER-phagy. **C.** Impact of UFMylation on cell cycle control. Note that by
815 regulating p53 levels, UFL1 likely regulates multiple check-point of the cell cycle. Parts of
816 the figure were created using BioRender.com.

817

818 **Figure I. Structure of human UFSP2 and impact of the indicated pathogenic**
819 **mutations.** The structure was obtained from AlphaFold. The V115E mutation destabilizes
820 the local structure which is mainly composed of hydrophobic residues. The D426A mutation
821 impairs 2 hydrogen bonds and one salt bridge interaction with residue H428. The H428R
822 not only impairs its interactions with D426 but it also forms two extra hydrogen bonds with
823 the sidechain of Y290. The Y290H mutation results in the formation of two extra hydrogen
824 bonds with the residue D297. For this figure (and figure 1), the indicated structures from the
825 Protein Data Bank (PDB) were downloaded and prepared using the Schrödinger protein
826 preparation wizard v2023-1 [116]. This included the incorporation of hydrogen atoms and

827 the modelling of possible missing sidechains and loops using Prime [117, 118]. The
828 hydrogen bonding network was optimized by adjusting the protonation and tautomeric states
829 of all residues at pH = 7.4 using PROPKA [116]. Epik [118, 119] was used to assign the
830 correct protonation states of the hetero entities at the same pH. Finally, the prepared
831 structures were subjected to geometry refinement using the OPLS4 force field [120] in a
832 restrained structural minimization. A local optimisation scheme was used to minimize the
833 structural energy of the mutated residues and surrounding neighbours within 5Å distance.
834 The graphics and interactions were visualized using Maestro GUI of Schrödinger v2023-1.
835

836 **Figure II. Reported modes of action for UFMylation-mediated control of DDR.** Parts of
837 the figure were created using BioRender.com. At DSB sites, the MRE11-RAD50-NBS1
838 (MRN) complex is recruited to the site of damage and triggers the activation of ATM. UFL1
839 is proposed to be rapidly recruited to these DNA damage sites upon UV or X-ray treatment,
840 where it is thought to UFMylate MRE11 and H4 and promotes ATM activation. However,
841 how UFL1 accesses, and most importantly, can be active inside the nucleus when the
842 essential partner of its E3 ligase activity DDRGK1 is ER-localised, is still puzzling.

843

844

845 **Outstanding questions**

846

847 • Can poly-UFMylation assemble in cells through other lysines than Lys69? Is
848 there any biological difference in the role(s) of mono- and poly-UFMylation?

849

850 • The expression level and activity of the UFMylation/deUFMylation machinery
851 components is tightly controlled at various levels (transcriptional, post-
852 transcriptional, translational and post-translational). Which specific cellular
853 contexts trigger these different modes of regulation?

854

855 • “Writers” (UBA5, UFC1, UFL1, DDRGK1 and CDK5RAP3) and “erasers (UFSP1
856 and 2) have been identified for UFM1. SAYSD1 is a putative “reader” of this PTM.
857 Are there any other proteins involved in shaping and detecting the UFMylation
858 code?

859

860 • Are there any other factors cooperating with UFL1 for UFM1 ligation beyond
861 DDRGK1 and CDK5RAP3? Which substrates would these “cooperators” affect
862 and what are their cellular functions?

863

864 • What are the structural determinants of UFMylation substrate recognition?

865

866 • How does UFM1 E3 ligase complex gain access to non-ER-associated substrates
867 (e.g. nuclear proteins)?

868

869 • How does UFL1 control DDRGK1 and CDK5RAP3 stability?

870

871 • How does CDK5RAP3 control UFL1 substrate specificity?

872

873 • Which impaired cellular function of UFMylation/deUFMylation drives the diseases
874 associated with mutations/altered expression of this machinery?

875

876 • In which pathological contexts is pharmacological targeting of
877 UFMylation/deUFMylation meaningful?

878

879 • Several somatic mutations of UFMylation/deUFMylation machinery are found in
880 tumours. Do, and how do, they contribute to cancer development and progression?
881
882

883 **Highlights**

884

885 • UFMylation is mediated by a simple cascade comprising solely one E1 (UBA5), one
886 E2 (UFC1) and one E3 (UFL1), whilst deUFMylation solely involves two UFSPs
887 (UFSP1 and 2).

888

889 • UFMylation displays several biochemical peculiarities, such as a trans-binding
890 mechanism required for both UFM1 activation by UBA5 and its conjugation by UFC1.
891 In addition, UFM1 ligation relies on the structural complementarity of UFL1 and
892 DDRGK1.

893

894 • The UFMylation machinery is induced by various cellular stress conditions and
895 controlled through multiple levels of regulation, such as alternative splicing and post-
896 translational modifications.

897

898 • UFMylation controls interconnected cellular processes, such as ER-phagy, the
899 unfolded protein response and the response to DNA damage.

900

901 • Mutations in components of the UFMylation/deUFMylation machinery contribute to
902 the development of diseases, principally related to the central nervous system.

903

904

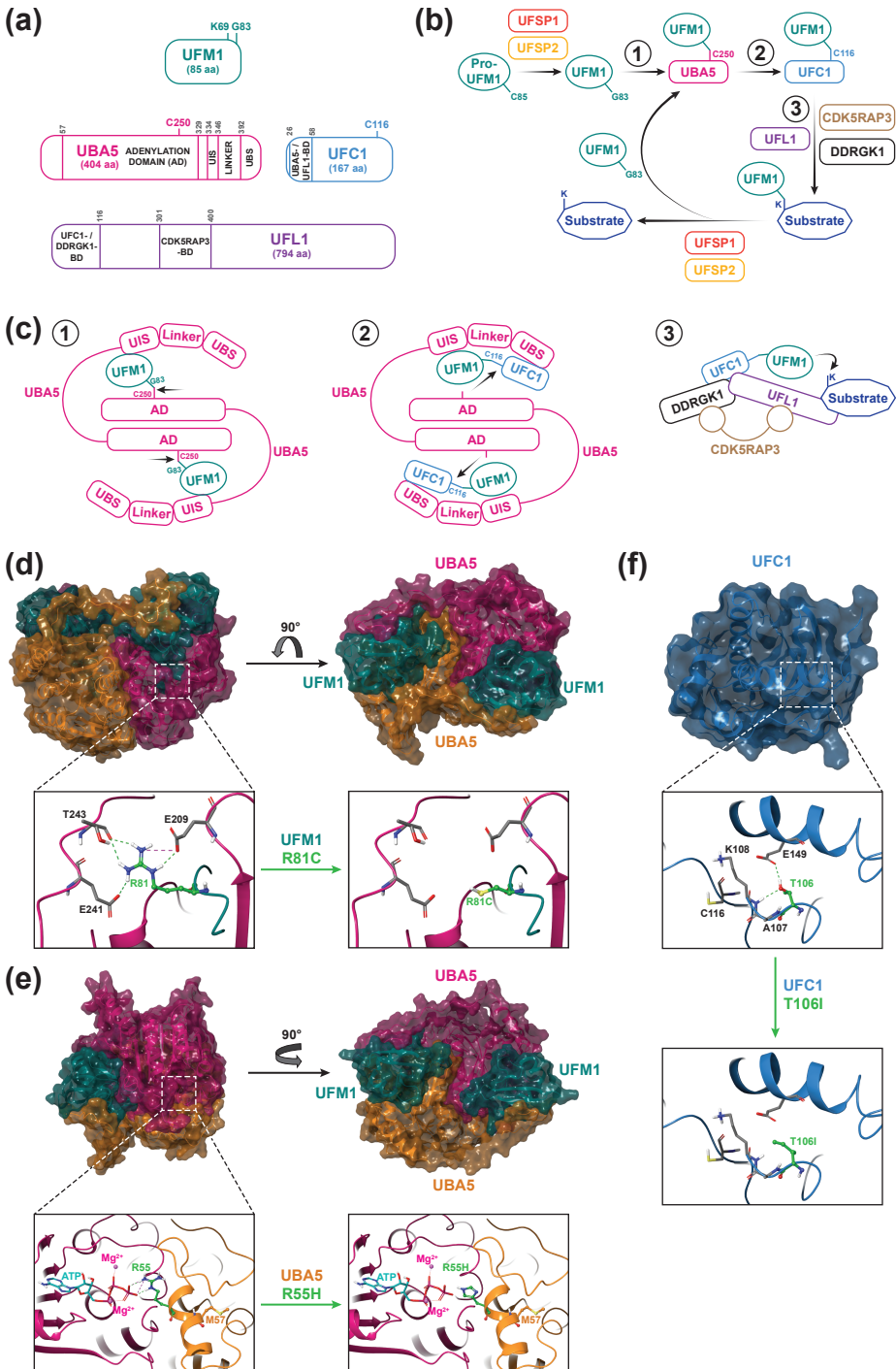


Figure 1

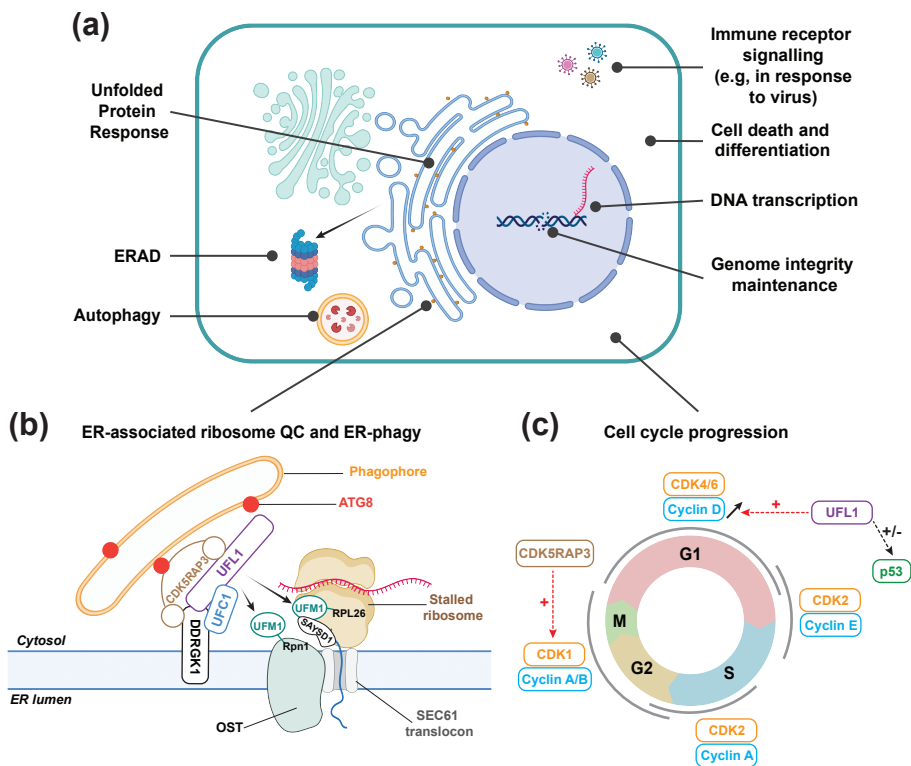


Figure 2

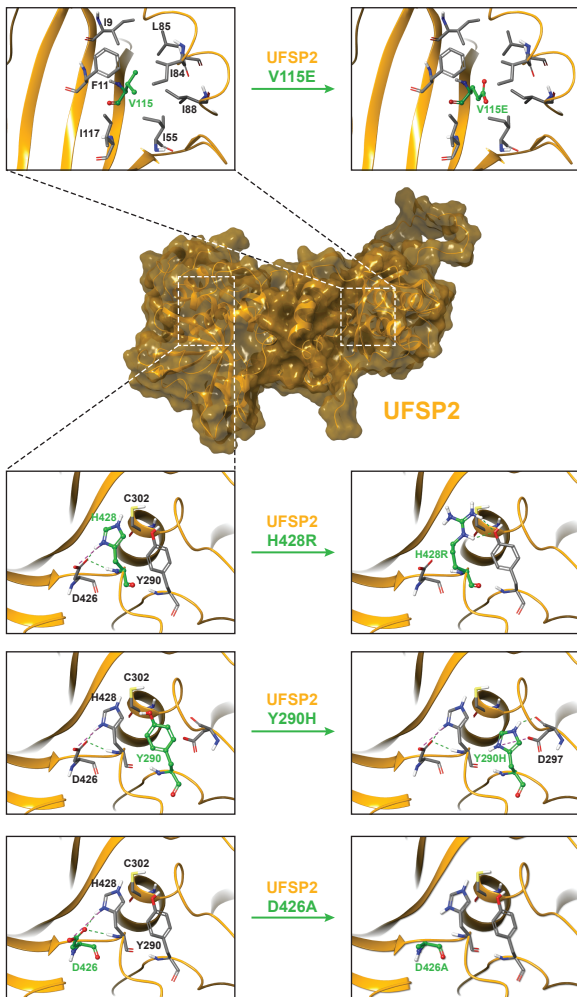


Figure 1
Accepted manuscript

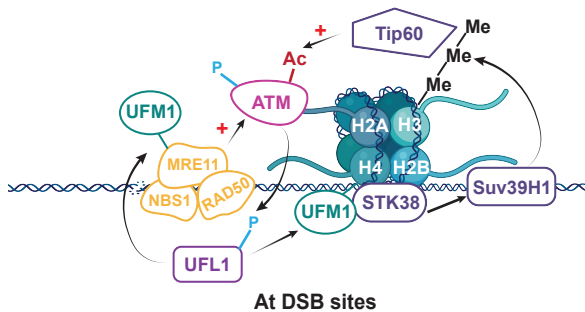


Figure II