

1 **Signaling from the RNA sensor RIG-I is regulated by ufmylation**

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27 **Keywords**

28 Retinoic acid-inducible gene I, RLR signaling, ubiquitin-like modifications, mitochondrial-  
29 associated ER membranes

30

31 **Abstract**

32 The RNA binding protein RIG-I is a key initiator of the antiviral innate immune response.  
33 The signaling that mediates the antiviral response downstream of RIG-I is transduced  
34 through the adaptor protein MAVS and results in the induction of type I and III interferons  
35 (IFN). This signal transduction occurs at endoplasmic reticulum (ER)-mitochondrial  
36 contact sites, to which RIG-I and other signaling proteins are recruited following their  
37 activation. RIG-I signaling is highly regulated to prevent aberrant activation of this pathway  
38 and dysregulated induction of IFN. Previously, we identified UFL1, the E3 ligase of the  
39 ubiquitin-like modifier conjugation system called ufmylation, UFL1, as one of the proteins  
40 recruited to membranes at ER-mitochondrial contact sites in response to RIG-I activation.  
41 Here, we show that UFL1, as well as the process of ufmylation, promote IFN induction in  
42 response to RIG-I activation. We find that following RNA virus infection, UFL1 is recruited  
43 to the membrane targeting protein 14-3-3 $\epsilon$ , and that this complex is then recruited to  
44 activated RIG-I to promote downstream innate immune signaling. Importantly, loss of  
45 ufmylation prevents 14-3-3 $\epsilon$  interaction with RIG-I, which abrogates the interaction of RIG-  
46 I with MAVS and thus downstream signal transduction that induces IFN. Our results define  
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48 post-translational control for IFN induction.

49

50 **Significance**

51 The viral RNA sensor RIG-I initiates the antiviral innate immune response by activating a  
52 signaling cascade that induces interferon. Activation of the RIG-I signaling pathway is  
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54 dysregulation that can lead to excessive inflammation or autoimmune disorders. Here, we  
55 characterize one such mechanism of regulation. We describe that UFL1, an E3 ligase for  
56 the ubiquitin-like modifier conjugation system called ufmylation, is important to promote  
57 RIG-I signaling. Using molecular approaches, we show that ufmylation promotes RIG-I  
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92

93 **Introduction**

94

95 Detection of RNA virus infection is initiated by cellular sensors such as RIG-I. RIG-I is a  
96 pattern recognition receptor that detects unique features of viral RNA that are generally

97 absent in cellular RNA, referred to as pathogen-associated molecular patterns (PAMPs)  
98 (1). Sensing of viral RNA PAMPs triggers RIG-I activation and induces a downstream  
99 signaling cascade that ultimately results in transcriptional induction of type I and type III  
100 interferons (IFN) and the antiviral response (2, 3). The RIG-I signaling cascade is carefully  
101 regulated by multiple mechanisms, including post-translational modifications that  
102 influence specific protein-protein interactions that can result in changes in protein  
103 localization to mediated signaling (3, 4). For example, following sensing of RNA PAMPs,  
104 RIG-I undergoes K63-linked polyubiquitination in order to transition to its fully active  
105 conformation, which promotes its interaction with the molecular trafficking protein 14-3-3 $\epsilon$   
106 (5–8). 14-3-3 $\epsilon$  facilitates the recruitment of activated RIG-I from the cytosol to intracellular  
107 membranes where it interacts with MAVS (7, 9, 10), which assembles other RIG-I pathway  
108 members to transduce the signals that induce IFN (7, 11). Importantly, many RNA viruses,  
109 including influenza A virus and some flaviviruses (dengue virus, Zika virus, and West Nile  
110 virus), prevent the interaction of RIG-I with 14-3-3 $\epsilon$  to limit IFN induction and evade the  
111 antiviral response (9, 10, 12).

112

113 In addition to RIG-I, a number of signaling proteins must be recruited to MAVS in order to  
114 propagate downstream IFN induction. Previously, we identified proteins that move to  
115 MAVS signaling sites at mitochondrial-associated endoplasmic reticulum (ER)  
116 membranes (MAM) during RNA virus infection (13, 14). These proteins likely aid in spatial  
117 organization of RIG-I pathway proteins during viral infection and include the GTPase  
118 RAB1B, which plays a role in recruiting TRAF3 to MAVS (15). In addition to RAB1B, we  
119 identified other proteins recruited to the MAM upon RIG-I signaling activation, one of which  
120 was UFL1 (referred to in our previous publication as KIAA0776) (14). UFL1 is an E3 ligase  
121 for UFM1, which is a ubiquitin-like modification of 85 amino acids. The process of  
122 ufmylation conjugates UFM1 covalently to lysine residues of target proteins through a  
123 process called ufmylation, which is similar to ubiquitination in that it also uses an E1, E2,  
124 and E3 ligase conjugation system (UBA5, UFC1, and UFL1; see Figure 2D). UFM1 is  
125 removed by the UFSP2 protease (16–20). The consequence of UFM1 addition to proteins  
126 is not fully understood, but the literature supports the idea that it can promote protein-  
127 protein interactions to regulate a number of biological processes (21–31). Here, we  
128 uncover a role for ufmylation in RIG-I activation. We found that the cellular proteins that  
129 catalyze ufmylation all promote RIG-I-mediated induction of IFN. Interestingly, we found  
130 that UFL1 interacts with both RIG-I and the molecular trafficking protein 14-3-3 $\epsilon$  following

131 RNA virus infection. Further, similar to RIG-I, UFL1 is recruited to intracellular membranes  
132 following RNA virus infection. Importantly, loss of ufmylation prevents the interaction of  
133 14-3-3 $\epsilon$  with RIG-I, which results in decreased MAVS activation and IFN induction in  
134 response to RNA virus infection. Thus, ufmylation can regulate RIG-I activation and  
135 downstream signaling of the intracellular innate immune system.

136

## 137 **Results**

138

139 **The ufmylation activity of UFL1 promotes RIG-I signaling.** Having found that the E3  
140 ligase of ufmylation UFL1 is recruited to MAVS signaling sites at the MAM in response to  
141 RIG-I signaling (14), we wanted to determine if UFL1 regulates RIG-I signaling. To test  
142 this, we measured induction of the IFN- $\beta$  promoter following UFL1 overexpression using  
143 an IFN- $\beta$  promoter luciferase reporter assay (32) and found that UFL1 increased activation  
144 of the IFN- $\beta$  promoter, similar to that of RIG-I expression, in a dose-dependent fashion in  
145 response to infection with Sendai virus (SenV) (Figure 1A). SenV is a murine  
146 paramyxovirus that specifically activates RIG-I (33). In support of UFL1 enhancing RIG-I  
147 signaling specifically, exogenous expression of UFL1 also increased IFN- $\beta$  promoter  
148 activity in response to transfection of 293T cells with a known RIG-I immunostimulatory  
149 RNA from hepatitis C virus (PAMP; Figure 1B) (34). However, UFL1 overexpression in  
150 293T cells did not lead to increased induction of IFN-stimulated genes (ISG), such as  
151 *ISG56* or *ISG15*, in response to exogenous IFN- $\beta$  treatment, indicating that UFL1 primarily  
152 regulates IFN induction and not the IFN response (Figure 1C). Next, we depleted UFL1  
153 by siRNA in two different cell types and measured SenV-induced activation of the RIG-I  
154 pathway. Depletion of UFL1 in 293T cells resulted in decreased phosphorylation of IRF3,  
155 a transcription factor for both type I and III IFNs, while exogenous expression of an siRNA-  
156 resistant UFL1 restored SenV-mediated IRF3 phosphorylation (Figure 1D). Depletion of  
157 UFL1 in primary neonatal human dermal fibroblasts (NHDFs) also reduced the SenV-  
158 mediated induction of both *IFNB1* and *IFNL1* transcripts, as measured by RT-qPCR  
159 (Figure 1E), as well as the production of IFN- $\beta$  protein, as measured by an enzyme-linked  
160 immunosorbent assay (ELISA) (Figure 1F).

161

162 To define the domains of UFL1 that regulate RIG-I signaling, we expressed a series of  
163 previously described UFL1 truncation mutants (diagrammed in Figure 1H) and measured  
164 SenV-mediated activation of the IFN- $\beta$  promoter in a luciferase reporter assay (16). The

165 ability of UFL1 to transfer UFM1 to a target protein has been suggested to require the first  
166 212 amino acids of the protein, as this domain interacts with the E2 ligase for ufmylation,  
167 UFC1 (16). The wild-type (WT) UFL1 (aa 1-794), as well as the C-terminal deleted mutants  
168 of UFL1, aa 1-212 and aa 1-452, which all have reported ufmylation activity (16),  
169 stimulated SenV-mediated induction of the IFN- $\beta$  promoter (Figure 1G). Interestingly, the  
170 N-terminal deleted mutant of UFL1 aa 213-794, that does not have reported ufmylation  
171 activity, also induced signaling, while the N-terminal deleted UFL1 mutant aa 453-794 did  
172 not (Figure 1G). However, our analysis of global UFM1 conjugates by these UFL1  
173 constructs revealed that while UFL1 WT, aa 1-212, and aa 1-452 all retain full ufmylation  
174 activity, aa 213-794 of UFL1 retain approximately 40% ufmylation activity, while aa 453-  
175 794 of UFL1 retain only about 20% activity (Figure 1H; Figure S1). Thus, taken together,  
176 this reveals that the ufmylation activity of UFL1 is required to promote RIG-I signaling that  
177 results in induction of IFN.

178

179 **The ufmylation machinery proteins positively regulate RIG-I signaling.** Having  
180 determined that the ufmylation activity of UFL1 is important for its role in RIG-I signaling,  
181 we hypothesized that UFM1 and the proteins required for UFM1 conjugation would also  
182 be required to promote this signaling. Similar to our results with UFL1, overexpression of  
183 UFM1 increased SenV-mediated activation of the IFN- $\beta$  promoter in a dose-dependent  
184 fashion (Figure 2A). Conversely, the activation of the IFN- $\beta$  promoter in response to SenV  
185 was significantly abrogated in 293T cells in which UFM1 was deleted by CRISPR/Cas9,  
186 as compared to WT 293T cells (Figure 2B). Importantly, this signaling was restored upon  
187 exogenous expression of UFM1 (Figure 2B). The absence of UFM1 expression also  
188 prevented the induction of IFN- $\beta$  protein in response to SenV infection, as measured by  
189 ELISA (Figure 2C). The process of ufmylation has 5 steps (Figure 2D). First, UFM1 is  
190 processed to expose the terminal glycine residue. Then, this mature UFM1 is added to the  
191 target protein by the actions of UBA5, which acts as an E1 ligase for UFM1; UFC1, the E2  
192 ligase; and UFL1, the E3 ligase (19). Finally, the UFSP2 protease removes UFM1, which  
193 enables recycling of mature UFM1 (18). We found that exogenous expression of each of  
194 the proteins involved in UFM1 conjugation, including the UFSP2 protease, positively  
195 regulated SenV-mediated induction of the IFN- $\beta$  promoter (Figure 2E). These results  
196 reveal that the proteins that catalyze ufmylation and the UFM1 modification itself promote  
197 RIG-I signaling.

198

199 **UFM1 is required for the RIG-I-driven transcriptional response.** After establishing that  
200 ufmylation promotes RIG-I activation, and in turn IFN expression, we next broadly  
201 measured the impact of ufmylation upon the transcriptional response to RIG-I signaling.  
202 Using RNA-sequencing, we analyzed gene expression in either WT or UFM1 KO 293T  
203 cells, following mock or SenV infection (Table S1.1; Table S1.2). Gene set enrichment  
204 analysis (Table S2.1; Table S2.2) of the transcripts significantly reduced (adjusted  $P < 0.01$ )  
205 by UFM1 KO in the absence of viral infection revealed previously described pathways  
206 regulated by ufmylation such as cytosolic ribosomes, ribosome assembly, and  
207 hematopoiesis (Figure S2A; Table S2.1) (21, 28, 29, 35). Following viral infection, the top  
208 10 gene categories negatively impacted by UFM1 KO, with a darker red color indicating  
209 more downregulation, were all related to the antiviral response, such as response to type  
210 I IFN and defense against virus (Figure 3A; Table S2.2). Indeed, of the top 50 most  
211 downregulated pathways impacted by UFM1 KO during infection, the majority were related  
212 to innate immune signaling or viral replication (Table S2.2), while upregulated gene  
213 categories were more diverse (Table S2.3; Table S2.4). Of the genes differentially  
214 expressed during UFM1 KO in response to SenV (adjusted  $P < 0.01$ ), the majority are  
215 downregulated (Figure 3B). Indeed, these downregulated genes included *IFNB1* and  
216 *IFNL1*, as well as other known ISGs (in red) (36) (Figure 3B; Figure 3C). These data are  
217 consistent with a model in which ufmylation-mediated regulation of IFN induction has  
218 broad consequences on genes induced by the IFN response.

219

220 **UFL1 is recruited to intracellular membranes and interacts with 14-3-3 $\epsilon$  and RIG-I**  
221 **during RNA virus infection.** Following the binding of RIG-I to non-self RNA, it interacts  
222 with several host proteins to facilitate its activation, localization to the MAM, and interaction  
223 with MAVS. These proteins include the E3 ligases for K63-linked ubiquitin TRIM25 and  
224 Riplet (5, 6, 37), as well as the molecular trafficking protein 14-3-3 $\epsilon$ . In particular, 14-3-3 $\epsilon$   
225 is required for RIG-I recruitment from the cytosol to MAVS signaling sites at intracellular  
226 membranes (5–7, 13); however, the mechanism underlying how 14-3-3 $\epsilon$  selects RIG-I as  
227 cargo has yet to be elucidated. Using a subcellular membrane fractionation assay (38),  
228 we confirmed that UFL1 increases its association with intracellular membranes in  
229 response to SenV, similar to RIG-I (Figure 4A; compare fraction #1, which has Cox-I and  
230 no GAPDH, with fractions #6-8, which are enriched for the cytosolic protein GAPDH) (7,  
231 12). This finding is consistent with our previous report that UFL1 is recruited to the MAM  
232 in response to either SenV or hepatitis C virus replication (14), suggesting that UFL1

233 recruitment occurs prior to MAVS activation, as MAVS is cleaved by the HCV NS3-NS4A  
234 protease (39–42). As the recruitment of RIG-I to intracellular membranes is known to  
235 require 14-3-3 $\epsilon$ , and, as both UFL1 and UFM1 have been shown to interact with 14-3-3 $\epsilon$   
236 (16), we hypothesized that UFL1 may interact with 14-3-3 $\epsilon$  to promote the IFN induction  
237 that we had observed in response to RNA virus infection. Thus, we first determined if the  
238 interaction of UFL1 with 14-3-3 $\epsilon$  is increased in response to RIG-I activation by SenV by  
239 performing co-immunoprecipitation. We found that Myc-14-3-3 $\epsilon$  did co-immunoprecipitate  
240 with Flag-UFL1, as reported previously (16), and that this interaction was increased by  
241 SenV (Figure 4B). Interestingly, the interaction of UFL1 with RIG-I also increased following  
242 SenV, both upon over-expression and at the level of the endogenous proteins (Figure 4C;  
243 Figure 4D). As RIG-I undergoes a series of modifications to become fully active (1, 4), we  
244 next used a panel of RIG-I mutants to define which stage of RIG-I activation promotes  
245 interaction with UFL1. These mutations prevent the distinct steps of RIG-I activation such  
246 as RIG-I binding to RNA (K888/907A), interacting with TRIM25 (T55I), or ubiquitination by  
247 Riplet and TRIM25 (K172/788R) (5, 43, 44). The interaction of UFL1 with RIG-I was  
248 significantly impaired by each of these mutations, suggesting that UFL1 regulates RIG-I  
249 function after it binds RNA and becomes ubiquitinated (Figure 4E). As this is the same  
250 step of activation at which 14-3-3 $\epsilon$  binds to RIG-I to promote its translocation to  
251 intracellular membranes (7), this suggests that RNA virus infection increases the  
252 interaction of 14-3-3 $\epsilon$  with UFL1, which then interacts with activated, K63-ubiquitinated  
253 RIG-I.

254

255 **UFL1 interaction with RIG-I requires 14-3-3 $\epsilon$  and UFM1.** Having determined that UFL1  
256 interacts with both activated RIG-I and 14-3-3 $\epsilon$  following RNA virus infection, we next  
257 defined the dynamics of this complex formation by testing two distinct models. In the first  
258 model, UFL1 would interact first with activated RIG-I, induce its ufmylation, and then the  
259 UFL1-RIG-I complex would interact with 14-3-3 $\epsilon$ . In this model, depletion of 14-3-3 $\epsilon$  or  
260 loss of UFM1 would not be expected to change the interaction of UFL1 with RIG-I. In the  
261 second model, UFL1 would interact first with 14-3-3 $\epsilon$  and induces its ufmylation, or that of  
262 another associated protein, and then the UFL1-14-3-3 $\epsilon$  complex would interact with  
263 activated RIG-I. In this second model, depletion of 14-3-3 $\epsilon$  would be expected to prevent  
264 UFL1 interaction with RIG-I, and loss of ufmylation would limit UFL1 interaction with RIG-  
265 I but would not affect UFL1 interaction with 14-3-3 $\epsilon$ . To elucidate these possibilities, first,



266 we used co-immunoprecipitation to measure the interaction of exogenously expressed  
267 Flag-UFL1 and HA-RIG-I in SenV-infected 293T lysates that had been depleted of 14-3-  
268 3 $\epsilon$  or CTRL by siRNA. This revealed that formation of the SenV-activated RIG-I-UFL1  
269 complex requires 14-3-3 $\epsilon$  (Figure 5A). Next, we tested if ufmylation was required for  
270 formation of the SenV-activated RIG-I-UFL1 complex by measuring this interaction in WT  
271 or UFM1 KO 293T cells. We found that UFM1 was required for SenV-activated RIG-I-  
272 UFL1 complex (Figure 5B). The results of these two experiments reveal that both 14-3-3 $\epsilon$   
273 and UFM1 are required for UFL1 to interact with RIG-I, supporting the second model of  
274 complex formation in which UFL1 interacts first with 14-3-3 $\epsilon$  and catalyzes its ufmylation,  
275 and then this complex associates with RIG-I. In support of this, we found that UFM1 was  
276 not required for UFL1 to interact with 14-3-3 $\epsilon$  (Figure 5C). Together, these data indicate  
277 that ufmylation promotes the interaction of UFL1 with 14-3-3 $\epsilon$  and activated RIG-I.

278

279 **Ufmylation promotes RIG-I interaction with 14-3-3 $\epsilon$  for MAVS activation.** Having  
280 found that that UFL1 requires 14-3-3 $\epsilon$  to interact with activated RIG-I, we next tested if  
281 UFL1 is required for the interaction of 14-3-3 $\epsilon$  with RIG-I, which is essential for activated  
282 RIG-I to translocate from the cytosol to intracellular membranes for interaction with MAVS  
283 (7). We performed a co-immunoprecipitation of Flag-RIG-I and Myc-14-3-3 $\epsilon$  from 293T  
284 cells and found that this SenV-mediated interaction was significantly decreased upon  
285 UFL1 depletion (Figure 6A). In addition, loss of UFM1 expression also decreased the  
286 SenV-induced interaction of RIG-I with 14-3-3 $\epsilon$  (Figure 6B). Importantly, we also found  
287 that UFM1 is required for the SenV-induced interaction of RIG-I with MAVS (Figure 6C)  
288 and MAVS higher-order oligomerization, which is a hallmark of MAVS activation (45, 46)  
289 (Figure 6D). In summary, these data reveal that UFL1 and UFM1 are required for the RIG-  
290 I interaction with 14-3-3 $\epsilon$ , for interaction with MAVS, and for MAVS activation by  
291 oligomerization.

292

## 293 **Discussion**

294

295 Regulation of RIG-I activation and downstream signaling is essential for proper induction  
296 and termination of IFN. Here, we show that both UFL1 and the process of ufmylation  
297 promote RIG-I pathway signaling that leads to IFN induction, uncovering an important step  
298 in the activation of the RIG-I pathway. RIG-I activation occurs upon RNA binding. Then,

299 RIG-I undergoes ATP hydrolysis. and interaction with K63-linked polyubiquitin chains,  
300 both covalently and non-covalently (5, 44, 47), which promotes formation of a RIG-I  
301 tetramer (48). This polyubiquitinated, activated RIG-I oligomer then interacts with the  
302 membrane trafficking protein 14-3-3 $\epsilon$  for translocation to MAVS at ER-mitochondrial  
303 contact sites (7). We found that UFL1 is recruited to 14-3-3 $\epsilon$  following RNA virus infection  
304 and that ufmylation facilitates the interaction between 14-3-3 $\epsilon$  and activated RIG-I.  
305 Importantly, this results in increased interaction of RIG-I with MAVS and MAVS  
306 oligomerization, ultimately promoting the downstream signal transduction which produces  
307 IFN.

308

309 Ufmylation is emerging as a post-translational modification that regulates diverse  
310 biological processes, including DNA repair, ER homeostasis, and even the replication of  
311 hepatitis A virus (21, 22, 24, 27, 28, 30, 35, 49). In these cases, UFL1, along with the other  
312 members of the ufmylation cascade, induce ufmylation of a target protein important for  
313 regulating these processes. For example, both MRE11 and histone H4 are ufmylated by  
314 UFL1 in the nucleus in response to DNA damage resulting in activation the key DNA repair  
315 kinase ATM (22, 24). UFL1 can also act at the ER, where it plays a role in ER protein  
316 quality control, where it ufmylates specific proteins, including ribosomal proteins RPL26,  
317 to induce lysosomal degradation of stalled peptides and/or the ER and prevent the  
318 unfolded protein response (27, 28, 49, 50). Interestingly, hepatitis A virus translation,  
319 which occurs in association with the ER, also requires ufmylation of RPL26 (30).  
320 Therefore, ufmylation can regulate several aspects of translation. It is possible that  
321 ufmylation regulates translation of certain mRNAs important for RIG-I signaling and  
322 subsequent IFN induction. However, we identified a role for ufmylation in regulating the  
323 interaction of RIG-I with 14-3-3 $\epsilon$ , one of the earliest known steps of RIG-I signaling,  
324 strongly supporting a mechanism in which following RIG-I activation, ufmylation is  
325 controlling this specific protein-protein interaction. The mechanisms by which the process  
326 of UFM1 addition regulates interactions between proteins or alters other aspects of protein  
327 function are largely unknown. Indeed, we found that UFSP2, the protease that removes  
328 UFM1 from proteins (18), promoted SenV-mediated IFN induction (Figure 2D), suggesting  
329 that we do not yet have a full grasp on the ufmylation process. It is possible that the  
330 dynamic process of ufmylation or the enhanced formation of mature UFM1 following  
331 deconjugation from targets or promote RIG-I signaling independent of deconjugation  
332 activity. Indeed, in support of this idea, others have shown that UFSP2 in myeloid cells is

333 required for influenza virus resistance in mice (31). It is also possible that UFSP2 acts on  
334 other members of the RIG-I pathway to alter their function. Future studies to define how  
335 the process of ufmylation regulates this and other aspects of the antiviral innate immune  
336 response will be of great interest as they may shed light broadly on how ufmylation  
337 regulates diverse cell biological processes that alter cellular signaling.

338

339 The mechanisms underlying how cytoplasmic UFL1 is recruited to its protein targets that  
340 reside in different subcellular compartments are not fully known. For example, we found  
341 that RIG-I activation induces UFL1 translocation to intracellular membranes (Figure 3A),  
342 and while we know that UFL1 is recruited to the MAM during infection, the mechanism by  
343 which UFL1 becomes membrane-associated remains unknown (14). DDRGK1 (UFBP1)  
344 may facilitate UFL1 targeting to the MAM, as DDRGK1 is localized to mitochondrial-ER  
345 contact sites (16, 51) and in some cases it is required for UFL1 recruitment to membranes  
346 (27, 28). Thus, both DDRGK1 and mitochondrial-ER contact sites could function as a  
347 regulatory hub that aids in the recruitment of UFL1 and RIG-I pathway signaling proteins.  
348 Interestingly, RAB1B, a GTPase that we found is recruited to the MAM and important for  
349 RIG-I signaling (14, 15) is ufmylated (52, 53), which reveals that ufmylation likely regulates  
350 a number of RIG-I pathway signaling proteins. As UFL1 contains no functional domains  
351 common to other E3 ligases that might allow one to predict how its targets are selected  
352 (16, 54, 55), defining the signals and features that control UFL1 localization, as well as  
353 the target proteins ufmylated in response to RIG-I activation, will undoubtedly reveal clues  
354 into how the process of ufmylation is activated and how specific targets are selected.

355

356 Our work revealed that 14-3-3 $\epsilon$  required ufmylation to interact with activated RIG-I. The  
357 details underlying how 14-3-3 $\epsilon$  interacts with activated RIG-I have not been fully  
358 elucidated, as it does not occur through the known phosphorylated amino acids on RIG-I,  
359 the typical recruitment signal of the 14-3-3 family of proteins (7, 56, 57). Interestingly,  
360 others have shown that 14-3-3 $\epsilon$  interacts with UFM1 and other members of the ufmylation  
361 pathway (16). Thus, taken together with our results, this suggests that ufmylation of 14-3-  
362 3 $\epsilon$  or a 14-3-3 $\epsilon$ -associated protein promotes the interaction between activated RIG-I and  
363 14-3-3 $\epsilon$ . In fact, a number of 14-3-3 family proteins are post-translationally modified by  
364 phosphorylation, acetylation, and oxidation (58). Therefore, post-translational modification  
365 of 14-3-3 $\epsilon$  by ufmylation could broadly define how cargo proteins, including RIG-I, are  
366 selected. Indeed, this mechanism could be shared with other RNA virus sensing

367 pathways, such as the RIG-I-like-receptor MDA5, which also interacts with a 14-3-3  
368 protein, 14-3-3 $\eta$ , by an unknown mechanism (59). Thus, ufmylation may broadly influence  
369 how 14-3-3 proteins or other host proteins interact with each other to regulate the  
370 intracellular innate immune response.

371

372 Overall, this work lays the groundwork for future studies to define how ufmylation of  
373 antiviral innate immune signaling proteins regulates their function and how specific  
374 signaling pathways are differentially activated through ufmylation. In addition, our work  
375 adds ufmylation to the growing list of ubiquitin-like and other modifications that regulate  
376 the intracellular innate immune response, including ISGylation, SUMOylation, FATylation,  
377 acetylation, phosphorylation, and others (4, 60, 61) broadening our understanding of how  
378 RIG-I signaling is activated and rapidly controlled by post-translational modifications in  
379 response to infection, leading to greater knowledge of the exquisite regulation of these  
380 pathways.

381

## 382 **Materials and Methods**

383

384 **Cell lines, viruses, and treatments.** Neonatal human dermal fibroblast (NHDF) cells and  
385 embryonic kidney 293T cells were grown in Dulbecco's modification of Eagle's medium  
386 (DMEM; Mediatech) supplemented with 10% fetal bovine serum (Thermo Fisher  
387 Scientific), 1X minimum essential medium non-essential amino acids (Thermo Fisher  
388 Scientific), and 25 mM HEPES (Thermo Fisher Scientific) (cDMEM). 293T (CRL-3216)  
389 were obtained from American Type Culture Collection (ATCC), NHDF cells (CC-2509)  
390 were obtained from Lonza. All cell lines were verified as mycoplasma free by the LookOut  
391 Mycoplasma PCR detection kit (Sigma). SenV Cantell strain was obtained from Charles  
392 River Laboratories and used at 200 hemagglutination units/mL (HAU). SenV infections  
393 were performed in serum-free media (30 minutes to 1 hour), after which complete media  
394 was replenished. IFN- $\beta$  (PBL Assay Science) was added to cells at a concentration of 50  
395 units/mL in cDMEM for 18 hours.

396

397 **Plasmids.** The following plasmids have been previously described: pEF-TAK-Flag, pEF-  
398 BOS-Flag-RIG-I (62), pIFN- $\beta$ -luc (63), pCMV-Renilla (Promega), pX459 (Addgene  
399 Plasmid #62988), pEF-BOS-Flag-RIG-I T55I (64), pEF-TAK-Myc-MAVS (32).  
400 pLJM1\_Flag-UFM1 was a gift from Dr. Craig McCormick at Dalhousie University. The

401 following plasmids were generated by insertion of PCR-amplified fragments into the NotI-  
402 to-PmeI digested pEF-TAK-Flag using InFusion cloning (Clontech), all primers are  
403 denoted in Table 1: pEF-TAK-Flag-UFL1 (GenBank: BC036379; GeneID: 23376), pEF-  
404 TAK-Flag-UBA5 (NM\_024818.6), pEF-TAK-Flag-UFC1 (NM\_016406.4), pEF-TAK-Flag-  
405 UFSP2 (NM\_018359.5), pEF-TAK-Flag-UFL1 1-212, pEF-TAK-Flag-UFL1 1-452, pEF-  
406 TAK-Flag-UFL1 213-794, and pEF-TAK-Flag-UFL1 453-794. Both pEF-TAK-Myc-14-3-3 $\epsilon$   
407 and pEF-TAK-Myc-UFL1 were generated by insertion of PCR-amplified fragments into the  
408 AgeI-NotI digested pEF-TAK-Myc (pEF-TAK-Myc-MAVS) by InFusion. The pEF-TAK-HA  
409 vector was generated by PCR to replace Flag with HA, and pEF-TAK-HA-RIG-I was  
410 generated by insertion of a PCR-amplified fragment into the NotI-AgeI digested pEF-TAK-  
411 HA vector. The following plasmids were generated by site-directed mutagenesis (see  
412 Table 1): pEF-TAK-Flag-UFL1<sup>siR</sup>, pEF-BOS-Flag-RIG-I K888/907A, and pEF-BOS-Flag-  
413 RIG-I K172/788R. To generate the CRISPR guide RNA plasmids px459-UFM1-E2 and  
414 px459-UFM1-B, sgRNA oligonucleotides were annealed and inserted into the BbsI-  
415 digested pX459 (30, 65). The plasmid sequences were verified by DNA sequencing and  
416 oligonucleotide sequences are available upon request.

417

418 **Generation of RNA PAMP.** Annealed oligonucleotides containing the sequence of the  
419 HCV 5'ppp poly-U/UC region (34) were *in vitro* transcribed using the MEGAshortscript T7  
420 transcription kit (Ambion) followed by ethanol precipitation, with the resulting RNA  
421 resuspended at 1  $\mu$ g/ $\mu$ L.

422

423 **Transfection.** DNA transfections were performed using FuGENE6 (Promega) or TransIT-  
424 LT1 (Mirus Bio). RNA PAMP transfections were done using the TransIT-mRNA  
425 Transfection kit (Mirus Bio). The siRNA transfections were done using Lipofectamine  
426 RNAiMax (Invitrogen). siRNAs directed against 14-3-3 $\epsilon$  (Dharmacon-L-017302-02-0005),  
427 UFL1 (Qiagen-SI04371318) or non-targeting AllStars negative control siRNA (Qiagen-  
428 1027280) were transfected into 293T cells (25 pmol of siRNA; final concentration of 0.0125  
429  $\mu$ M) or NHDF cells (250 pmol of siRNA; final concentration of 0.25  $\mu$ M). Media was  
430 changed 4-24 hours post-transfection, and cells were incubated for 36-48 h post-  
431 transfection prior to each experimental treatment. IFN- $\beta$ -promoter luciferase assays were  
432 performed as previously described at 18-24 hours post treatment and normalized to the  
433 *Renilla* luciferase transfection control (33).

434

435 **ELISA.** IFN- $\beta$  ELISAs were performed using Human IFN-beta DuoSet (R&D Systems)  
436 with supernatants collected from cultured cells.

437

438 **Generation of KO cell lines.** UFM1 KO 293T cells were generated by CRISPR/Cas9,  
439 using two guides targeting exon 2 and 3, similar to others, as we have done previously  
440 (15, 30).

441

442 **RNA analysis.** Total cellular RNA was extracted using the RNeasy Plus mini kit (Qiagen).  
443 RNA was then reverse transcribed using the iScript cDNA synthesis kit (BioRad), as per  
444 the manufacturer's instructions. The resulting cDNA was diluted 1:3 in ddH<sub>2</sub>O. RT-qPCR  
445 was performed in triplicate using the Power SYBR Green PCR master mix (Thermo-  
446 Fisher) and QuantStudio 6 Flex RT-PCR system. Oligonucleotide sequences for qPCR  
447 are available upon request.

448

449 **RNA-seq.** WT and UFM1 KO 293T cells were mock or SenV-infected (18 h) and  
450 harvested in biological duplicate, followed by total RNA extraction via TRIzol reagent  
451 (Thermo Fisher Scientific). Sequencing libraries were prepared using the KAPA Stranded  
452 mRNA-Seq Kit (Roche) and sequenced on an Illumina Novaseq 6000 with 50 bp paired-  
453 end reads (>20 million reads per sample) in an S1 flow cell by the Duke University Center  
454 for Genomic and Computational Biology.

455 RNA-seq data was processed using the TrimGalore toolkit (66) which employs Cutadapt  
456 (67) to trim low-quality bases and Illumina sequencing adapters from the 3' end of the  
457 reads. Only reads that were 20nt or longer after trimming were kept for further analysis.  
458 Reads were mapped to the GRCh38v93 version of the human genome and transcriptome  
459 (68) using the STAR RNA-seq alignment tool (69). Reads were kept for subsequent  
460 analysis if they mapped to a single genomic location. Gene counts were compiled using  
461 the HTSeq tool (70). Only genes that had at least 10 reads in any given library were used  
462 in subsequent analysis. Normalization and differential expression was carried out using  
463 the DESeq2 (71) Bioconductor (72) package with the R statistical programming  
464 environment. The false discovery rate was calculated to control for multiple hypothesis  
465 testing. Gene set enrichment analysis (73) was performed to identify gene ontology terms  
466 and pathways associated with altered gene expression for each of the comparisons  
467 performed. All RNA-seq data are deposited in the GEO database under GSE186287.

468

469 **Immunoblotting.** Cells were lysed in a modified radioimmunoprecipitation assay (RIPA)  
470 buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 0.5% sodium deoxycholate, and 1% Triton X-  
471 100) supplemented with protease inhibitor cocktail (Sigma) and Halt Phosphatase Inhibitor  
472 (Thermo-Fisher), and post-nuclear lysates were isolated by centrifugation. Quantified  
473 protein (between 5 -15 µg) was resolved by SDS/PAGE, transferred to nitrocellulose or  
474 polyvinylidene difluoride (PVDF) membranes in a 25 mM Tris-192 mM glycine-0.01% SDS  
475 buffer. Membranes were stained with Revert 700 total protein stain (LI-COR Biosciences)  
476 and then blocked in 3% BSA in Tris-buffered saline containing 0.01% Tween-20 (TBS-T).  
477 After washing with PBS-T or TBS-T (for phosphoproteins) buffer, following incubation with  
478 primary antibodies, membranes were incubated with species-specific horseradish  
479 peroxidase-conjugated antibodies (Jackson ImmunoResearch, 1:5000) or fluorescent  
480 secondaries (LI-COR Biosciences), followed by treatment of the membrane with Clarity  
481 Western ECL substrate (BioRad) and imaging on a LICOR Odyssey FC. The following  
482 antibodies were used for immunoblotting: R-anti-SenV (MBL, 1:1000), M-anti-Tubulin  
483 (Sigma, 1:1000), R-anti-GAPDH (Cell Signaling Technology, 1:1000), R-anti-p-IRF3 (Cell  
484 Signaling Technology, 1:1000), R-anti-IRF3 (Cell Signaling Technology, 1:1000), R-anti-  
485 UFL1 (Novus Biologicals, 1:1000), R-anti-UFM1 (Abcam, 1:1000), anti-RIG-I (M-  
486 AdipoGen, R-Abcam, 1:1000), R-anti-14-3-3ε (Cell Signaling Technology, 1:1000), M-anti-  
487 Flag M2 (Sigma, 1:1000), anti-Flag-HRP (Sigma, 1:1000-1:5000), R-anti-Flag (Sigma,  
488 1:1000), anti-HA (M- and R-Sigma, 1:1000), and anti-Myc (M-Santa Cruz or R-Cell  
489 Signaling Technology, 1:1000).

490

491 **Immunoprecipitation.** Cells were lysed in RIPA buffer with or without 10% glycerol.  
492 Quantified protein (between 100-500 µg) was incubated with protein-specific, isotype  
493 control antibody (R-Cell Signaling Technology or M-Thermo Fisher), or anti-Flag M2  
494 magnetic beads (Sigma), in lysis buffer either at room temperature for 2 h or at 4°C  
495 overnight with head over tail rotation. The lysate/antibody mixture was then incubated with  
496 Protein G Dynabeads (Invitrogen) for 1 h. Beads were washed 3X in PBS or RIPA buffer  
497 and eluted in 2X Laemmli Buffer (BioRad) with or without 5% 2-Mercaptoethanol at 95°C  
498 for 5 min. Proteins were resolved by SDS/PAGE and immunoblotting, as above.

499

500 **Subcellular membrane fractionation.** Membrane fractionation was performed as  
501 previously described (7, 12, 38, 74). Cells were lysed in hypotonic buffer (10 mM Tris-HCL  
502 (pH 7.5), 10 mM KCl, and 5 mM MgCl<sub>2</sub> supplemented with protease inhibitor cocktail) for

503 10 minutes on ice followed by 20 passages through a 20-gauge needle. Nuclei and  
504 unbroken cells were removed by centrifugation at 1000xg for 5 min at 4°C. The resulting  
505 supernatants were mixed thoroughly with 72% sucrose and overlaid with 55% sucrose,  
506 followed by 10% sucrose, all in low-salt buffer (2 nM EDTA, 20 nM HEPES (pH 8.0), 150  
507 mM NaCl, 0.1% SDS, 1% Triton X-100). The gradients were subjected to centrifugation at  
508 38,000 RPM in a Beckman SW41 Ti Rotor for 14 h at 4°C. 1 mL fractions were collected  
509 using a BioComp piston gradient fractionator and resulting fractions were divided in half  
510 and mixed with 2 parts 100% methanol and precipitated overnight at -80°C. Protein pellets  
511 were collected by centrifugation and resuspended in 2X Laemmli buffer and heated for 5  
512 min at 95°C for immunoblot analysis. 10% pre-fractionated cells from each condition were  
513 collected as the input.

514

515 **Semi-denaturing detergent agarose gel electrophoresis.** SDD-AGE was performed as  
516 described (45, 46). Briefly, crude mitochondria (P5 fraction) were isolated from an equal  
517 number of WT or UFM1 KO 293T cells that were mock or SenV infected (12 h),  
518 resuspended in hypotonic buffer (10 mM Tris, pH 7.5, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.5  
519 mM EDTA). Resulting samples were split and 2X SDD-AGE sample buffer (0.5X TBE,  
520 10% glycerol, 2% SDS, 0.2 mM Bromophenol Blue) buffer with or without 5% 2-  
521 Mercaptoethanol was added, and samples were loaded onto a vertical 1.5% agarose gel.  
522 Electrophoresis was performed with a constant voltage of 70 V at 4 °C in SDD-AGE  
523 running buffer (1X TBE and 0.1% SDS). Gels were transferred onto a nitrocellulose  
524 membrane overnight on ice at 25 V. Membranes were fixed in 0.25% glutaraldehyde in  
525 PBS and immunoblotting was performed as usual. 15% of the SDD-AGE samples were  
526 reserved for input.

527

528 **Quantification of immunoblots.** Immunoblots imaged using the LICOR Odyssey FC  
529 were quantified by ImageStudio software, and raw values were normalized to relevant  
530 controls for each antibody. Phosphoprotein values were normalized to Tubulin and  
531 displayed as the percentage of signal from WT. Relative membrane association of UFL1  
532 was quantified as the ratio of UFL1 to Cox-1 in fraction 1 normalized to total protein levels  
533 of UFL1 in the input and displayed as the percentage of UFL1 membrane association  
534 normalized to mock values.

535



536 **Statistical analysis.** Student's unpaired t-test, one-way ANOVA, or two-way ANOVA  
537 were implemented for statistical analysis of the data followed by appropriate post-hoc test  
538 (as indicated) using GraphPad Prism software. Graphed values are presented as mean  $\pm$   
539 SD or SEM (n = 3 or as indicated); \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, and \*\*\*p  $\leq$  0.001.

540

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542

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552

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557

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561 Health.

562

563 **Figure Legends**

564 **Figure 1. The ufmylation activity of UFL1 promotes RIG-I signaling.**

565 A) IFN- $\beta$ -promoter reporter luciferase expression (rel. to *CMV-Renilla*) from 293T cells  
566 expressing vector, Flag-UFL1, or Flag-RIG-I, followed by mock or SenV infection (18 h) or  
567 in B) 293T cells transfected with vector (Vec) or Flag-UFL1, followed by mock or HCV  
568 PAMP RNA transfection (24 h). C) RT-qPCR analysis (rel. to *GAPDH*) of RNA extracted  
569 from 293T cells transfected vector or Flag-UFL1 that were treated with IFN- $\beta$  (18 h). D)  
570 Immunoblot analysis of p-IRF3 following siRNA transfection along with expression of  
571 vector or Flag-UFL1<sup>siR</sup>, which has point mutations in the siRNA seed sequence.  
572 Quantification of p-IRF3/Tubulin is shown on the right. E) RT-qPCR analysis (rel. to *18S*)  
573 of RNA extracted from primary neonatal human dermal fibroblasts (NHDFs) transfected  
574 with either siCTRL or siUFL1 followed by mock or SenV infection (8 h). F) ELISA for IFN-  
575  $\beta$  of supernatants harvested from NHDFs transfected with siCTRL or siUFL1 and infected  
576 with SenV for the indicated times. G) Relative IFN- $\beta$ -promoter reporter luciferase  
577 expression (rel. to *CMV-Renilla*) from 293T cells expressing indicated constructs followed  
578 by mock or SenV infection (12-18 h), with results graphed as relative SenV fold change  
579 for each. H) Diagram of UFL1 truncation constructs and corresponding ufmylation activity  
580 represented as the mean of mock and SenV values normalized to WT. For A) mean  $\pm$   
581 SD, n=3 technical replicates and representative of n=3 independent experiments. For all  
582 others, mean  $\pm$  SEM, n=3 or n=5 (1G) biological replicates. \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, and  
583 \*\*\*p  $\leq$  0.001 determined by two-way ANOVA followed by Šidák's multiple comparisons  
584 test (B, E), Student's t-test (C, D, F), or one-way ANOVA followed by Dunnett's multiple  
585 comparisons test (G).

586

587 **Figure 2. The ufmylation machinery proteins positively regulate RIG-I signaling.**

588 A) IFN- $\beta$ -promoter reporter luciferase expression (rel. to *CMV-Renilla*) from 293T cells  
589 expressing vector or Flag-UFM1, followed by mock or SenV infection (18 h) or in B) WT  
590 or CRISPR/CAS9 UFM1 KO 293T cells transfected with vector (Vec) or Flag-UFM1 (for  
591 KO), followed by mock or SenV infection (18 h). C) ELISA for IFN- $\beta$  of supernatants  
592 harvested from WT or CRISPR/CAS9 UFM1 KO 293T cells that were SenV infected (18  
593 h). D) Diagram of UFM1 conjugation. E) Relative IFN- $\beta$ -promoter reporter luciferase  
594 expression (rel. to *CMV-Renilla*) from 293T cells expressing indicated constructs followed  
595 by mock or SenV infection (18 h), with results graphed as relative SenV fold change for  
596 each. Represented as mean  $\pm$  SEM, n=3 biological replicates. \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, and

597 \*\*\* $p \leq 0.001$  determined by two-way ANOVA followed by Tukey's multiple comparisons  
598 test (A-B), Student's t-test (C), or one-way ANOVA followed by Dunnett's multiple  
599 comparisons test (E).

600

601 **Figure 3. UFM1 is required for the RIG-I driven transcriptional response.**

602 RNA-seq analysis WT or UFM1 KO 293T cells following mock or SenV infection (18 h).  
603 A) Gene set enrichment analysis of negatively regulated differentially expressed genes in  
604 SenV-infected 293T cells represented by normalized enrichment score (UFM1 KO / WT).  
605 B) Volcano plot of differentially expressed genes (adj  $P < 0.01$ ) shown in grey, with ISGs  
606 shown in red, in SenV-infected 293T cells (UFM1 KO / WT). C) Heatmap of the effect of  
607 UFM1 KO on the fold change of the 50 most induced IFN and ISGs (UFM1 KO / WT)  
608 following SenV infection (adj  $P < 0.01$ ).

609

610 **Figure 4. UFL1 is recruited to intracellular membranes and interacts with 14-3-3 $\epsilon$**   
611 **and RIG-I during RNA virus infection.**

612 A) Immunoblot analysis of inputs and subcellular membrane flotation of 293T cell extracts  
613 that were mock or SenV-infected (4 h) followed by sucrose gradient fractionation, with  
614 fraction numbers indicated from the top of the gradient (1) to bottom (8). Fractionation  
615 controls, GAPDH for cytosol and Cox-I for membranes, are indicated and reveal that the  
616 membranes are localized to fraction #1. Relative quantification of the ratio of UFL1 to a  
617 membrane marker (Cox-I) in fraction 1 normalized to total protein levels in inputs are  
618 shown on the right. B) Immunoblot analysis of anti-Flag immunoprecipitated extracts and  
619 inputs from 293T cells expressing Myc-14-3-3 $\epsilon$  and Flag-UFL1 that were mock- or SenV-  
620 infected (4 h), with relative quantification on right. C) Immunoblot analysis of anti-Flag  
621 immunoprecipitated extracts and inputs from 293T cells expressing Myc-UFL1 and Flag-  
622 RIG-I that were mock- or SenV-infected (4 h), with relative quantification with IP values  
623 normalized to inputs values on right. D) Immunoblot analysis of anti-RIG-I  
624 immunoprecipitated (or anti-IgG) extracts and inputs from 293T cells that were mock- or  
625 SenV-infected (4 h), with relative quantification with IP values normalized to inputs values  
626 on right. E) Immunoblot analysis of anti-Flag immunoprecipitated extracts and inputs from  
627 293T cells expressing Myc-UFL1 and Flag-RIG-I constructs that were mock- or SenV-  
628 infected (4 h), with results quantified as relative fold change (SenV to Mock) for each. The  
629 graphs are represented as the mean  $\pm$  SEM,  $n=3$  (A-B, D-E) or  $n=4$  (C) biological

630 replicates and  $*p \leq 0.05$ ,  $**p \leq 0.01$ , and  $***p \leq 0.001$  determined by Student's t-test (A-D)  
631 or one-way ANOVA followed by Dunnett's multiple comparisons test (E).

632

633 **Figure 5. UFL1 interaction with RIG-I requires 14-3-3 $\epsilon$  and ufmylation.**

634 A) Immunoblot analysis of anti-HA immunoprecipitated extracts and inputs from 293T cells  
635 transfected with siCTRL or si14-3-3 $\epsilon$  followed by SenV infection (4h). B) Immunoblot of  
636 anti-HA immunoprecipitated extracts and inputs from 293T WT or UFM1 KO cells  
637 transfected with HA-RIG-I and Flag-UFL1. C) Immunoblot of anti-Flag immunoprecipitated  
638 extracts and inputs from 293T WT or UFM1 KO cells transfected with Flag-UFL1 and Myc-  
639 14-3-3 $\epsilon$ . In (A-C), SenV infection was for 4 hours, and relative quantification is shown on  
640 the right, indicating the mean  $\pm$  SEM (A, B),  $n=3$  (A, B) biological replicates. For (C)  
641 values shown are SD of IP values adjusted for input expression, with  $n=2$  biological  
642 replicates.  $*p \leq 0.05$ ,  $**p \leq 0.01$ , and  $***p \leq 0.001$  determined by Student's t-test.

643

644 **Figure 6. Ufmylation promotes RIG-I interaction with 14-3-3 $\epsilon$  for MAVS activation.**

645 A) Immunoblot of anti-Flag immunoprecipitated extracts and inputs from 293T cells  
646 transfected with siCTRL or siUFL1 and indicated constructs. B) Immunoblot of anti-Flag  
647 immunoprecipitated extracts and inputs from 293T WT or UFM1 KO cells. C) Immunoblot  
648 of anti-Myc immunoprecipitated extracts from 293T WT or UFM1 KO cells. D) 293T WT or  
649 UFM1 KO were mock or SenV-infected (12 h). Immunoblotting shows endogenous MAVS  
650 in input samples and MAVS aggregation from P5 fractions, in the presence or absence of  
651 denaturing reagent ( $\beta$ -mercaptoethanol). SenV infection was for 4 h (A-C) or 12 h (D). In  
652 (A-C), relative quantification of indicated protein in the IP is shown on the right; in (D) SDD-  
653 AGE MAVS values are normalized to corresponding SDS-PAGE values. Graphs show the  
654 mean  $\pm$  SEM for  $n=3$  biological replicates.  $*p \leq 0.05$ ,  $**p \leq 0.01$ , and  $***p \leq 0.001$   
655 determined by Student's t-test.

656

657 **Figure S1. The domains of UFL1 that form UFM1 conjugates in cells.**

658 Quantification of immunoblots from 293T cells expressing indicated Flag-UFL1  
659 constructs or vector followed by mock or SenV infection (18 h) represented as the ratio  
660 of UFM1 conjugates (approximately 25-50 kDa) to Flag-UFL1 expression in each lane

661 normalized to loading control with WT Flag-UFL1 mock set to 100. Graph indicates the  
662 mean +/- SEM for n=3 biological replicates.

663

664 **Fig. S2. Transcriptional response of genes negatively regulated by UFM1.**

665 RNA-seq analysis of WT versus UFM1 KO 293T cells showing the gene set enrichment  
666 analysis (top 10 categories) of negatively regulated differentially expressed genes  
667 represented by normalized enrichment score to identify gene ontology terms and  
668 pathways associated with altered gene expression for each of the comparisons  
669 performed (adj P<0.01).

670

671 **Dataset S1.** Differential expression analysis from RNA-seq analysis for UFM1 KO / WT  
672 293T cells

673 Table S1.1: UFM1 KO / WT Mock

674 Table S1.2 UFM1 KO / WT SenV (18 h)

675

676 **Dataset S2.** Gene Set Enrichment Analysis for UFM1 KO / WT 293T cells

677 Table S2.1: UFM1 KO / WT Mock- negative direction

678 Table S2.2 UFM1 KO / WT SenV (18 h)- negative direction

679 Table S2.3 UFM1 KO / WT Mock- positive direction

680 Table S2.4 UFM1 KO / WT SenV (18 h)- negative direction

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