1 # Self-stabilization mechanism encoded by a bacterial toxin facilitates reproductive
2 parasitism

3

4 Toshiyuki Harumoto[1,2]*

5

6 [1]Hakubi Center for Advanced Research, Kyoto University, Yoshida-honmachi, Sakyo-ku, Kyoto,
7 606-8501, Japan.

8 [2]Graduate School of Biostudies, Kyoto University, Yoshida-Konoe-cho, Sakyo-ku, Kyoto 606-8501,
9 Japan.

10

11 Lead Contact: Toshiyuki Harumoto (harumoto.toshiyuki.5c@kyoto-u.ac.jp)

12

13

SUMMARY

A wide variety of maternally transmitted endosymbionts in insects are associated with reproductive parasitism, whereby they interfere with host reproduction to increase the ratio of infected females and spread within populations[1,2]. Recent successes in identifying bacterial factors responsible for reproductive parasitism[3–7] as well as further omics approaches[8–12] have highlighted the common appearance of deubiquitinase domains, although their biological roles, in particular, how they link to distinct manipulative phenotypes remain poorly defined. *Spiroplasma poulsonii* is a helical and motile bacterial endosymbiont of *Drosophila*[13,14], which selectively kills male progeny with a male-killing toxin Spaid (*S. poulsonii* androcidin) that encodes an ovarian tumor (OTU) deubiquitinase domain[6]. Artificial expression of Spaid in flies reproduces male-killing-associated pathologies that include abnormal apoptosis and neural defects during embryogenesis[6,15–19]; moreover, it highly accumulates on the dosage-compensated male X chromosome[20], congruent with cellular defects like the DNA damage/chromatin bridge-breakage specifically induced upon that chromosome[6,21–23]. Here I show that without the function of OTU, Spaid is polyubiquitinated and degraded through the host ubiquitin-proteasome pathway, leading to the attenuation of male-killing activity as shown previously[6]. Furthermore, I find that Spaid utilizes its OTU domain to deubiquitinate itself in an intermolecular manner. Collectively, the deubiquitinase domain of Spaid serves as a self-stabilization mechanism to facilitate male killing in flies, optimizing a molecular strategy of endosymbionts that enables the efficient manipulation of the host at low energetic cost.

Keywords: symbiosis, reproductive parasitism, male killing, deubiquitinase, *Drosophila*, *Spiroplasma*

RESULTS

The OTU deubiquitinase domain predicted in Spaid

Full-length (FL) Spaid is a 1,065 amino acid (aa) protein. Conserved domain searches by InterPro[24] predicted two eukaryotic-like domains, the ankyrin repeat[25,26] and the OTU domain (spanning residues 279-465), in the Spaid aa sequence (Figure 1A). The OTU family proteins are cysteine proteases, whose catalytic Cys-His dyad constitutes the active site of the deubiquitinating enzyme that reverses ubiquitination, an essential post-translational modification in eukaryotes virtually affecting every aspect of proteins, such as stability, interactions, activity, and localization[27–29]. In host-microorganism interactions, protein ubiquitination is crucial as a host defense; therefore, invading pathogens employ deubiquitinase domain-containing proteins to counteract and exploit it[30–33]. OTU is one of seven deubiquitinase family members in eukaryotes; meanwhile, a plethora of viruses as well as a few pathogenic bacteria, including *Chlamydia* and *Legionella*, also utilize OTU domains for immune evasion[32,34–40]. The multiple alignment of the predicted OTU in Spaid with representative family members from both eukaryotes and bacteria revealed putative catalytic residues Cys-290 and His-458 (Figure S1A). To further scrutinized the above domain annotation, two distinct methods of protein structure modeling were applied. First, I utilized the Phyre2 homology modeling server[41], and found that the high-ranked 3D fold models (with confidence values of > 90%) are all generated using the OTU family proteins as templates (Data S1A). Next, I employed ColabFold software[42] whose structural prediction is powered by the AlphaFold2 program, a neural network model integrating physical and biological features of protein structure[43]. Structural comparison of the ColabFold model against the Protein Data Bank (PDB) by a DALI server search[44] successfully retrieved OTU family proteins as top hits (with Z scores of 6.2-14.1) (Data S1B). In the above two 3D models, Cys-290 and His-458 near the N- and C-terminal domain boundaries were in close vicinity to each other, which was very likely to constitute the catalytic Cys-His dyad (Figures S1B and S1C). These data, together with the following genetic/biochemical analyses, reinforce the idea that Spaid possesses deubiquitinase activity through its OTU domain.

To address the function of the Spaid OTU in male killing, a deletion construct lacking residues 264-465, encompassing the OTU domain, was generated previously (ΔOTU, Figure 1A)[6]. In this new study, two additional amino acid substitution constructs were produced by replacing presumed catalytic Cys-290 and non-catalytic Cys-358 with alanine, expecting to abolish deubiquitinase activity or to serve as a control, respectively (C290A and C358A, Figure 1A). I utilized these Spaid derivative constructs to confirm the deubiquitinase activity of Spaid and explore its exact role in male killing.

70

71 ***C290A mimics the phenotypes of the OTU deletion***

72 In previous work , WT and ΔOTU Spaid were expressed by the GAL4/UAS system in *D.*
73 *melanogaster* as GFP-tagged proteins to compare their male-killing activity and subcellular
74 localization patterns[6]. I reproduced the results as follows: i) strong expression of WT and ΔOTU by
75 the *actin-GAL4* driver eliminated male progeny regardless of the presence or absence of the OTU
76 domain (Figure S2A); ii) alternatively, when weakly expressed by the *armadillo-GAL4* driver, WT
77 Spaid still killed all males, but the ΔOTU construct could no longer kill them (Figure S2B). A re-
78 examination of the subcellular distribution of GFP-tagged WT and ΔOTU Spaid in larval salivary
79 gland cells reconfirmed their distribution in the cytoplasm and/or more likely in the intracellular
80 membrane system including the endoplasmic reticulum (ER), Golgi apparatus, and plasma
81 membrane irrespective of sexes (Figures S3A and S3B). As shown previously, a striking difference
82 was evident in the nuclear localization in cells, where the ΔOTU Spaid-GFP signals were reduced
83 (Figures S3A and S3B); additionally, the accumulation on the male X chromosome and resultant
84 DNA damage were almost eliminated by the deletion of the OTU domain (Figures S3B and S3C).

85 I then repeated the above experiments with the newly designed C358A and C290A constructs
86 and obtained comparable results: i) strong expression of both constructs eliminated male offspring
87 completely (Figure S2A), while weak expression of C358A but not C290A showed a substantial
88 male killing phenotype (Figure S2B), ii) the distribution patterns of C358A and C290A were almost
89 the same as those of WT and ΔOTU, respectively (Figures S3A-C). Thus, a single amino acid
90 mutation at Cys-290 closely mimics the phenotypes observed with the entire deletion of the OTU
91 domain, confirming Cys-290 as a key residue in the active site, which works as a cysteine-type
92 deubiquitinase. High-level expression of OTU deficient Spaid still induced male killing, suggesting
93 that the OTU deubiquitinase activity is not essential for the male-killing activity itself. Instead, it is
94 assumed to be involved in the nuclear translocation of the protein or the enhancement of protein
95 stability to facilitate male killing activity.

96

97 ***Loss of OTU function reduces Spaid protein levels***

98 During the initial characterization of the *spaid* locus, a spontaneously mutated *Spiroplasma* strain
99 with reduced male-killing ability was obtained[6]. Comparison of the genomic sequence with that of
100 the original strain revealed an 828-bp deletion, leading to a C-terminally truncated form of Spaid
101 (ΔC Spaid) lacking the hydrophobic region (HR) (Figure 1A)[6]. In the same manner as for FL Spaid,
102 WT, ΔOTU, C358A, and C290A derivatives of ΔC Spaid (Figure 1A) were constructed and

103 expressed with GAL4 drivers to obtain results similar to those with FL Spaid (Figures 1B and 1C),
104 indicating that the HR is dispensable for the male killing activity under the artificial expression
105 condition, although its loss in *Spiroplasma* reduces male-killing ability by potentially affecting
106 membrane targeting and/or extracellular secretion of Spaid within bacterial cells.
107 Next, I examined the subcellular localization pattern of ΔC Spaid in larval salivary gland cells
108 and found marked differences. Unlike FL Spaid, the GFP signals of WT and C358A ΔC Spaid were
109 predominantly detected within nuclei but were negligible in the intracellular membrane system
110 (Figures 2A and 2B). Most strikingly, the GFP signals of ΔOTU and C290A ΔC Spaid were
111 diminished in the cytoplasm, and the intranuclear signals were far more affected in both sexes
112 (Figure 2). The distinct distribution patterns of the FL and ΔC forms indicated that the HR likely
113 dispatches Spaid to the host intracellular membrane system when expressed in fly tissues. As
114 expected, WT/C358A forms of ΔC Spaid strongly accumulated on the male X chromosome and
115 induced DNA damage, similar to FL Spaid (Figure 2B). In contrast, the ΔOTU and C290A forms of
116 ΔC, but not FL Spaid induced DNA damage on the male X chromosome to some extent (compare
117 Figures 2B and 2C with S3B and S3C), probably due to the higher accumulation of ΔC Spaid inside
118 the nucleus, hence on the male X chromosome.
119 The above results strongly suggested that the OTU domain is neither involved in the DNA
120 damaging activity nor the nuclear translocation of Spaid, but rather is required for its stable
121 expression. In the following experiments, I tested this possibility by mainly using the ΔC forms of
122 Spaid, whose distribution patterns seemed to be more amenable to the analysis of protein stability.
123
124 ***The deubiquitinase activity of Spaid counteracts host proteasome-dependent degradation***
125 To assess whether the low abundance of OTU-deficient Spaid (ΔOTU/C290A) is related to the
126 ubiquitin-proteasome degradation pathway, ΔC Spaid-GFP was transiently expressed in *Drosophila*
127 S2 cells and the host proteasomal activity was blocked by a proteasome inhibitor MG-132. In the
128 absence of MG-132, the levels of GFP fluorescence of OTU-deficient constructs were significantly
129 lower than those of control constructs, reminiscent of the results in the larval salivary gland cells,
130 whereas they were substantially recovered after the treatment with MG-132 (Figures 3A and S4A).
131 Quantification of the relative GFP fluorescence revealed that it is elevated with MG-132 treatment in
132 general; however, the GFP fluorescence ratio between with/without MG-132 treatment was higher
133 for OTU-deficient constructs than for control constructs (Figures 3B and S4B), indicating that the
134 stability of OTU-deficient Spaid is much more sensitive to the host proteasomal activity. Closer
135 examination of the distribution pattern of GFP fluorescence confirmed the reduced signal of OTU-

136 deficient Spaid within cells, in particular in nuclei, while MG-132 treatment markedly improved the
137 fluorescence (Figure 3C).
138 Then how does Spaid counteract host proteasomal degradation with the help of its OTU
139 deubiquitinase activity? The simplest scenario is that Spaid can remove the attached ubiquitin
140 conjugation by itself (auto-deubiquitination). In that case, OTU-deficient Spaid, which is
141 polyubiquitinated and degraded in the absence of MG-132, would accumulate and be detected in the
142 presence of MG-132. To test this, ΔC Spaid-GFP was expressed and precipitated from whole cell
143 extracts with GFP-Trap magnetic beads, and bound proteins were isolated by highly stringent washes
144 (8M Urea, 1% SDS in PBS)[45,46]. The precipitated ΔC Spaid-GFP was analyzed for the ubiquitination
145 state using a ubiquitin-specific antibody for Western blot analysis. As expected, a high molecular
146 weight smear was detected only in the samples expressing OTU-deficient constructs treated with
147 MG-132, corresponding to the polyubiquitinated fraction of ΔC Spaid (Figures 3D and S4C). No
148 obvious polyubiquitinated signals were observed in OTU active control samples, biochemically
149 confirming the deubiquitinase activity of Spaid OTU. Besides, polyubiquitination of Spaid was
150 verified in FL Spaid-GFP as well (Figure S3D). These results together with the harsh conditions used
151 in the immunoprecipitation experiments supported the idea that Spaid is auto-deubiquitinated to
152 protect itself from proteolysis by the host proteosome.
153
154 ***Self-stabilization of Spaid through intermolecular deubiquitination***
155 Several mammalian deubiquitinases have been shown to deubiquitinate themselves to change their
156 own ubiquitination states and resultant fates[47–52]. More recently, a comprehensive library screening
157 identified a set of mammalian deubiquitinases that auto-deubiquitinate themselves either in an
158 intramolecular (within the protein) or intermolecular (between the proteins) manner to increase their
159 own stability[53]. To better understand the behavior of Spaid OTU deubiquitination and determine
160 which strategy is applicable, I designed co-transfection experiments with two differently tagged ΔC
161 Spaid constructs with/without deubiquitinase activity: C290A-GFP expressing cells were co-
162 transfected with either a C358A-HA or a C290A-HA construct. I first analyzed the expression level
163 of C290A-GFP and found that the GFP fluorescence was relatively higher in the co-transfection with
164 C358A-HA than with C290A-HA, even without the addition of MG-132 (Figures 4A and 4B),
165 implying that the proteolysis of C290A is blocked in the presence of C358A. Next, C290A-GFP was
166 precipitated from the respective whole cell extracts in denaturing conditions, and its
167 polyubiquitination state was analyzed. Remarkably, polyubiquitination of C290A-GFP disappeared
168 when the cells were co-transfected with C358A-HA, but was not affected by co-transfection with
169 C290A-HA (Figure 4C). These data support the notion that the auto-deubiquitination of Spaid occurs

170 in an intermolecular manner, although they did not necessarily exclude the possibility that some
171 intramolecular deubiquitination can happen.
172 If intermolecular auto-deubiquitination of Spaid occurs, a direct homomeric interaction of Spaid
173 would be expected. To test this, cells were co-transfected with ΔC Spaid-GFP and ΔC Spaid-HA,
174 and their direct interaction was analyzed by a co-immunoprecipitation assay. Cells co-transfected
175 with GFP and ΔC Spaid-HA served as a negative control. Western blot analyses of GFP-Trap
176 precipitates with an anti-HA antibody showed that the HA signal was detected from the ΔC Spaid-
177 GFP and ΔC Spaid-HA co-transfected co-immunoprecipitation, but not from the negative control,
178 demonstrating the homomeric interaction of Spaid *in vivo* (Figure 4D).
179 The two eukaryotic-like domains encoded in the N-terminal half of Spaid, ankyrin repeats and
180 the OTU domain, are involved in targeting Spaid to the male X chromosome[6] and protein self-
181 stabilization (this study), respectively. Therefore, the remaining C-terminal half without any
182 functional prediction could be involved in the homomeric interaction of Spaid described above. I
183 generated a construct lacking almost the entire sequence following the OTU domain (ΔC2 Spaid;
184 Figure 1A) and performed a co-immunoprecipitation assay. Similar to ΔC Spaid-GFP, ΔC2 Spaid-
185 GFP was also precipitated together with ΔC Spaid-HA (Figure 4E), thus excluding the possibility
186 that the C-terminal half of the protein is solely required for the homomeric interaction. Rather,
187 homomeric interfaces could be located within the N-terminal half containing the two eukaryotic-like
188 domains.
189

190 DISCUSSION

191 In this study, I have confirmed i) the male-killing toxicity of Spaid is attenuated but not eliminated
192 without a functional OTU; then shown that: ii) the OTU-deficient Spaid is unstable within host cells,
193 because it is polyubiquitinated and degraded through the host ubiquitin-proteasome system; iii) the
194 OTU domain works intermolecularly to deubiquitinate Spaid; and iv) Spaid monomers directly
195 interact to form homomers, though its C-terminal region is dispensable for the interaction. Taken
196 together, these data lead to a model in which the OTU deubiquitinase of Spaid serves as a self-
197 stabilization mechanism to facilitate male killing in flies (Figure 4F). In the artificial expression
198 experiments using the weak GAL4 driver (*armadillo-GAL4*), the OTU-deficient Spaid failed to kill
199 males (Figures 1C and S2B)[6]. Assuming that even the weak GAL4 driver would produce much more
200 Spaid than the endogenous bacteria within host cells, increasing protein stability through the OTU
201 deubiquitinase domain is likely indispensable for the efficient killing of male flies in the natural
202 symbiotic relationship. Given that the available resources are limited in endosymbiosis, the presence
203 of a self-stabilization mechanism would be fundamental for reproductive parasitism.

204 In the previous study[6], it was hypothesized that the OTU domain of Spaid is involved in nuclear
205 translocation, based on the localization analysis of FL Spaid and its deletion constructs. The present
206 study using ΔC Spaid and derivatives describes the deubiquitinase activity of OTU and its biological
207 role in evading the host ubiquitin-proteasome degradation, though other enzymatic targets except for
208 Spaid itself cannot be ruled out with the current data. In general, active proteasomes are ubiquitous
209 except in the luminal side of membranous organelles[54]. It is likely that OTU-deficient FL Spaid is
210 degraded by proteasomes within the nucleus and cytoplasm; however, the fraction targeted to the
211 intracellular membrane system with the help of HR is not exposed to active proteasomes for
212 degradation.

213 A number of bacterial deubiquitinases, like OTU, are distantly related members of eukaryotic
214 deubiquitinase families, although most of them belong to a distinct cysteine peptidase family of a
215 different origin, categorized as the clan CE[32,55,56]. One of the best characterized proteins containing a
216 CE peptidase domain in reproductive parasites is a *Wolbachia* factor responsible for cytoplasmic
217 incompatibility (CI) (killing of progeny from matings between uninfected females and infected
218 males)[1]. CI factors (Cifs) consist of a binary gene product of CifA and CifB, and the latter contains
219 the Ulp1 (ubiquitin-like-specific protease 1) domain whose deubiquitinase activity is biochemically
220 validated though its direct substrate for deubiquitination is still unknown[3–5,57]. A recent *in vivo*
221 expression study of CifB in *D. melanogaster* revealed that its protein level in spermatids declined
222 without the intrinsic deubiquitinase activity, whereas high-level expression is required for the
223 induction of sterility[58]. A new study of the same group reinforced the existence of a similar self-
224 stabilization function as demonstrated here for Spaid[59]. Very recently, a novel *Wolbachia* protein
225 designated Oscar was identified as a causative factor of male killing in Lepidoptera[7]. The authors
226 showed that the expression of Oscar kills males by directly targeting and degrading host
227 Masculinizer protein required for masculinization and dosage compensation[7,60,61]. Interestingly,
228 Oscar contains a CifB C-terminus-like domain, yet it does not seem to be essential for the male-
229 killing function of Oscar[7]. Although detailed biochemical validation is still awaited, these *Wolbachia*
230 proteins might commonly employ deubiquitinase domains to facilitate distinct reproductive
231 phenotypes, analogous to the Spaid OTU.

232 The present study raises further questions. First, the identity of the host pathways involved in the
233 ubiquitination/degradation of Spaid are unknown. Protein ubiquitination is catalyzed by a cascade of
234 E1, E2, and E3 enzymes, and E3s are responsible for the substrate recognition and specificity[27,62]. It
235 would be useful to further investigate whether a specific E3 ligase for Spaid is dispatched, or
236 whether other conventional E3s that can recognize analogous host protein sequence motifs are
237 applied for the process. In relation to this, the deubiquitinase properties of Spaid OTU, such as

substrate selectivity, cross-reactivity, and enzyme kinetics, should also be assessed by producing recombinant proteins. Second, the extent to which deubiquitinase-dependent stabilization mechanisms operate in other types of reproductive parasitism, including feminization (conversion of genetic males into fertile females) and parthenogenesis (enabling reproduction without mating with males), is unclear, because our knowledge about the molecular aspects of reproductive parasitism is still very limited. Third, the origin and evolution of these bacterial toxin/effector proteins is obscure. Multiple lines of evidence have suggested that the bacterial deubiquitinases were acquired from eukaryotic genomes via lateral gene transfer[9,32]. It is possible that the horizontally acquired deubiquitinase-containing locus was used as a scaffold for protein evolution[9]. Alternatively, the deubiquitinase domain could have been acquired afterwards as the result of an evolutionary arms race between hosts and bacterial endosymbionts. Further studies are needed to provide an overall picture of reproductive parasitism in arthropods, which would be facilitative in developing innovative approaches to manipulate and control arthropods that could play both beneficial and harmful roles in natural and agricultural settings.

ACKNOWLEDGMENTS

I thank the Bloomington *Drosophila* Stock Center in the USA and the Department of *Drosophila* Genomics and Genetic Resources at Kyoto Institute of Technology in Japan for fly stocks, and the Developmental Studies Hybridoma Bank at the University of Iowa in the USA for monoclonal antibodies. I also thank Catherine Regnard and Peter Becker for providing plasmid vectors and comments, Masayuki Miura for the cell stock, Makoto Hayashi for comments and suggestions on the work, James Alan Hejna for comments and English editing, Natsuya Oura and the members of Tadashi Uemura's laboratory for discussion and support. This work was supported by the Hakubi Project of Kyoto University, JST ERATO Grant Number JPMJER1902, Nagase Science and Technology Foundation, Japan Society for the Promotion of Science 22K19352.

AUTHOR CONTRIBUTIONS

TH conceived the study, performed the experiments, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

The author declares no competing interests.

INCLUSION AND DIVERSITY

271 I support inclusive, diverse, and equitable conduct of research.

272

273 MAIN FIGURE TITLES & LEGENDS

274 **Figure 1. OTU-deficiency attenuates the male-killing activity of ΔC Spaid.** (**A**) Schematic
275 representation of the protein structure of FL, ΔC, and ΔC2 Spaid. An N-terminal signal peptide (SP),
276 ankyrin repeats (ANK, red), the OTU deubiquitinase domain (OTU, blue; see also Figure S1 and
277 Data S1), and a C-terminal hydrophobic region (HR) are depicted as shown in the box. Asterisks
278 represent the positions of amino acid substitutions. The entire amino acid sequence of OTU and key
279 residues (catalytic Cys-290, His-458 and non-catalytic Cys-358) are also shown at the bottom.
280 Presumptive deubiquitinase (DUB) activity of each derivative is indicated by "+" (active) and "–"
281 (inactive). See text for other explanations. (**B, C**) *UAS-GFP* (negative control), *UAS-ΔC Spaid* (WT)
282 and its derivative lines (ΔOTU, C358A and C290A) were crossed with the *actin-GAL4* (**B**, strong
283 expression; n = 5 independent crosses) and *armadillo-GAL4* (**C**, weak expression; n = 7 independent
284 crosses) driver lines, respectively. The numbers of female (red) and male (blue) progeny obtained
285 from the crosses are shown. In **B**, offspring with both *GAL4* and *UAS* (+) or with only *UAS* (–) were
286 counted separately. Different letters (Steel–Dwass test, **B**) and asterisks (two-tailed Mann–Whitney
287 U test, **C**) represent statistically significant differences ($P < 0.05$). NS, not significant ($P > 0.05$) (see
288 also Table S1). Box plots indicate the median (bold line), upper and lower quartiles (box edges), and
289 the maximum/minimum values (whiskers). Dot plots show individual data points. The total counts
290 for each genotype and sex are shown at the bottom. See Figures S2 and S3 for the analysis of FL
291 Spaid.

292

293 **Figure 2. OTU-deficiency reduces the expression level of ΔC Spaid.** Female (**A**) and male (**B**)
294 larval salivary gland cells expressing ΔC Spaid-GFP (green). A recombined *actin-GAL4, tubulin-*
295 *GAL80^{ts}* driver line was used to circumvent male lethality (see STAR Methods for details). WT
296 (female, n = 11; male, n = 8), ΔOTU (female, n = 13; male, n = 8), C358A (female, n = 14; male, n =
297 9), and C290A (female, n = 12; male, n = 12) expressing cells were immunostained for DNA damage
298 (pH2Av, magenta) and histone H4 lysine 16 acetylation (H4K16ac, blue), a marker of the dosage-
299 compensated male X chromosome. Yellow arrows represent the accumulation of ΔC Spaid-GFP and
300 DNA damage on the male X chromosome with strong H4K16ac signals. (**C**) High-gain images of
301 ΔOTU and C290A reveal faint GFP signals on the male X chromosome. See Figures S3A-C for the
302 analysis of FL Spaid.

303

304 **Figure 3. The OTU deubiquitinase of Spaid stabilizes itself by counteracting the host ubiquitin-**
305 **proteasome pathway.** (**A**) S2 cells expressing ΔC Spaid-GFP. The raw GFP signals of C358A and
306 C290A derivatives are indicated. Cells were treated with DMSO (top) or proteasome inhibitor MG-
307 132 (bottom) for 9 h. (**B**) The relative GFP fluorescence of C358A and C290A derivatives of ΔC
308 Spaid-GFP expressed in S2 cells cultured without/with MG-132, respectively. The same data sets
309 used in (**A**) were analyzed (n = 6 areas per well in each condition). As a transfection marker, FLAG-
310 tagged mCherry (mCherry-FLAG) was co-transfected and its fluorescence was used to normalize the
311 GFP fluorescence (see STAR Methods). The median values and the ratios derived from GFP
312 fluorescence in the absence and presence of MG-132 are shown at the bottom. Different letters
313 (Steel–Dwass test) represent statistically significant differences ($P < 0.05$) (see also Table S1). (**C**)
314 High magnification images of S2 cells expressing ΔC Spaid-GFP derivatives (green, raw GFP
315 signals) treated with DMSO or MG-132. C358A (DMSO, n = 18; MG-132, n = 14) and C290A
316 (DMSO, n = 20; MG-132, n = 23) expressing cells were stained for DNA (magenta). (**D**)
317 Ubiquitination assay using GFP-Trap. C358A and C290A derivatives of ΔC Spaid-GFP were
318 immunoprecipitated (IP) from S2 cells cultured without/with MG-132. Immunoprecipitates were
319 washed with stringent buffer and analyzed by Western blotting to detect GFP (represents native ΔC
320 Spaid) and ubiquitin (Ub; a high-molecular-weight smear represents the polyubiquitinated fraction).
321 Input samples were probed with anti-FLAG antibody. mCherry-FLAG and non-specific bands
322 (“FLAG” and “*”) are shown as transfection and loading controls, respectively. See also Figures
323 S3D and S4.
324
325 **Figure 4. Self-stabilization of Spaid by intermolecular deubiquitination.** (**A**) S2 cells expressing
326 the C290A derivative of ΔC Spaid-GFP, co-transfected with a C290A or C358A derivative tagged
327 with HA, respectively. The raw GFP signals of C290A are indicated. Cells were treated with DMSO
328 (top) or MG-132 (bottom) for 9 h. (**B**) The relative GFP fluorescence of the C290A derivative of ΔC
329 Spaid-GFP, co-transfected with a C290A or C358A derivative tagged with HA in S2 cells cultured
330 without or with MG-132. The same data sets used in (**A**) were analyzed (n = 6 areas per well in each
331 condition). Other explanations are the same as in Figure 3B. Different letters (Steel–Dwass test)
332 represent statistically significant differences ($P < 0.05$) (see also Table S1). (**C**) Ubiquitination assay
333 using GFP-Trap. The same technique used in Figure 3D was applied. Input samples were
334 immunoblotted and probed with anti-HA and anti-β-Actin antibodies to show the transfection
335 efficiency and the loading control. (**D**) Homomeric interaction of ΔC Spaid in S2 cells. ΔC Spaid-
336 GFP was co-immunoprecipitated with ΔC Spaid-HA and analyzed by Western blotting to detect HA
337 and GFP. Free GFP was used as a negative control. Input samples were immunoblotted with anti-HA

338 and anti-β-Actin antibodies to show the transfection efficiency and the loading control. (**E**) Direct
339 interaction of ΔC and ΔC2 Spaid in S2 cells. ΔC2 Spaid-GFP was co-immunoprecipitated with ΔC
340 Spaid-HA. ΔC Spaid-GFP was used as a positive control. Other explanations are the same as in (**D**).
341 (**F**) A proposed model for the self-stabilization mechanism of Spaid to facilitate male killing in flies.
342 See text for details.
343
344
345 **STAR METHODS**
346 **RESOURCE AVAILABILITY**
347 **Lead contact:** Further information and requests for materials should be addressed to and will be
348 fulfilled by the lead contact, Toshiyuki Harumoto (harumoto.toshiyuki.5c@kyoto-u.ac.jp).
349
350 **Materials availability:** All new materials generated in this study will be made available from the
351 lead contact upon request.
352
353 **Data and code availability**
354 • All data reported in this paper will be shared by the lead contact upon request.
355 • This paper does not report original code.
356 • Any additional information required to reanalyze the data reported in this paper is available from
357 the lead contact upon request.
358
359 **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
360 **Fly stocks and husbandry**
361 The stocks of *D. melanogaster* were reared at 25 °C with standard cornmeal food. The absence of
362 *Spiroplasma* and *Wolbachia* were tested by a diagnostic PCR with specific primers (SpoulF 5'-GCT
363 TAA CTC CAG TTC GCC-3' and SpoulR 5'-CCT GTC TCA ATG TTA ACC TC-3' for
364 *Spiroplasma*; *wsp*_81F 5'-TGG TCC AAT AAG TGA TGA AGA AAC-3'and *wsp*_691R 5'-AAA
365 AAT TAA ACG CTA CTC CA-3' for *Wolbachia*)[63,64]. Some of them were treated with tetracycline
366 before the test[6]. The following lines were provided by the Bloomington *Drosophila* Stock Center at
367 Indiana University (BDSC) and the Department of *Drosophila* Genomics and Genetic Resources at
368 Kyoto Institute of Technology (DGGR): *actin-GAL4* (BDSC #4414), *tubulin-GAL80^{ts}* (BDSC
369 #7108), *armadillo-GAL4* (BDSC #1560), *UASp-EGFP* (DGGR #116071), and *CyO, ActGFP*
370 (DGGR #107783). *UASp-FL Spaid-EGFP* and *UASp-FL Spaid.ΔOTU-EGFP* transgenic lines were
371 generated in the previous study[6]. Other original *UAS* transgenic lines listed below were generated

372 using germline transformation by P-element based plasmid vectors (BestGene Inc.): *UASp-FL*
373 *Spaid.C290A-EGFP*, *UASp-FL Spaid.C358A-EGFP*, *UASp-ΔC Spaid-EGFP*, *UASp-ΔC*
374 *Spaid.ΔOTU-EGFP*, *UASp-ΔC Spaid.C290A-EGFP*, and *UASp-ΔC Spaid.C358A-EGFP*. For the
375 plasmid construction, please see below for details.
376 To artificially express *spaid* in flies by the GAL4/UAS system[65], homozygous *UAS-Spaid* transgenic
377 lines listed above were crossed with the *actin-GAL4*/*CyO* (for the strong expression in Figures 1B
378 and S2A) or *armadillo-GAL4* (for the weak expression in Figures 1C and S2B) driver lines.
379 Emerging adult flies were segregated by sexes and genotypes (in the crosses with *actin-GAL4*/*CyO*)
380 and counted separately until the 18-19th day in each vial. In the latter crosses with the *armadillo-*
381 *GAL4* driver line, *UAS-Spaid* female flies were mated with GAL4 driver male flies to avoid the
382 effect of maternally loaded GAL4[66], which caused substantial male death during embryonic stages.
383 In the former crosses with the *actin-GAL4* driver line, no obvious differences were observed in the
384 reciprocal mating, suggesting no/negligible maternal contribution in the experiments. For the
385 expression of *spaid* in larval salivary gland cells (Figures 2 and S3A-C), male lethality was
386 circumvented by using a recombined *actin-GAL4, tubulin-GAL80^{ts}*/*CyO* line. Crosses were
387 maintained at 20 °C for 6-7 days to let the larvae grow until the early 3rd instar stage, then were
388 shifted up to 29 °C and kept for 1 day before dissection at the wandering 3rd instar stage. Only GFP-
389 positive larvae were dissected.
390
391 **Cell line and maintenance**
392 *Drosophila* S2 cells (provided by Masayuki Miura) were maintained at 25 °C with Schneider's
393 *Drosophila* Medium (Gibco, 21720024) supplemented with Fetal Bovine Serum (Cytiva,
394 SH30071.03) and penicillin-streptomycin (FUJIFILM Wako Pure Chemical, 168-23191). For
395 plasmid DNA transfection, cells were seeded in multi-well plates (24-well plate with 500 µl medium
396 for imaging; 6-well plate with 2 mL medium for biochemical assays) at 1.0 x 10^6 cells/ml one day
397 before transfection. The cells were transfected with pMT plasmid vectors (encoding ΔC or FL Spaid
398 derivatives tagged with GFP and mCherry as a transfection control; see below for details) using
399 HilyMax transfection reagent (Dojindo, H357) following the manufacturer's protocol. A few hours
400 after transfection, the medium was changed to avoid toxicity. About 20 h after transfection, the
401 metallothionein promoter was induced by adding 1 mM $CuSO_4$ to the medium. About 20 h after the
402 induction, 20 µM MG-132 (Calbiochem, 474790) diluted in DMSO was added to inhibit the
403 proteasomal activity. As a negative control, DMSO was added. 9 h after the treatment, the cells were
404 observed or harvested for protein extraction.
405

METHOD DETAILS

Plasmid construction

The codon-optimized *ΔC spaid* sequence (2,367 bp) was synthesized and cloned into the pDONR221 vector by GeneArt service (Thermo Fisher Scientific) in the previous study[6]. The *FL spaid* sequence (3,195 bp) was generated and cloned into the pENTR vector as described previously[6]. To generate the ΔOTU deletion in ΔC Spaid, two PCR fragments (nucleotide positions 1-789 and 1,396-2,367)) were fused by PCR and cloned into the pENTR vector, using a pENTR/D-TOPO cloning kit (Thermo Fisher Scientific, K240020) as described previously[6]. The C290A and C358A single amino acid substitution constructs were generated by the SPRINP (Single-Primer Reactions IN Parallel) method[67]. Briefly, the template plasmid DNA (pENTR-FL Spaid and pDONR221-ΔC Spaid, respectively) was amplified with either the forward or reverse primer separately (C290A forward primer 5'-GGC TCC GCC CTG TTT TGG AGT GTG GCC-3', C290A reverse primer 5'-AAA CAG GGC GGA GCC ATC CTC GAC CAC-3'; C358A forward primer 5'-GCC AAC GCC CTG ATC CGC GAT ATC TTC-3', C358A reverse primer 5'-GAT CAG GGC GTT GGC GGT CTG ATC GCT-3; the underscores represent the nucleotide substitution introduced into the primers). The two single-primer PCR reactions were mixed and denatured, then cooled down gradually to reanneal the complementary strands. After purification with the Wizard SV Gel and PCR Clean-Up System (Promega, A9282), the template plasmid DNA was digested with DpnI and used in the transformation reaction.

UAS-Spaid plasmids generated for this study were as follows: pUASp-FL Spaid.C290A-EGFP; pUASp-FL Spaid.C358A-EGFP; pUASp-ΔC Spaid-EGFP; pUASp-ΔC Spaid.ΔOTU-EGFP; pUASp-ΔC Spaid.C290A-EGFP; pUASp-ΔC Spaid.C358A-EGFP; and pUASp-ΔC Spaid-3xHA. To construct these plasmids, the Gateway cassettes containing the *spaid* open reading frames (ORFs) in the pDONR221/pENTR vectors were transferred into the destination vectors pPWG (#1078; containing a C-terminal EGFP tag) and pPWH (#1102; containing a C-terminal 3xHA tag), obtained from the *Drosophila* Genomics Resource Center (DGRC) by the LR clonase II enzyme mix kit (Thermo Fisher Scientific, 11791020). *UAS-Spaid* transgenic lines used in the fly genetics experiments (Figures 1, 2, S2 and S3) were generated by microinjection of the above plasmids into *D. melanogaster* embryos (except for the pUASp-ΔC Spaid-3xHA plasmid used only for the vector construction below).

In the ubiquitination assay in S2 cells (Figures 3, 4A-4C, S3D, and S4), the following ΔC and FL Spaid plasmids were used: pMT-ΔC Spaid-EGFP; pMT-ΔC Spaid.ΔOTU-EGFP; pMT-ΔC Spaid.C290A-EGFP; pMT-ΔC Spaid.C358A-EGFP; pMT-ΔC Spaid.C290A-3xHA; pMT-ΔC Spaid.C358A-3xHA; pMT-FL Spaid.C290A-EGFP; and pMT-FL Spaid.C358A-EGFP. To construct

440 these plasmids, the Gateway cassettes containing *ΔC* and *FL spaid* fragments in the
441 pDONR221/pENTR vectors were transferred into the pMTWG destination vector containing the
442 metallothionein promoter and a C-terminal EGFP tag (a gift from Catherine Regnard and Peter
443 Becker). To replace the EGFP tag with the 3xHA tag, a PCR fragment containing a 969-bp sequence
444 of *ΔC spaid* (nucleotide position 1,399-2,367, containing an AgeI site) followed by the 3xHA
445 sequence was PCR amplified from the pUASp-ΔC Spaid-3xHA plasmid (forward primer 5'-AAC
446 ATC CGC ATG ATC AAC GAG-3', reverse primer 5'-CTA GCT AGC TTA GTG TCC GCC ATG
447 AGC AGC GTA ATC-3'; the underscore represents the NheI site added to the primer). Then, the
448 AgeI-NheI fragment (1,079 bp) was inserted into the pMT-ΔC Spaid.C290A/C358A-EGFP plasmid,
449 which had been digested with the corresponding restriction enzymes. To make a transfection control
450 plasmid (pMT-mCherry-FLAG), the ORF of mCherry was amplified with primers containing
451 restriction enzyme sites and the FLAG tag sequence (forward primer 5'-CCG GAT ATC CAA CAT
452 GGT GAG CAA GGG CGA GGA G-3', reverse primer 5'-CTA GCT AGC TTA CTT GTC ATC
453 GTC GTC CTT GTA ATC CTT GTA CAG CTC GTC CAT GC-3'; the underscores represent the
454 EcoRV and NheI sites added to the primers). The EcoRV-NheI fragment (743 bp) was inserted into
455 the corresponding sites of the pMTWG vector.
456 In the homomeric interaction assay in S2 cells (Figures 4D and 4E), the following ΔC and ΔC2
457 Spaid plasmids were used: pMT-ΔC Spaid-EGFP.3xFLAG; pMT-ΔC Spaid-3xHA; and pMT-ΔC2
458 Spaid-EGFP.3xFLAG. To construct the ΔC Spaid-EGFP.3xFLAG plasmid, the pMT-ΔC Spaid-
459 EGFP plasmid was digested with NcoI and NheI to swap the EGFP tag with a gBlocks gene
460 fragment (IDT) encoding the EGFP.3xFLAG tag sequence. The ΔC Spaid-3xHA plasmid was
461 generated by replacing the EcoRI-SacI fragment (1,473 bp) of the pMT-ΔC Spaid.C358A-3xHA
462 plasmid with the corresponding fragment from pMT-ΔC Spaid-EGFP. To construct the ΔC2 Spaid
463 (1,425 bp) plasmid, a PCR fragment containing a 525-bp portion of *ΔC2 spaid* (nucleotide position
464 901-1,425, containing an EcoRI site) was amplified from the pMT-ΔC Spaid-EGFP plasmid
465 (forward primer 5'-CTG CAA GTG CGC AAC AAT ATC-3', reverse primer 5'-CAC GAG CTC
466 ACC ACT TTG TAC AAG AAA GCT GGG TCA TTG ATC TCG TTG ATC ATG CG-3'; the
467 underscore represents the SacI site added to the primer). The EcoRI-SacI fragment (531 bp) was
468 inserted into the pMT-ΔC Spaid-EGFP.3xFLAG plasmid digested with corresponding restriction
469 enzymes. To make a control GFP plasmid (pMT-EGFP.3xFLAG), the pMT-ΔC Spaid-
470 EGFP.3xFLAG plasmid was digested with EcoRV and SacI. After blunting the SacI site by the
471 Quick Blunting Kit (NEB, E1201S), the digested plasmid was self-ligated.
472 PrimeSTAR Max DNA Polymerase (Takara Bio, R045A) and Mighty Mix DNA ligation kit
473 (Takara Bio, 6023) were used for PCR and DNA ligation reactions, respectively.

474

475 **Staining and imaging**

476 Salivary glands were dissected out from wandering third instar larvae and fixed in PBS with 4%
477 paraformaldehyde (EM Grade; Electron Microscopy Sciences, 15710) at room temperature for 20
478 min with gentle rocking. After washing with PBT (PBS containing 0.1% Triton X-100), tissues were
479 treated with a blocking buffer [PBT containing 1% bovine serum albumin (BSA, heat shock fraction;
480 Sigma-Aldrich, A7906)] for 30 min, and incubated with primary antibodies at 4 °C overnight,
481 washed three times in PBT, and incubated with secondary antibodies at room temperature for 90
482 min, then washed three times in PBT. Antibodies were diluted in the blocking buffer. Transfected S2
483 cells were diluted 6-fold and seeded in a 2 well chamber slide (Nunc Lab-Tek, 177380) coated with
484 concanavalin A (0.5 mg/ml) and incubated at room temperature for 30 min. After fixation at room
485 temperature for 30 min, cells were permeabilized with PBT for 15 min, stained for DNA, and
486 washed once in PBS. For GFP, raw fluorescent signals without antibody staining were detected.

487 The following primary antibodies were used: rabbit anti-acetyl-Histone H4 (Lys16) (H4K16ac,
488 1:2,000; Upstate, 07-329); mouse anti-γ-H2Av UNC93-5.2.1 (pH2Av, 1:500; Developmental Studies
489 Hybridoma Bank). The following secondary antibodies were used at a 1:2,000 dilution: donkey anti-
490 mouse IgG Alexa Fluor Plus 555 conjugate (Thermo Fisher Scientific, A32773), donkey anti-rabbit
491 IgG Alexa Fluor Plus 647 conjugate (Thermo Fisher Scientific, A32795). DNA staining was carried
492 out with DAPI (0.5 μg/ml; Nacalai tesque, 19178-91) together with secondary antibody staining.

493 Stained salivary glands and S2 cells were mounted in ProLong Glass Antifade Mountant
494 (Thermo Fisher Scientific, P36980) and observed under a FV3000 confocal laser scanning
495 microscope (Evident). In Figures 2 and S3A-C, images of salivary glands were acquired using a 40x
496 oil immersion objective (UPLXAPO40XO, NA 1.4) with 2x zoom scan (1,024 x 1,024 frame size;
497 about 20-30 sections in 0.42 μm optimal intervals). In Figure 3C, images of S2 cells were acquired
498 using a 100x oil immersion objective (UPLXAPO100XO, NA 1.45) with 1x zoom scan (1,024 x 685
499 frame size; about 10-20 sections in 0.4 μm intervals). In Figures 3A, 4A, and S4A, transfected S2
500 cells cultured in plastic multi-well plates were directly observed under the FV3000 confocal
501 microscope with a 10x objective (UPLXAPO10X, NA 0.4). Images were acquired at six areas per
502 well (800 x 800 frame size; 11 sections in 6.0 μm intervals) for quantitative analysis.

503

504 **Biochemical assays**

505 For the ubiquitination assay, transfected S2 cells were pelleted at 1,000 g and rinsed twice with ice-
506 cold PBS. The cells were lysed in 200 μl RIPA buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1%
507 NP-40 substitute, 0.5% sodium deoxycholate, 0.1% SDS; Nacalai tesque, 16488-34) containing 1

mM EDTA, 5 mM N-ethylmaleimide (NEM, Nacalai tesque, 15512-24), 40 µM PR-619 (LifeSensors, SI9619), 1x cOmplete ULTRA (Roche, 5892791001), and 1 mM Pefabloc SC (Roche, 11429868001). After a 15 min incubation on ice with periodic mixing, the samples were pulse sonicated, then incubated on ice for another 15 min. Cell lysates were cleared by centrifugation at 13,000 g at 4 °C for 10 min and the supernatant was collected and stored at -80 °C until use. Protein concentration was quantified using the BCA protein assay kit (Takara Bio, T9300A). Cell lysates (about 200 µl) were diluted 2-fold with dilution buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) supplemented with 5 mM NEM, 40 µM PR-619, 1x cOmplete ULTRA, and 1 mM Pefabloc SC. For immunoprecipitation, 6 µl GFP-Trap magnetic agarose (ChromoTek, gtmak-20) pre-equilibrated with dilution buffer was added to the diluted lysates, then gently rotated at 4 °C for 2 h. Magnetically separated beads were washed once with dilution buffer, three times with stringent washing buffer (8M Urea, 1% SDS in PBS, prepared just before use), and once with 1% SDS in PBS[45,46]. Bound proteins were eluted with 2x sample buffer (Bio-Rad, 1610747) containing 0.1M TCEP-HCl (Nacalai tesque, 07277-61), incubated at 70 °C for 30 min, then resolved by SDS-PAGE on 4-15% precast gradient gels (Mini-PROTEAN TGX Gels, Bio-Rad, 4561084) in Tris/Glycine/SDS buffer (Bio-Rad, 1610732) for 30 min with 200V constant voltage. Proteins were transferred to 0.2 µm PVDF membranes (Trans-Blot Turbo Transfer Pack, Bio-Rad, 1704156) using the "High MW" setting of the Trans-Blot Turbo Transfer System (Bio-Rad). Blotted membranes were rinsed briefly with TBS-T [TBS (20 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 0.1% Tween 20], treated with a blocking buffer (TBS-T containing 1% BSA) at room temperature for 30 min, or Bullet Blocking One (Nacalai tesque, 13779-14) at room temperature for 5 min, and incubated with primary antibodies at 4 °C overnight; the membranes were then washed three times in TBS-T and incubated with peroxidase-conjugated secondary antibodies at room temperature for 1 h. Primary and secondary antibodies were diluted in the blocking buffer. For the analysis of FL Spaid (Figure S3D), primary and secondary antibodies were diluted in Signal Enhancer HIKARI Solution A and B, respectively (Nacalai tesque, 02267-41). After washing three times with TBS-T, the membranes were incubated with ECL Prime Western Blotting Detection Reagent (Amersham, RPN2232) at room temperature for 5 min, and chemiluminescent signals were detected with a ChemiDoc Imaging System (Bio-Rad).

For the co-immunoprecipitation assay, cell lysates were prepared as indicated above except that cells were lysed with RIPA buffer without SDS (Nacalai tesque, 08714-04) and lysates were passed through a 27G needle 10 times; the volume was then adjusted to 400 µl with dilution buffer prior to immunoprecipitation using GFP-Trap magnetic agarose beads. After protein binding, the beads were washed three times with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton X-100,

0.5 mM EDTA) containing 1x cOmplete ULTRA and 1 mM Pefabloc SC, and bound proteins were analyzed by Western blotting.

The following antibodies were used: mouse anti-Ubiquitin P4D1 (1/1,000; Santa Cruz, sc-8017); rabbit anti-GFP (1/10,000; Invitrogen, A-11122); mouse anti-FLAG M2 (1/10,000; Sigma-Aldrich, F1804); rat anti-HA 3F10 (1/5,000; Roche, 11867423001); mouse anti-β-Actin C4 (1/5,000; Santa Cruz, sc-47778). The following peroxidase-conjugated secondary antibodies were used (1/10,000; purchased from Jackson ImmunoResearch): donkey anti-mouse IgG (715-035-150); donkey anti-rabbit IgG (711-035-152); donkey anti-rat IgG (712-035-153).

Protein domain search, multiple alignment, and 3D modeling

The amino acid sequence of FL Spaid was analyzed by the InterPro website[24] with default parameters. The following three predictions associated with OTU/cysteine protease were obtained: SSF54001 ("Cysteine proteinases", amino acids 264-461, e-value: 1.18E-15), PF02338 ("OTU-like cysteine protease", amino acids 344-438, e-value: 6.50E-06), and PS50802 ("OTU domain profile", amino acids 279-465, score: 13.786528). In this paper, the last prediction was adopted according to the results of the OTU domain alignment shown in Figure S1A.

For the multiple alignment of the OTU domain sequence, Jalview v2.11.2.4 was used. I also referred to the alignment presented in ref. 32. Protein sequences of OTU family proteins from *Homo sapiens* (Hs), *Drosophila melanogaster* (Dm), *Chlamydia pneumoniae* (Cp), *Legionella pneumophila* (Lp; only the N-terminal OTU domain sequence was used) were obtained from UniProtKB (accession numbers in parentheses): OTU1_Hs (Q5VVQ6); OTUB1_Hs (Q96FW1); OTUB2_Hs (Q96DC9); OTUD1_Hs (Q5VV17); OTUD3_Hs (Q5T2D3); OTUD4_Hs (Q01804); OTUD5_Hs (Q96G74); OTU6A_Hs (Q7L8S5); OTU6B_Hs (Q8N6M0); OTU7A_Hs (Q8TE49); OTU7B_Hs (Q6GQQ9); TNAP3_Hs (P21580); VCIP1_Hs (Q96JH7); ZRAN1_Hs (Q9UGI0; a.k.a. TRABID); ALG13_Hs (Q9NP73); OTULIN_Hs (Q96BN8); OTU_Dm (P10383); YOD1_Dm (Q9VRJ9); TRBID_Dm (Q9VH90); ChlaOTU_Cp (Q9Z868); LotA_Lp (Q5ZTB4).

3D models of the OTU domain of Spaid (279-465 aa) were generated by the Phyre2 server using the intensive mode[41] (Figure S1B and Data S1A) and ColabFold software[42] powered by the AlphaFold2 program[43] (Figure S1C), respectively. Structural comparison of the ColabFold model was performed by the DALI server[44] (Data S1B). Default parameters were utilized in all programs. Obtained 3D models were visualized by PyMOL v2.5.0 (Schrödinger).

QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis and processing

576 Two confocal z-sections of salivary gland cells were selected and max-projected in Figures 2 and
577 S3A-C. For the images of S2 cells, all confocal z-sections were max-projected in Figures 3A, 4A,
578 and S4A, or single z-section was selected and indicated in Figure 3C. The brightness and contrast of
579 the presented images were adjusted by Fiji software (Fiji Is Just ImageJ)[68]. The adjustment was
580 performed uniformly on the entire images.
581 For the relative quantification of GFP signals of transfected S2 cells (Figures 3B, 4B, and S4B),
582 all z-sections were max-projected by a custom macro with Fiji software. Image analysis was
583 performed by custom R scripts with the EBImage package[69]. The maximum projection images of
584 GFP and mCherry were smoothed by a Gaussian filter and binarized by the moving average method.
585 GFP and mCherry signals were measured by summation, and the value of GFP was divided by that
586 of mCherry to calculate the relative fluorescence of GFP.
587
588 **Statistical analysis**
589 R software v4.0.3 (the R Foundation) was used for all statistical analyses. Multiple comparisons in
590 Figures 1B, 3B, 4B, S2A and S4B were performed using the Steel-Dwass test by the pSDCFlig
591 function in the NSM3 R package[70]. The Mann-Whitney U test (two-tailed) was used in Figures 1C
592 and S2B. *P*-values less than 0.05 were considered as significant. Exact *P*-values are listed in Table
593 S1. Sample sizes are indicated in the corresponding figure legends.
594
595
596 **SUPPLEMENTAL DATA TITLE & LEGEND**
597 **Data S1. The results of the 3D structural modeling of Spaid OTU, related to Figures 1 and S1.**
598 (**A**) The output of the Phyre2 server search. The top 40 templates used for the 3D model prediction
599 of the Spaid OTU sequence (279-465 aa) are listed. Confidence of the modeling (100% maximum,
600 shown in a red-white scale), sequence identity (% i.d.) between Spaid OTU, Protein Data Bank
601 (PDB) IDs and associated information, names of molecules, and source organisms are indicated.
602 vOTU, viral OTU. The Phyre2 server selected six templates (colored in yellow) to generate the final
603 3D model indicated in Figure S1B. (**B**) The output of the DALI server search. The top 20 proteins
604 with high structural similarities between the ColabFold model of Spaid OTU (Figure S1C) are listed.
605 Z scores (optimized similarity score, shown in the red-white scale), RMSD (root-mean-square
606 deviation), sequence identity (% i.d.) between Spaid OTU, PDB IDs and associated titles, names of
607 molecules, and source organisms are indicated.
608

609 **REFERENCES**

610 1. Werren, J.H., Baldo, L., and Clark, M.E. (2008). *Wolbachia*: master manipulators of invertebrate
611 biology. Nat. Rev. Microbiol. *6*, 741–751. 10.1038/nrmicro1969.

612 2. Hurst, G.D.D., and Frost, C.L. (2015). Reproductive parasitism: maternally inherited symbionts
613 in a biparental world. Cold Spring Harb. Perspect. Biol. *7*, a017699.
614 10.1101/cshperspect.a017699.

615 3. Beckmann, J.F., and Fallon, A.M. (2013). Detection of the *Wolbachia* protein WPIP0282 in
616 mosquito spermathecae: implications for cytoplasmic incompatibility. Insect Biochem. Mol.
617 Biol. *43*, 867–878. 10.1016/j.ibmb.2013.07.002.

618 4. Beckmann, J.F., Ronau, J.A., and Hochstrasser, M. (2017). A *Wolbachia* deubiquitylating
619 enzyme induces cytoplasmic incompatibility. Nat. Microbiol. *2*, 17007.
620 10.1038/nmicrobiol.2017.7.

621 5. LePage, D.P., Metcalf, J.A., Bordenstein, S.R., On, J., Perlmutter, J.I., Shropshire, J.D., Layton,
622 E.M., Funkhouser-Jones, L.J., Beckmann, J.F., and Bordenstein, S.R. (2017). Prophage WO
623 genes recapitulate and enhance *Wolbachia*-induced cytoplasmic incompatibility. Nature *543*,
624 243–247. 10.1038/nature21391.

625 6. Harumoto, T., and Lemaitre, B. (2018). Male-killing toxin in a bacterial symbiont of *Drosophila*.
626 Nature *557*, 252–255. 10.1038/s41586-018-0086-2.

627 7. Katsuma, S., Hirota, K., Matsuda-Imai, N., Fukui, T., Muro, T., Nishino, K., Kosako, H., Shoji,
628 K., Takanashi, H., Fujii, T., et al. (2022). A *Wolbachia* factor for male killing in lepidopteran
629 insects. Nat. Commun. *13*, 6764. 10.1038/s41467-022-34488-y.

630 8. Bordenstein, S.R., and Bordenstein, S.R. (2016). Eukaryotic association module in phage WO
631 genomes from *Wolbachia*. Nat. Commun. *7*, 13155. 10.1038/ncomms13155.

632 9. Gillespie, J.J., Driscoll, T.P., Verhoeve, V.I., Rahman, M.S., Macaluso, K.R., and Azad, A.F.
633 (2018). A tangled web: origins of reproductive parasitism. Genome Biol. Evol. *10*, 2292–2309.
634 10.1093/gbe/evy159.

635 10. Schubert, A.F., Nguyen, J.V., Franklin, T.G., Geurink, P.P., Roberts, C.G., Sanderson, D.J.,
636 Miller, L.N., Ovaa, H., Hofmann, K., Pruneda, J.N., et al. (2020). Identification and
637 characterization of diverse OTU deubiquitinases in bacteria. EMBO J. *39*, e105127.
638 10.15252/embj.2020105127.

639 11. Gerth, M., Martinez-Montoya, H., Ramirez, P., Masson, F., Griffin, J.S., Aramayo, R., Siozios,
640 S., Lemaitre, B., Mateos, M., and Hurst, G.D.D. (2021). Rapid molecular evolution of
641 *Spiroplasma* symbionts of *Drosophila*. Microb. Genom. *7*. 10.1099/mgen.0.000503.

642 12. Massey, J.H., and Newton, I.L.G. (2022). Diversity and function of arthropod endosymbiont
643 toxins. Trends Microbiol. *30*, 185–198. 10.1016/j.tim.2021.06.008.

644 13. Malogolowkin, C., and Poulson, D.F. (1957). Infective transfer of maternally inherited abnormal
645 sex-ratio in *Drosophila willistoni*. Science *126*, 32.

646 14. Poulson, D.F. (1963). Cytoplasmic inheritance and hereditary infections in *Drosophila*. In
647 Methodology in Basic Genetics (Burdette W.J. ed.) Appendix III (Holden-Day), pp. 404–424.

648 15. Counce, S.J., and Poulson, D.F. (1962). Developmental effects of the sex-ratio agent in embryos
649 of *Drosophila willistoni*. J. Exp. Zool. *151*, 17–31. 10.1002/jez.1401510103.

650 16. Tsuchiyama-Omura, S., Sakaguchi, B., Koga, K., and Poulson, D.F. (1988). Morphological
651 features of embryogenesis in *Drosophila melanogaster* infected with a male-killing *Spiroplasma*.
652 Zoolog. Sci. *5*, 375–383.

653 17. Bentley, J.K., Veneti, Z., Heraty, J., and Hurst, G.D.D. (2007). The pathology of embryo death
654 caused by the male-killing *Spiroplasma* bacterium in *Drosophila nebulosa*. BMC Biol. *5*, 9.
655 10.1186/1741-7007-5-9.

656 18. Martin, J., Chong, T., and Ferree, P.M. (2013). Male killing *Spiroplasma* preferentially disrupts
657 neural development in the *Drosophila melanogaster* embryo. PLoS ONE *8*, e79368.
658 10.1371/journal.pone.0079368.

659 19. Harumoto, T., Anbutsu, H., and Fukatsu, T. (2014). Male-killing *Spiroplasma* induces sex-
660 specific cell death via host apoptotic pathway. PLoS Pathog. *10*, e1003956.
661 10.1371/journal.ppat.1003956.

662 20. Lucchesi, J.C., and Kuroda, M.I. (2015). Dosage compensation in *Drosophila*. Cold Spring Harb.
663 Perspect. Biol. *7*, a019398. 10.1101/cshperspect.a019398.

664 21. Veneti, Z., Bentley, J.K., Koana, T., Braig, H.R., and Hurst, G.D.D. (2005). A functional dosage
665 compensation complex required for male killing in *Drosophila*. Science *307*, 1461–1463.
666 10.1126/science.1107182.

667 22. Cheng, B., Kuppanda, N., Aldrich, J.C., Akbari, O.S., and Ferree, P.M. (2016). Male-killing
668 *Spiroplasma* alters behavior of the dosage compensation complex during *Drosophila*
669 *melanogaster* embryogenesis. Curr. Biol. *26*, 1339–1345. 10.1016/j.cub.2016.03.050.

670 23. Harumoto, T., Anbutsu, H., Lemaitre, B., and Fukatsu, T. (2016). Male-killing symbiont
671 damages host's dosage-compensated sex chromosome to induce embryonic apoptosis. Nat.
672 Commun. *7*, 12781. 10.1038/ncomms12781.

673 24. Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G.,
674 Paysan-Lafosse, T., Qureshi, M., Raj, S., et al. (2021). The InterPro protein families and domains
675 database: 20 years on. Nucleic Acids Res. *49*, D344–D354. 10.1093/nar/gkaa977.

676 25. Al-Khodor, S., Price, C.T., Kalia, A., and Abu Kwaik, Y. (2010). Functional diversity of ankyrin
677 repeats in microbial proteins. Trends Microbiol. *18*, 132–139. 10.1016/j.tim.2009.11.004.

678 26. Jernigan, K.K., and Bordenstein, S.R. (2014). Ankyrin domains across the Tree of Life. PeerJ *2*,
679 e264. 10.7717/peerj.264.

680 27. Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–
681 479. 10.1146/annurev.biochem.67.1.425.

682 28. Reyes-Turcu, F.E., Ventii, K.H., and Wilkinson, K.D. (2009). Regulation and cellular roles of
683 ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. *78*, 363–397.
684 10.1146/annurev.biochem.78.082307.091526.

685 29. Komander, D., and Rape, M. (2012). The ubiquitin code. Annu. Rev. Biochem. *81*, 203–229.
686 10.1146/annurev-biochem-060310-170328.

687 30. Randow, F., and Lehner, P.J. (2009). Viral avoidance and exploitation of the ubiquitin system.
688 Nat. Cell Biol. *11*, 527–534. 10.1038/ncb0509-527.

689 31. Lin, Y.-H., and Machner, M.P. (2017). Exploitation of the host cell ubiquitin machinery by
690 microbial effector proteins. J. Cell Sci. *130*, 1985–1996. 10.1242/jcs.188482.

691 32. Hermanns, T., and Hofmann, K. (2019). Bacterial DUBs: deubiquitination beyond the seven
692 classes. Biochem. Soc. Trans. *47*, 1857–1866. 10.1042/BST20190526.

693 33. Tripathi-Giesgen, I., Behrends, C., and Alpi, A.F. (2021). The ubiquitin ligation machinery in the
694 defense against bacterial pathogens. EMBO Rep. *22*, e52864. 10.15252/embr.202152864.

695 34. Makarova, K.S., Aravind, L., and Koonin, E.V. (2000). A novel superfamily of predicted
696 cysteine proteases from eukaryotes, viruses and *Chlamydia pneumoniae*. Trends Biochem. Sci.
697 *25*, 50–52.

698 35. Bailey-Elkin, B.A., van Kasteren, P.B., Snijder, E.J., Kikkert, M., and Mark, B.L. (2014). Viral
699 OTU deubiquitinases: a structural and functional comparison. PLoS Pathog. *10*, e1003894.
700 10.1371/journal.ppat.1003894.

701 36. Furtado, A.R., Essid, M., Perrinet, S., Balañá, M.E., Yoder, N., Dehoux, P., and Subtil, A.
702 (2013). The chlamydial OTU domain-containing protein *Chla*OTU is an early type III secretion
703 effector targeting ubiquitin and NDP52. Cell. Microbiol. *15*, 2064–2079. 10.1111/cmi.12171.

704 37. Kubori, T., Kitao, T., Ando, H., and Nagai, H. (2018). LotA, a *Legionella* deubiquitinase, has
705 dual catalytic activity and contributes to intracellular growth. Cell. Microbiol. *20*, e12840.
706 10.1111/cmi.12840.

707 38. Ma, K., Zhen, X., Zhou, B., Gan, N., Cao, Y., Fan, C., Ouyang, S., Luo, Z.-Q., and Qiu, J.
708 (2020). The bacterial deubiquitinase Ceg23 regulates the association of Lys-63-linked
709 polyubiquitin molecules on the *Legionella* phagosome. J. Biol. Chem. *295*, 1646–1657.
710 10.1074/jbc.RA119.011758.

711 39. Liu, S., Luo, J., Zhen, X., Qiu, J., Ouyang, S., and Luo, Z.-Q. (2020). Interplay between bacterial
712 deubiquitinase and ubiquitin E3 ligase regulates ubiquitin dynamics on *Legionella* phagosomes.
713 Elife *9*, e58114. 10.7554/eLife.58114.

714 40. Shin, D., Bhattacharya, A., Cheng, Y.-L., Alonso, M.C., Mehdipour, A.R., van der Heden van
715 Noort, G.J., Ovaa, H., Hummer, G., and Dikic, I. (2020). Bacterial OTU deubiquitinases regulate
716 substrate ubiquitination upon *Legionella* infection. Elife *9*, e58277. 10.7554/eLife.58277.

717 41. Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., and Sternberg, M.J.E. (2015). The Phyre2
718 web portal for protein modeling, prediction and analysis. Nat. Protoc. *10*, 845–858.
719 10.1038/nprot.2015.053.

720 42. Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S., and Steinegger, M. (2022).
721 ColabFold: making protein folding accessible to all. Nat. Methods *19*, 679–682. 10.1038/s41592-
722 022-01488-1.

723 43. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool,
724 K., Bates, R., Žídek, A., Potapenko, A., et al. (2021). Highly accurate protein structure prediction
725 with AlphaFold. Nature *596*, 583–589. 10.1038/s41586-021-03819-2.

726 44. Holm, L. (2022). Dali server: structural unification of protein families. Nucleic Acids Res.,
727 gkac387. 10.1093/nar/gkac387.

728 45. Mayor, U., and Lindon, C. (2014). One step purification to test ubiquitination of GFP-tagged
729 proteins. In Application Note; Ubiquitination of GFP-tagged proteins (ChromoTek).

730 46. Lee, S.Y., Ramirez, J., Franco, M., Lectez, B., Gonzalez, M., Barrio, R., and Mayor, U. (2014).
731 Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates
732 protein homeostasis through the proteasomal shuttle Rpn10. Cell. Mol. Life Sci. *71*, 2747–2758.
733 10.1007/s00018-013-1526-7.

734 47. Shen, C., Ye, Y., Robertson, S.E., Lau, A.W., Mak, D.-O.D., and Chou, M.M. (2005).
735 Calcium/calmodulin regulates ubiquitination of the ubiquitin-specific protease TRE17/USP6. J.
736 Biol. Chem. *280*, 35967–35973. 10.1074/jbc.M505220200.

737 48. Wada, K., and Kamitani, T. (2006). UnpEL/Usp4 is ubiquitinated by Ro52 and deubiquitinated
738 by itself. Biochem. Biophys. Res. Commun. *342*, 253–258. 10.1016/j.bbrc.2006.01.144.

739 49. Denuc, A., Bosch-Comas, A., Gonzàlez-Duarte, R., and Marfany, G. (2009). The UBA-UIM
740 domains of the USP25 regulate the enzyme ubiquitination state and modulate substrate
741 recognition. PLoS One *4*, e5571. 10.1371/journal.pone.0005571.

742 50. Huang, X., Summers, M.K., Pham, V., Lill, J.R., Liu, J., Lee, G., Kirkpatrick, D.S., Jackson,
743 P.K., Fang, G., and Dixit, V.M. (2011). Deubiquitinase USP37 is activated by CDK2 to
744 antagonize APC(CDH1) and promote S phase entry. Mol. Cell *42*, 511–523.
745 10.1016/j.molcel.2011.03.027.

746 51. Mashtalir, N., Daou, S., Barbour, H., Sen, N.N., Gagnon, J., Hammond-Martel, I., Dar, H.H.,
747 Therrien, M., and Affar, E.B. (2014). Autodeubiquitination protects the tumor suppressor BAP1
748 from cytoplasmic sequestration mediated by the atypical ubiquitin ligase UBE2O. Mol. Cell *54*,
749 392–406. 10.1016/j.molcel.2014.03.002.

750 52. Wijnhoven, P., Konietzny, R., Blackford, A.N., Travers, J., Kessler, B.M., Nishi, R., and
751 Jackson, S.P. (2015). USP4 auto-deubiquitylation promotes homologous recombination. Mol.
752 Cell *60*, 362–373. 10.1016/j.molcel.2015.09.019.

753 53. Hou, Z., Shi, W., Feng, J., Wang, W., Zheng, E., Lin, H., Yu, C., and Li, L. (2021). Self-
754 stabilizing regulation of deubiquitinating enzymes in an enzymatic activity-dependent manner.
755 Int. J. Biol. Macromol. *181*, 1081–1091. 10.1016/j.ijbiomac.2021.04.073.

756 54. Wójcik, C., and DeMartino, G.N. (2003). Intracellular localization of proteasomes. Int. J.
757 Biochem. Cell Biol. *35*, 579–589. 10.1016/s1357-2725(02)00380-1.

758 55. Barrett, A.J., and Rawlings, N.D. (1996). Families and clans of cysteine peptidases. Perspect.
759 Drug Discov. Des. *6*, 1–11. 10.1007/BF02174042.

760 56. Rawlings, N.D., Barrett, A.J., Thomas, P.D., Huang, X., Bateman, A., and Finn, R.D. (2018).
761 The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a
762 comparison with peptidases in the PANTHER database. Nucleic Acids Res. *46*, D624–D632.
763 10.1093/nar/gkx1134.

764 57. Beckmann, J.F., Van Vaerenberghe, K., Akwa, D.E., and Cooper, B.S. (2021). A single mutation
765 weakens symbiont-induced reproductive manipulation through reductions in deubiquitylation
766 efficiency. Proc. Natl. Acad. Sci. U. S. A. *118*, e2113271118. 10.1073/pnas.2113271118.

767 58. Horard, B., Terretaz, K., Gosselin-Grenet, A.-S., Sobry, H., Sicard, M., Landmann, F., and
768 Loppin, B. (2022). Paternal transmission of the *Wolbachia* CidB toxin underlies cytoplasmic
769 incompatibility. Curr. Biol., S0960-9822(22)00114-2. 10.1016/j.cub.2022.01.052.

770 59. Terretaz, K., Horard, B., Weill, M., Loppin, B., and Landmann, F. (2023). Functional analysis of
771 *Wolbachia* Cid effectors unravels cooperative interactions to target host chromatin during
772 replication. PLoS Pathog. *19*, e1011211. 10.1371/journal.ppat.1011211.

773 60. Kiuchi, T., Koga, H., Kawamoto, M., Shoji, K., Sakai, H., Arai, Y., Ishihara, G., Kawaoka, S.,
774 Sugano, S., Shimada, T., et al. (2014). A single female-specific piRNA is the primary determiner
775 of sex in the silkworm. Nature *509*, 633–636. 10.1038/nature13315.

776 61. Fukui, T., Kawamoto, M., Shoji, K., Kiuchi, T., Sugano, S., Shimada, T., Suzuki, Y., and
777 Katsuma, S. (2015). The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by
778 targeting the masculinizing gene. PLoS Pathog. *11*, e1005048. 10.1371/journal.ppat.1005048.

779 62. Zheng, N., and Shabek, N. (2017). Ubiquitin ligases: structure, function, and regulation. Annu.
780 Rev. Biochem. *86*, 129–157. 10.1146/annurev-biochem-060815-014922.

781 63. Montenegro, H., Solferini, V.N., Klaczko, L.B., and Hurst, G.D.D. (2005). Male-killing
782 *Spiroplasma* naturally infecting *Drosophila melanogaster*. Insect Mol. Biol. *14*, 281–287.
783 10.1111/j.1365-2583.2005.00558.x.

784 64. Zhou, W., Rousset, F., and O'Neil, S. (1998). Phylogeny and PCR-based classification of
785 *Wolbachia* strains using *wsp* gene sequences. Proc. Biol. Sci. *265*, 509–515.
786 10.1098/rspb.1998.0324.

787 65. Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates
788 and generating dominant phenotypes. Development *118*, 401–415.

789 66. Rørth, P. (1998). Gal4 in the *Drosophila* female germline. Mech. Dev. *78*, 113–118.

790 67. Edelheit, O., Hanukoglu, A., and Hanukoglu, I. (2009). Simple and efficient site-directed
791 mutagenesis using two single-primer reactions in parallel to generate mutants for protein
792 structure-function studies. BMC Biotechnol. *9*, 61. 10.1186/1472-6750-9-61.

793 68. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch,
794 S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for
795 biological-image analysis. Nat. Methods *9*, 676–682. 10.1038/nmeth.2019.

69. Pau, G., Fuchs, F., Sklyar, O., Boutros, M., and Huber, W. (2010). EBImage—an R package for image processing with applications to cellular phenotypes. Bioinformatics *26*, 979–981. 10.1093/bioinformatics/btq046.

70. Hollander, M., A. Wolfe, D., and Chicken, E. (2013). Nonparametric Statistical Methods, 3rd Edition 3rd ed. (John Wiley & Sons, Inc.) 10.1002/9781119196037.fmatter.