

E4 ligase–specific ubiquitination hubs coordinate DNA double-strand-break repair and apoptosis

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E4 LIGASE SPECIFIC UBIQUITYLATION HUBS COORDINATE DNA DOUBLE STRAND BREAK REPAIR AND APOPTOSIS

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37 Multiple protein ubiquitylation events at DNA double strand breaks (DSBs) regulate 38 damage recognition, signaling and repair. It has remained poorly understood how the 39 repair process of DSBs is coordinated with the apoptotic response. Here, we identified 40 the E4 ubiquitin ligase UFD-2 as a mediator of DNA damage-induced apoptosis in a 41 genetic screen in *Caenorhabditis elegans*. We demonstrate that upon initiation of 42 homologous recombination by RAD-51, UFD-2 forms foci that contain substrate 43 processivity factors including the ubiquitin-selective segregase CDC-48(p97), the 44 deubiquitylation enzyme ATX-3(Ataxin-3), and the proteasome. In the absence of UFD-45 2, RAD-51 foci persist and DNA damage-induced apoptosis is prevented. In contrast, 46 UFD-2 foci are retained until recombination intermediates are removed by the Holliday 47 junction processing enzymes GEN-1, MUS-81 or XPF-1. UFD-2 foci formation also 48 requires pro-apoptotic CEP-1(p53) signaling. Our findings establish a central role for 49 UFD-2 in the coordination between the DNA repair process and the apoptotic response.

50

51 INTRODUCTION

52 DNA double strand breaks (DSBs) are highly cytotoxic and require the assembly of DNA damage signaling complexes and the DSB repair machinery at the DNA breaks 1 . In the C. 53 elegans germline DSBs are mainly repaired by homologous recombination (HR)². After 54 initial processing of the damaged site, RAD-51 accumulates on single stranded DNA 55 56 (ssDNA) overhangs and mediates strand invasion into the undamaged template, thus 57 facilitating recombination and repair. Ultimately cruciform recombination intermediates called Holliday junctions (HJ) are formed ³. HJs can be processed by two major pathways: HJ 58 59 dissolution via the combined action of the Bloom's syndrome helicase and Topoisomerase TopoIII α^4 , or by resolution of HJs by nucleases acting as resolving enzymes ⁵. While HJ 60 dissolution predominates in most systems 6,7 , in *C. elegans* the GEN-1 resolvase is needed for 61

completion of HR repair of DSBs⁸. The resolution of HR intermediates is important for the 62 63 apoptotic response to DSBs as GEN-1 and HJ processing factors are required for DNA 64 damage-induced programmed cell death. While the mechanisms for such regulation are not 65 known yet, the C-terminal non-catalytic domain of GEN-1 appears to be important for DNA damage signaling ^{8,9}. The apoptotic response to persistent DSBs facilitates the removal of 66 67 germ cells in *C. elegans* when DSBs or meiotic recombination intermediates are not repaired, and occurs in the meiotic pachytene zone of the nematode germline ¹⁰. DNA damage 68 checkpoint signaling leads to the activation of the C. elegans p53 homolog CEP-1 followed 69 by the induction of apoptosis ^{11,12}. CEP-1/p53 protein becomes available in the late pachytene 70 71 region of the germline, leading to apoptosis competency of these germ cells. CEP-1 72 expression in earlier stages of meiosis is translationally repressed by the conserved mRNA binding protein GLD-1¹³. Thus, apoptosis is only initiated when aberrant meiotic 73 74 recombination intermediates or ionizing radiation (IR)-induced DSBs persist in late pachytene 75 cells. It remains, however, unclear how DNA damage processing by recombination repair is 76 coordinated with the apoptosis pathway to allow sufficient time to resolve HR intermediates.

77 In order to better understand how the apoptotic response to DSBs is regulated, we undertook a 78 genetic screen in C. elegans for defects in the IR-induced germ cell apoptosis. RNAi 79 knockdown and genetic mutation of ufd-2 resulted in a reduced apoptotic response. We 80 demonstrate that upon initiation of HR by the recombinase RAD-51, UFD-2 forms foci that 81 we define as ubiquitylation hubs as they also contain substrate CDC-48, ATX-3, and the 82 proteasome. In the absence of UFD-2 or its catalytic activity, RAD-51 foci persist. Similarly 83 to *ufd-2* deficiency, elevated RAD-51 levels result in reduced apoptosis. When the resolution 84 of HJs is hampered due to the absence of GEN-1, MUS-81, or XPF-1, UFD-2 foci persist. 85 UFD-2 foci formation not only requires RAD-51 but also pro-apoptotic signaling through the 86 C. elegans p53 homolog CEP-1. We thus propose that UFD-2 specific ubiquitylation hubs

87 link pro-apoptotic and DNA repair signaling to coordinate the apoptotic response with88 ongoing DSB repair activity.

89

90 **RESULTS**

91 Ligase activity of UFD-2 triggers DSB-induced apoptosis

92 To identify new regulators of the apoptotic response to DNA damage, we performed an RNA 93 interference (RNAi) screen targeting 770 genes whose transcription is enriched in the C. *elegans* germline 14 (Fig. 1a). We focused on those candidate genes because in C. *elegans* 94 DNA damage induced apoptosis only occurs in germ cells ^{10,15}. We identified the E4 ubiquitin 95 96 ligase UFD-2 as the most prominent hit resulting from our screen. RNAi against ufd-2 led to a 97 dose dependent reduction of IR induced apoptosis (Fig. 1b), a phenotype confirmed by 98 analyzing the two different null alleles ufd-2(tm1380) and ufd-2(hh1) (Fig. 1c, d). In contrast, 99 neither developmental apoptosis that occurs during the somatic development of the worm, nor 100 physiological germ cell apoptosis, a background level of germ cell apoptosis that occurs independently of DNA damage, was defective in ufd-2 mutants (Supplementary Fig. 1a, b). 101

102 UFD-2 participates in the ubiquitin fusion degradation (UFD) pathway that was first identified in budding yeast ¹⁶. Substrate ubiquitylation involves E1 ubiquitin activating, E2 103 104 ubiquitin conjugating, and E3 ubiquitin ligase enzymes. UFD-2 defines a class of so-called E4 105 enzymes, which further elongate pre-existing ubiquitin chains to facilitate efficient proteasomal degradation ¹⁷⁻²⁰. It preferentially targets lysine residues 29 and 48 of ubiquitin 106 107 for autoubiquitylation (Supplementary Fig. 1e). A P951A point mutation in the U-box domain completely blocks the ligase activity of UFD-2²¹ (Fig. 1e). To determine if UFD-2 108 109 catalytic activity was required for DNA damage-induced apoptosis, we transgenically expressed UFD-2::GFP or UFD-2^{P951A}::GFP in the germline of wild-type or the *ufd-2* deletion 110

background. Importantly, UFD-2::GFP expression fully restored the apoptotic DNA damage
response in *ufd-2(tm1380)* mutant animals (Fig. 1f). In contrast, the catalytically dead mutant
UFD-2^{P951A}::GFP showed strongly reduced apoptosis after treatment with 60 Gy IR
comparable to *ufd-2* deletion mutant. Overexpression of UFD-2^{P951A}::GFP in the wild-type
background also caused defective apoptosis, which indicates that the inactive U-box mutant
acts dominant-negatively in response to DNA damage (Fig. 1f).

117

118 UFD-2 forms focal accumulations upon DSB induction

119 To determine *in vivo* localization, we raised polyclonal antibodies that specifically recognize 120 UFD-2 both by western blot analysis and immunofluorescence staining (Fig. 2a and 121 **Supplementary Fig. 2a**). Using immunostaining, we found that under unperturbed conditions 122 the protein was evenly distributed in the C. elegans germ line syncytium (Supplementary 123 Fig. 2b). Commencing from late pachytene cells, UFD-2 accumulated at the nuclear periphery 124 resulting in a ring-shaped staining pattern. After IR treatment, UFD-2 foci of varying size and 125 number became detectable within the nucleoli (Fig. 2a, b and Supplementary Fig. 2b). The 126 pattern of antibody staining was confirmed by GFP-tagged UFD-2 transgenes (Fig. 2c, d). 127 These UFD-2 foci occurred in the mitotic zone (data not shown) as well as in the mid-late 128 pachytene zone of the germline after IR (Supplementary Fig 2b). Given our interest in 129 apoptosis we focused on UFD-2 foci formation in the pachytene region. Pachytene cells elicit 130 DNA damage-induced apoptosis upon DNA damage checkpoint activation, whereas mitotic nuclei in the distal germ line compartment are subjected to cell cycle arrest ¹⁰. In contrast to 131 132 the IR-induced apoptosis defect, the cell cycle arrest, which can be monitored by scoring the 133 number of mitotic nuclei that are enlarged due to continuous growth of cellular and nuclear compartments in the absence of cell division (Supplementary Fig. 1c, d)^{10,22}, was normally 134 135 induced in *ufd-2* mutant animals, suggesting that the DNA damage checkpoint in general was

136 functional. Unlike IR-induced RAD-51 repair foci, which accumulate immediately upon 137 damage induction, UFD-2 foci were not yet detectable 5 hrs following damage (Supplementary Fig. 2c, d). We therefore scored UFD-2 foci formation 24 hrs after IR, a 138 time concurrent with full apoptosis activation ¹⁰, using both antibodies and GFP transgenes. 139 140 The number of foci observed in pachytene cells increased from 0-5 foci per germline to more 141 than 15 upon treatment with 60 Gy of IR (Fig. 2a-d and Supplementary Fig. 2b, c). 142 Surprisingly, the ubiquitin ligase mutant was equally efficient in UFD-2 foci formation as the 143 wild-type ligase (Fig. 2 c, d). Together, these data indicate that the UFD-2 ligase activity is 144 required to trigger DNA damage-induced apoptosis (Fig. 1f), but is not necessary for UFD-2 145 foci formation (Fig. 2c, d).

146

147 Ubiquitin signaling fine-tunes the apoptotic response

Given that UFD-2 triggers protein degradation ^{16,17,23}, we examined if factors associated with 148 the ubiquitin-proteasome system (UPS) might associate with UFD-2 foci ^{17,24,25}. Hence, we 149 analyzed ubiquitin localization 24 hrs after irradiation. In fact, an antibody recognizing 150 151 conjugated mono- and polyubiquitin chains co-stained UFD-2 foci (Fig. 3a and Supplementary Fig. 3e). Additional staining experiments detected co-localization of the 152 153 proteasome and the ubiquitin-selective segregase CDC-48 with UFD-2 foci (Fig. 3a). Among 154 other processes, CDC-48/p97 has been established to coordinate the degradation of 155 chromatin-associated proteins during DNA replication or DNA repair by extracting ubiquitylated substrate proteins from higher order complexes ²⁶⁻²⁸. As CDC-48 has been 156 shown to interact with UFD-2²⁰, we wondered if the interaction was necessary for UFD-2 157 dependent apoptotic signaling. Transgenic overexpression of UFD-2^{C448Y}::GFP, a mutant 158 version that provides ligase activity but is not able to interact with CDC-48²⁹, led to UFD-2 159 foci even without IR treatment (Supplementary Fig. 3f-j). However, UFD-2^{C448Y}::GFP failed 160

161 to rescue the apoptosis phenotype displayed by ufd-2 deletion worms (Fig. 3g), suggesting 162 that in addition to ligase activity also the interaction with CDC-48 is a prerequisite for the 163 apoptotic function of UFD-2. CDC-48 has been demonstrated to guide ubiquitin chain 164 topology by coordinating different substrate processing enzymes such as UFD-2 and the deubiquitylation enzyme ATX-3²⁰. Intriguingly, we also found that ATX-3 localized to UFD-165 166 2 foci (Fig. 3a and Supplementary Fig. 3c, d), which indicates an orchestrated action of 167 UFD-2, ATX-3, and CDC-48 at ubiquitylation hubs triggered by DNA damage. The 168 ubiquitylation activity of UFD-2 was dispensable for the recruitment of the proteasome, ATX-169 3, and CDC-48 (Fig. 3b). In contrast, apoptosis induction required the catalytic activity of 170 UFD-2 as well as its interaction with CDC-48 (Fig. 1e, f, and 3g).

171 Given that in yeast and humans, Ufd2/UBE4B mediates elongation of preformed 172 ubiquitin chains, we tested whether UFD-2 cooperates with the E3 ligase HECD-1, the 173 ortholog of budding yeast Ufd4 and human HECTD1 or TRIP12, to trigger DNA damage induced apoptosis ^{17,30-32}. Indeed, loss of HECD-1 prevented UFD-2 foci formation, 174 175 suggesting ubiquitin-dependent recruitment of UFD-2 (Fig. 3c, d). Apoptosis was reduced in 176 hecd-1 mutants, which implicates a role of UFD-2 focal accumulation in response to DNA 177 damage (Fig. 3e). The apoptosis defect was even more pronounced in ufd-2; hecd-1 double 178 mutants, indicating that the activity of both enzymes is required to achieve apoptosis (Fig. 179 **3e**). In contrast, the deubiquitylation enzyme ATX-3 counteracted UFD-2 recruitment as both 180 UFD-2 foci formation and apoptosis were increased in atx-3 mutants (Fig. 3c, d, f). 181 Accordingly, the excessive DNA damage-induced apoptosis occurring in *atx-3* mutants was 182 suppressed in *ufd-2; atx-3* double mutant worms (Fig. 3f). The number of ubiquitin foci per 183 germline was decreased in *hecd-1*, whereas it was increased in atx-3 (Fig. 3c, d and 184 Supplementary Fig. 3k). This observation suggests ubiquitin dependent formation of UFD-2 185 foci, determined by ubiquitin-mediated recruitment signals fine-tuned by HECD-1 and ATX-

3. We therefore conclude that the apoptotic response to DNA damage is coordinated byubiquitylation signals defined by UFD-2 in cooperation with HECD-1 and ATX-3.

188

189 UFD-2 supports RAD-51 dissociation from DNA repair sites

190 Next we analyzed if UFD-2 also affects the DNA repair process in addition to apoptosis. In 191 contrast to DSB induction by IR, UV irradiation did not result in formation of UFD-2 foci 192 consistent with a specific role of UFD-2 in responding to DSBs (Supplementary Fig. 3a). In 193 line with this observation, we found that RPA-1::GFP and BRD-1::GFP HR fusion proteins ^{33,34} accumulate in UFD-2 foci 24 hrs after IR treatment (Fig. 4a, b). Furthermore, IR of L4 194 staged *ufd-2* mutant larvae resulted in reduced embryonic survival in the ensuing generation 195 196 (Supplementary Fig. 3b). To establish whether *ufd-2* promotes the processing of DNA repair 197 intermediates, we analyzed the kinetics of RAD-51 foci. While both wild-type and ufd-2 198 mutants accumulated an equal amount of RAD-51 positive nuclei one hour after IR, twice as 199 many RAD-51 stained nuclei persisted 16 hrs later in *ufd-2* mutants (Fig. 4c, d). This delay in 200 RAD-51 foci dissociation that temporally coincides with UFD-2 foci formation suggests that 201 UFD-2 contributes to resolution of repair intermediates.

202

203 UFD-2 acts downstream of pro-apoptotic signaling

We next wished to further investigate the role of the DSB repair process in UFD-2 foci formation (**Fig. 4a**). Impairment of HR in *rad-51* deletion mutant worms blocked UFD-2 foci formation (**Fig. 5b**). Conversely, *rad-54* deletion defective in removal of RAD-51 from DNA during HR repair ³⁵ caused an accumulation of UFD-2 foci (**Fig. 5b**). The nucleases GEN-1, MUS-81 and XPF-1 are required for the resolution of HJs in order to complete the HR repair process of IR-induced DSBs ^{8,36,37}. Deletion of the *gen-1*, *mus-81*, and/or *xpf-1* HJ processing enzymes also led to focal accumulation of UFD-2 (**Fig. 5c and Supplementary Fig. 4a**). Of note, *mus-81* and *xpf-1* mutant animals showed elevated numbers of UFD-2 foci also in the absence of IR-induced DSBs consistent with the function of MUS-81/XPF-1 in meiotic HJ resolution 36,37 . These results indicate that HR needs to commence for UFD-2 foci to form, which persist until HR is completed (**Fig. 5b, c**).

215 As ufd-2 mutant worms displayed reduced apoptosis, we assessed whether apoptotic 216 signaling was affected in *ufd-2* mutant worms. The apoptotic core machinery is conserved 217 from C. elegans to the mammalian system. The p53 homologue CEP-1 induces transcription of the two BH3-only proteins EGL-1 and CED-13^{13,38}, which bind to the only Bcl2-like 218 protein CED-9. As a consequence, the inhibitory effect of CED-9 on the Apaf1-like CED-4 is 219 alleviated and CED-4 activates the caspase CED-3, which executes cell death (Fig. 5a)³⁹. In 220 221 view of the ubiquitin ligase activity, we tested whether CEP-1 protein might accumulate upon 222 DNA damage in the absence of UFD-2. However, in wild-type and ufd-2 mutant worms CEP-223 1 protein was equally expressed following 60 Gy irradiation (Supplementary Fig. 4b-d). 224 Additional evaluation of mRNA transcripts of the CEP-1 target gene egl-1 showed a 225 comparable transcriptional regulation in both genotypes 4 and 24 hrs after damage infliction 226 (Supplementary Fig. 4d). Having established that CEP-1 activation occurs independently of 227 *ufd-2*, we wondered if UFD-2 foci formation might be dependent on CEP-1. Strikingly, loss 228 of CEP-1 prevented UFD-2 foci induction after IR (Fig. 5d), whereas UFD-2 protein 229 expression remained unaffected (Supplementary Fig. 5b). Consistently, a double mutant of the two pro-apoptotic CEP-1 effectors, egl-1; ced-13, which is similarly defective in DNA 230 damage-induced apoptosis as *cep-1* mutants⁴⁰, phenocopied the *cep-1* defect in UFD-2 foci 231 232 formation after DNA damage (Fig. 5d and Supplementary Fig. 5a). To further confirm a 233 direct role of CEP-1 in UFD-2 foci formation, we enhanced CEP-1 activity by employing a gld-1(op236) mutation, previously shown to increase CEP-1 levels ¹³. gld-1 mutants indeed 234 235 displayed strongly elevated UFD-2 foci, supporting the idea that CEP-1 promotes UFD-2 236 focal accumulation. The *cep-1*; *gld-1* double mutant displayed a similar number of UFD-2 237 foci as wild-type germ cells (Fig. 5d and Supplementary Fig. 5a). One potential explanation 238 for the failure of *cep-1* to completely suppress the foci formation in *gld-1* might be the numerous additional target mRNAs of GLD-1^{41,42}. Of note, the failure of *cep-1* to initiate 239 240 apoptosis does not affect repair activity as IR-induced embryonic lethality has previously been shown to remain unaffected ¹³. Consistently, we found that RAD-51 foci disassembly 16 241 242 hrs after damage induction was as efficient in *cep-1* and *gld-1* mutants as in wild-type 243 (Supplementary Fig. 5c). In contrast to the loss of CEP-1 signaling, UFD-2 foci formation 244 was unaltered in apoptosis deficient *ced-3* and *ced-4* mutant worms (Fig. 5d), emphasizing 245 the necessity of CEP-1 activity for UFD-2 foci formation rather than the apoptotic process in 246 general. Taken together, UFD-2 seems to act downstream of the pro-apoptotic signaling 247 cascade.

248

249 Resolution of RAD-51 is linked to apoptotic signaling

250 We next wished to investigate the role of UFD-2 in the removal of RAD-51 foci and its 251 consequence on apoptosis. Germline-specific expression of UFD-2::GFP in transgenic ufd-2 252 deletion mutants rescued the delay of RAD-51 removal from DNA (Fig. 6a). Increased RAD-253 51 retention occurred in moderately RAD-51::GFP overexpressing worms after 24 hrs of IR 254 compared to wild-type (Fig. 6a). Importantly, the retention of RAD-51 filaments either by 255 loss of *ufd-2* or by RAD-51 overexpression strictly correlated with reduced apoptosis levels 256 (Fig. 6b). Despite the elevated RAD-51 protein levels, the GFP transgenic line possessed 257 normal repair capacity as assessed by embryonic survival after IR thus suggesting that the reduced apoptosis is not related to enhanced removal of DSBs (Supplementary Fig. 6a). 258 259 Conversely, the *atx-3* mutant, which displayed increased UFD-2 foci and apoptosis after DNA 260 damage, showed decreased RAD-51 retention 16 hrs after IR (Supplementary Fig. 6b). To 261 test whether elevated RAD-51 levels might directly account for the reduced apoptosis 262 observed in ufd-2 mutants or upon overexpression of RAD-51, we depleted RAD-51 by RNAi knockdown. Indeed, rad-51(RNAi) in ufd-2 mutant or RAD-51::GFP expressing worms 263 264 reverted the apoptosis defect following IR treatment (Fig. 6c). Importantly, rad-51(RNAi) also reduced embryonic survival after IR in wild-type and rad-51 mutant worms 265 266 (Supplementary Fig. 6c). We further validated the role of RAD-51 filaments in suppressing 267 the apoptotic response by inhibition of RAD-51 filament formation with the RAD-51 inhibitor B02⁴³. Similarly to reduced RAD-51 levels, treatment with B02 reverted the apoptosis 268 phenotype of *ufd-2* deletion mutants or the RAD-51 overexpression line (Fig. 6d), suggesting 269 270 that RAD-51 accumulation directly antagonizes apoptotic signaling. Moreover, rad-51 271 heterozygous mutants with reduced RAD-51 levels reverted the apoptosis defect of ufd-2 272 mutants (Fig. 6e). In summary, these observations support the idea that UFD-2 contributes to 273 resolution of DNA repair sites and that retention of RAD-51 filaments leads to inhibition of 274 apoptosis (Fig. 6f).

275

276 **DISCUSSION**

277 In this study we uncovered a ubiquitin dependent process that facilitates the communication 278 between DNA repair and the apoptotic response. We implicated the E4 ubiquitin ligase UFD-279 2 as a central regulator for the spatiotemporal coordination of both processes. Our data 280 suggest that defects in timely proceeding of HR either by failure to resolve HJs as previously demonstrated ^{8,9} or by aberrant retention of RAD-51 foci at the chromatin caused by loss of 281 282 UFD-2 as shown here, halt the apoptotic response. Conversely, RAD-51 filament assembly and pro-apoptotic signaling by the p53 tumor suppressor homolog CEP-1 are both required 283 284 for the formation of UFD-2-specific hubs that we define by the presence of proteolytic factors 285 of the UPS machinery (Fig. 6f). We propose that these degradation hubs calibrate the 286 proceeding of the DNA repair machinery with apoptotic activity via modulation of ubiquitin 287 signaling. Such a calibration might allow gaining time for ongoing HR repair when CEP-1 288 dependent apoptotic signaling has already been triggered. Indeed, CEP-1 activity can be detected within the first hour following IR treatment ⁴⁰, while the rapidly formed RAD-51 foci 289 are turned over in the course of 24 hrs. To prevent the precocious demise of cells that are 290 engaged in the process of repairing DSBs, the simultaneous presence of pro-apoptotic 291 292 signaling and ongoing HR requires coordination, which we propose is orchestrated at the HR 293 repair sites through the UFD-2-ubiquitin hubs that might thus provide feedback to the 294 apoptotic signaling on the status of the damage removal. The fine-tuning of ubiquitin chain 295 topology by concerted action of UFD-2, the E3 ligase HECD-1 and the hydrolase ATX-3 at 296 HR sites might indeed constitute a versatile signaling tool to enable communication between 297 the apoptotic response and DNA damage (Fig. 6f). Since the E3 ligase HECD-1 is required for UFD-2 hub formation and apoptosis execution, we propose that E4 activity ^{17,31,44} is 298 299 providing an additional layer of regulation by editing ubiquitin chain topology. The human E4 300 homolog UBE4B cooperates similarly with the HECT domain E3 ligase TRIP12 in substrate ubiquitylation, suggesting the existence of a conserved signaling pathway³⁰. In support of this 301 idea, TRIP12 fine-tunes ubiquitin controlled events at DSBs ⁴⁵ and recent reports linked 302 303 UBE4B to different cancer types, highlighting the relevance of ubiquitin signaling in the decision between DNA damage and apoptosis response ⁴⁶⁻⁴⁹. Disassembly of RAD-51 304 305 filaments might involve the ubiquitin-selective segregase CDC-48/p97, which was recently implicated in chromatin associated protein degradation^{26,27}. Moreover, Cdc48 was shown to 306 limit RAD51 occupancy on DNA ⁵⁰. In agreement with this notion, CDC-48 binding is 307 308 required for UFD-2 to trigger DNA damage induced cell death (Fig. 3g). Defects in DNA 309 repair and apoptosis are especially relevant in tumor formation. Thus, understanding the 310 conserved role of UFD-2/UBE4B in response to IR induced DNA damage might open new 311 therapeutic directions for drug development and cancer treatment.

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328

Author Contributions L.A. and M.S. designed, performed and analyzed the experiments. W.P. performed *in vitro* ubiquitylation assays. É.K. generated ATX-3 antibody. A.G. and B.S. designed and performed the RNAi screen. B.S. and T.H. supervised the design and data interpretation. L.A., B.S., and T.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

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466 FIGURE LEGENDS

467 Figure 1 Ubiquitin ligase activity of UFD-2 is required for apoptosis execution. (a) 468 Schematic illustration of RNAi screen for identification of DNA damage-induced apoptosis 469 mediators. After RNAi treatment worms were subjected to IR and scored for apoptotic corpses (indicated by filled arrowheads) 24 hrs later by differential interference contrast 470 471 (DIC) microscopy. Representative images of 3 independent experiments. (b) Apoptotic 472 corpses in worms treated with indicated RNAi constructs and exposed to different IR doses, 473 24 hrs after treatment. Data represent mean \pm s.e.m. of 3 independent experiments. *n* varied 474 from 2-11 animals, see **Supplementary Table 1**. (c) Representative images of late pachytene 475 cells of C. elegans germline 24 hrs after IR treatment (0, 60 Gy). Filled arrowheads indicate 476 apoptotic corpses. Scale bar 5 μ m. Representative images of 3 independent experiments. (d) 477 Analysis of DNA damage induced apoptosis 24 hrs after IR treatment (0, 30 or 60 Gy) of 478 indicated genotypes. Center lines show the medians; box limits indicate the 25th and 75th 479 percentiles as determined by R software; whiskers extend 1.5 times the interquartile range 480 from the 25th and 75th percentiles, outliers are represented by dots. The notches are defined 481 as +/-1.58*IQR/sqrt(n) and represent the 95% confidence interval for each median. Nonoverlapping notches give roughly 95% confidence that two medians differ. Data of 5 482 483 independent experiments. n varied from 69-80 animals, see Supplementary Table 1. (e) Auto-ubiquitylation of UFD-2 with UFD-2 (wild-type) or UFD-2^{P951A} as ubiquitin ligases. 484 485 Representative immunoblot of 3 independent experiments. (f) Analysis of DNA damage 486 induced apoptosis 24 hrs after IR treatment (0, 60 Gy) of indicated genotypes. Statistics as in 487 Fig. 1d. Data of 3 independent experiments. n varied from 36-63 animals, see 488 Supplementary Table 1.

Figure 2 UFD-2 forms foci late after IR treatment. (a) Representative images of worm
germlines of indicated genotypes stained with α-UFD-2 antibody and DAPI 24 hrs after IR

491 treatment (60 Gy). Filled arrowhead indicate nuclei with UFD-2 foci. Scale bar, 5 µm. 492 Representative images of 3 independent experiments and (b) corresponding quantification of 493 UFD-2 foci in pachytene region of germlines. Data show means \pm s.e.m. of 12 independent 494 experiments. n = 231 animals (wt 0 Gy) and n = 280 animals (wt 60 Gy). (c) Representative 495 images of worm germlines of indicated genotypes stained with GFP-booster and DAPI 24 hrs 496 after IR treatment (60 Gy). Filled arrowheads indicate nuclei with UFD-2 foci. Scale bar, 5 497 μ m. Representative images of 3 independent experiments and (d) corresponding 498 quantification of UFD-2 foci in pachytene region of germlines. Data show means \pm s.e.m. of 3 499 independent experiments. *n* varied from 34-51 animals, see **Supplementary Table 1**.

Figure 3 UPS factors accumulate in UFD-2 hubs and balance apoptotic signaling. 500 Representative images of (a) ufd-2(tm1380); UFD-2::GFP and (b) ufd-2(tm1380); UFD-501 2^{P951A}::GFP immunostained with indicated antibodies 24 hrs after IR (Gy 60). The boxed area 502 503 is three times magnified (3x zoom). α -alpha SU, α -Proteasome 20S alpha subunits. Scale bars, 504 5 μ m. Representative images of 3 independent experiments. (c) Representative images of 505 worm germlines of indicated genotypes immunostained with α -UFD-2 antibody and DAPI 24 506 hrs after IR treatment (60 Gy). Filled and empty arrowhead indicated nuclei positive or 507 negative for UFD-2 foci, respectively. Scale bar, 5 µm Representative images of 3 508 independent experiments and (d) corresponding quantification of UFD-2 foci in pachytene 509 region of germlines. Data show means \pm s.e.m. of 3 independent experiments. *n* varied from 510 36-107 animals, see Supplementary Table 1. (e,f,g) Analysis of DNA damage induced 511 apoptosis 24 hrs after IR treatment (0, 60 Gy) of indicated genotypes. Statistics as in **Fig. 1d**. 512 Data of 3 independent experiments. n varied from 39-52 animals for (e), 38-51 animals for (f) 513 and 34-74 animals for (g), see Supplementary Table 1.

Figure 4 Loss of *ufd-2* delays DSB repair processing. (a) Schematic illustration of DNA DSB

515 repair by HR in C. elegans. Upon DSB induction RPA binds resected single stranded DNA,

516 BRD-1 acts together with BRCA-1 at DSB site, RPA is exchanged for RAD-51, which 517 mediates strand invasion, Gen-1 resolves HJ resulting in repaired DSB. Names in brackets 518 indicate human homologues. (b) Representative images of BRD-1::GFP and RPA-1::GFP 519 germlines stained with α -UFD-2 and DAPI 24 hrs after IR treatment (60 Gy). Scale bar, 5 μ m. 520 Representative images of 3 independent experiments. (c) Representative images of wild-type 521 and ufd-2(tm1380) germlines isolated 16 hrs after IR treatment (20 Gy), stained with α -RAD-522 51 and DAPI. Filled arrowheads indicate nuclei positive for RAD-51 staining. Scale bar, 10 523 um. Representative images of 3 independent experiments. (d) Quantification of germ cells 524 positive for RAD-51 staining of wild-type and ufd-2(tm1380) worms treated with IR (0 Gy) 525 and isolated after 1hr or treated with IR (20 Gy) and isolated after 1, 7, 16, 48 hrs. Data show 526 means \pm s.e.m. of 3 independent experiments. *n* varied from 35-43 animals, see 527 **Supplementary Table 1.** The triple asterisk indicates P value of ≤ 0.001 in two-tailed 528 Student's *t*-test.

Figure 5 UFD-2 foci in repair and apoptosis after DNA damage. (a) Schematic illustration of apoptosis pathway in *C. elegans*. Names in brackets indicate human homologues. (**b,c,d**) Quantification of UFD-2 foci in pachytene region of germlines of indicated genotypes isolated 24 hrs after irradiation (60 Gy). Data show means \pm s.e.m. of 3 independent experiments. *n* varied from 30-113 animals for (**b**), 34-92 animals for (**c**) and 20-202 animals for (**d**), see **Supplementary Table 1**.

Figure 6 UFD-2 coordinates communication between repair and apoptosis after DNA damage. (a) Quantification of germ cells positive for RAD-51 staining of indicated genotypes treated with IR (0 or 20 Gy) and isolated after 24 hrs. Data show means \pm s.e.m. of 3 independent experiments. *n* varied from 34-63 animals, see **Supplementary Table 1**. The triple asterisk indicates *P* value of \leq 0.001 in two-tailed Student's *t*-test. (b) Analysis of DNA damage induced apoptosis 24 hrs after IR treatment (0, 60 Gy) of indicated genotypes. 541 Statistics as in Fig. 1d. Data of 3 independent experiments. *n-values* varied from 61-82 542 animals, see Supplementary Table 1. (c,d,e) Analysis of DNA damage induced apoptosis 24 543 hrs after IR treatment (0, 60 Gy) of (c) ufd-2 and RAD-51::GFP treated with rad-51 or control 544 RNAi or (d) wild-type, ufd-2, and RAD-51::GFP worms treated with RAD51 inhibitor B02 545 (200 mM) or (e) indicated genotypes. Statistics as in Fig. 1d. Data of 3 independent 546 experiments. *n-values* varied from 30-47 animals for (c), 28-69 animals for (d) and 29-40 547 animals for (e), see Supplementary Table 1. (f) Model for the coordination between HR and 548 apoptotic signaling by UFD-2. DSB repair triggers RAD-51 accumulation at ssDNA to 549 facilitate homology pairing. After efficient strand invasion RAD-51 is removed and HJ are 550 resolved by HJ processing enzymes (GEN-1, MUS-81 and XPF-1). UFD-2 supports RAD-51 551 dissociation from DSB at advanced time points. Ongoing repair is reflected by the presence of 552 UFD-2 containing hubs late after IR. These ubiquitylation hubs contain processivity factors 553 like CDC-48 and proteasome (not shown). Interaction between UFD-2 and CDC-48 is 554 necessary to transduce a pro-apoptotic signal. UFD-2 hub formation is fine-tuned by the E3 555 ligase HECD-1, the DUB ATX-3, and pro-apoptotic CEP-1/p53 signaling.

556 METHODS

C. elegans strains. C. elegans strains were cultured at 20 °C on nematode growth medium 557 (NGM) and fed with Escherichia coli (E. coli) strain OP50 according to standard procedures 558 559 ⁵¹. The Bristol strain N2 was used as wild-type. Mutants and transgenic animals used in this study are listed in the following: mus-81(tm1937) I, rad-54&snx-3(ok615) I/hT2 [bli-4(e937)] 560 561 let-?(q782) qIs48] (I;III), cep-1(lg12501)I, ced-1(e1735)I, gld-1(op236)I, ufd-2(tm1380)II, 562 ufd-2(hh1)II, xpf-1(tm2842) II, gen-1(tm2940)III, ced-4(n1162) III, hecd-1(tm2371)IV, rad-563 51(ok2218) IV/nT1[qIs51](IV;V), ced-3(n717) IV, atx-3(gk193)V, egl-1(n1084n3082)V; ced-13(tm536)X, , Is[rad-51::GFP:3xFLAG], gla-3(op216)I, hus-1(op241)I, unc-119(ed3)III; 564 565 gtIs[unc-119(+), Ppie-1::GFP::rpa-1::pie-1-3'UTR], hhIs121[unc-119(+), Pmex-5::ufd-2::GFP::tbb-2 3'UTR], hhIs135[unc-119(+), Pmex-5 (w/o ATG)::ufd-2 (w/o TAA, 566 567 P951A)::(Gly)5Ala::gfp F64LS65T(w introns/stop)::tbb-2 3'UTR], hhls134[unc-119(+), 568 Pmex-5::ufd-2 (C448Y)::GFP::tbb-2 3'UTR].

The transgenic lines *hhIs121*, *hhIs134*, and *hhIs135* were generated for this study. Briefly, fosmid WRM0621dE05 was used as template to obtain the genomic sequence of *ufd-2* that was cloned together with ppJA252, pJA257 into pCG150 containing the *unc-119(+)* marker for selection of transgenic worms ⁵². *ufd-2* was modified by directed mutagenesis to create $ufd-2^{P951A}$ or $ufd-2^{C448Y}$. The constructs were bombarded into *unc-119(ed4)III* mutants as described previously ⁵³.

575 No statistical method was used to predetermine sample size. The experiments were not
576 randomized and were not performed with blinding to the conditions of the experiments. All *n*577 values are specified in Supplementary Table 1 (for data in Fig. 1-6) and Supplementary
578 Table 2 (for data in Supplementary Fig. 1-6).

Ionizing radiation. Synchronized hermaphrodites were grown until L4 stage and irradiated
with the corresponding dose (Radiation source: 120-kV X-rays (25 mA; 0.5mm Alu-filter;
ISOVOLT 160 M1/10-55, GE Sensing & Inspection Technologies) or Biobeam 8000 using
Cs137 as radiation source).

RNAi treatment. RNA interference was performed using the feeding method ⁵⁴. Three P0 583 584 worms were placed on IPTG (isopropylthiogalactoside) and ampicillin-containing NGM-585 plates seeded with E. coli [HT115(DE3)] expressing double-stranded RNA (dsRNA) and 586 incubated at 15°C for 72 hrs. Three single F1 worms were transferred each to a new, freshly seeded plate and allowed to lay eggs for approximately 20 hrs. F1 worms were removed and 587 588 F2 worms were allowed to grow up to the L4 stage, treated with ionizing radiation and 589 analyzed for radiation induced apoptosis. Clones in RNAi feeding vectors were provided by 590 Marc Vidal of Dana Farber Cancer Center.

591 **Apoptotic corpses.** For physiological apoptosis analysis, synchronized L1 larvae were grown 592 until L4 stage. Apoptotic corpses were scored 24 hrs later. For this, worms were mounted on 3% agar pads, paralyzed with 60 nM NaN₃ and analysed via DIC microscopy ⁵⁵. For DNA 593 594 damage induced apoptosis worms were subjected to IR at L4 stage before apoptosis was 595 evaluated 24 hrs later. Developmental apoptosis was assessed in L1 larvae. Therefore worms 596 were grown until day one adulthood. 100 worms were transferred to a NGM-agar plate 597 without E. coli and allowed to lay eggs until they were removed after 1 hr. Freshly hatched L1 larvae were scored for apoptotic corpses ⁵⁶. 598

599 UFD-2 foci. Synchronized worms were grown until L4 larvae stage and irradiated with 0 and
600 Gy. 24 hrs later, germlines were isolated and immunostained. Number of UFD-2 foci was
601 scored in all focal planes in pachytene germ cells. One germline per worm was scored.

602 **Protein expression and purification.** cDNAs encoding ufd-2b, $ufd-2b^{C448Y}$, and $ufd-2b^{P951A}$ 603 were cloned into the pET-21d expression vector (Novagen) and pGex4T1 (GE Healthcare). 604 Recombinant proteins were expressed in *E. coli* strain BL21 Codon Plus (Novagen) and 605 purified using the ÄKTA purifier system (GE Healthcare).

606 Antibody production. His-tagged purified proteins (UFD-2, ATX-3²⁰) were used for 607 immunization of rabbits and anti-sera were affinity purified using respective GST-tagged 608 recombinant proteins (BioGenes). For validation see **Supplementary Fig. 2a** and **3d**, 609 respectively.

610 Preparation of worm lysates. Synchronized L1 larvae were grown on NGM-agar plates with 611 OP50 bacteria until they reached adulthood. Worm lysates used for SDS-PAGE were either 612 prepared from a distinct number of worms (n=150) or by washing worms from NGM-agar 613 plates followed by multiple washing step with M9 buffer [3 g/l KH2P04, 6 g/l Na2 HPO4, 5 614 g/l NaCl, 1 mM Mg S04 (added after sterilization)], until bacteria were removed. The samples 615 were heated to 95°C for 5 min and subsequently shock-frozen in liquid nitrogen. After 616 thawing, samples were subjected to sonication (two times for 15 s, on ice; 50% power; 617 Sonopuls UW 2200, Bandelin) and taken up in 4 x SDS sample buffer followed by 618 centrifugation at 15,000 rpm for 10 min.

Immunotechniques. Immunostaining of isolated germlines was done according to the 'freeze-crack' protocol. Worms were dissected onto polylysine-coated slides (Thermo Scientific) in 60 nM NaN₃ to isolate germlines and fixed in fixation buffer (3.7 % Formaldehyde, 0.2 % Tween 20) for 10 min with subsequent shock freezing in liquid nitrogen. This was followed by incubation in 1:1 mixture of methanol and acetone at -20 °C for 10 min. Germlines were permeabilized 3 times in 1 % PBS-Triton X-100 for 20 min followed by washing in 0.1 % PBS-Tween 20 (PBS-T) for 10 min and blocking in 10 % goat 626 serum in 0.1 % PBS-T. A specific staining protocol was followed for GFP-expressing lines 627 avoiding freezing. Isolated germlines were fixed with fixation buffer for 10 min in PCR tubes, 628 directly followed by permeabilization and blocking as described above. Germlines were incubated with primary antibody overnight at 4 °C (anti-UFD-2 1:3,000, anti-CDC-48 629 1:12,000⁵⁷, anti-RAD-51 1:350 (14B4, #NB100-148, Novus Biologicals⁵⁸), anti-FK2-630 631 ubiquitin 1:100 (AB 612093, #04-263, Millipore, validation on manufacturer's website), anti-Proteasome 20S alpha 1+2+3+5+6+7 antibody 1:300 (MCP231, #ab22674, abcam ⁵⁹), anti-632 633 ATX-3 1:700). Incubation with the fluorescently labeled secondary antibodies (#A-11037, #R37117, Life Technologies; 1:200) or GFP-booster (#GBA-488, ChromoTek; 1:400⁶⁰) was 634 635 done at room temperature for 1 hr. Germlines were mounted in DAPI Fluoromount-G 636 medium (SouthernBiotech). For western blotting, worm lysates were separated by SDS-637 polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes 638 (Whatman, Protran). Membranes were blocked in 1x Roti-Block (Roth) and incubated with 639 the primary antibodies overnight at 4 °C in Roti-Block (Roth; anti-ATX-3 1:10,000, anti-CDC-48 1:50,000⁵⁷, anti-UFD-2 1:20,000, anti-CEP 1:15,000¹³, anti-tubulin 1:5000 (clone 640 DM1A, Sigma-Aldrich⁶¹). Incubation with fluorescently labeled secondary antibodies (LiCor 641 642 IRDye 680, #926-32222 and #926-32223, LiCor IRDye 800, #926-32212 and #926-32213, 643 1:10,000) was done at room temperature, before detection of signals using the Li-Cor 644 Odyssey scanner. Quantification of signal intensities was done using the Odyssey V4.0 645 software (Li-Cor). The uncropped versions of western blots that have been used to assemble 646 the main figures are collected in Supplementary Fig. 7.

647 Immunoprecipitation *in vivo* and *in vitro*. Worm lysates were prepared as described above 648 and the protein concentration was determined by measuring absorption at 260nm with 649 Nanodrop 800 UV/Vis Spectrometer. 250 µl of Dynabeads (Invitrogen) were used per 650 reaction, washed twice with conjugation buffer [100 mM sodium phosphate, 0,15 M NaCl]

and resuspended in 1 ml 5 mM crosslinking reagent BS³ (Thermo Scientific). Crosslinking 651 652 was performed on rotation wheel for 30 min at room temperature. The reaction was stopped by adding 50 µl quenching buffer [1 M Tris/HCL, pH 7.5] followed by 15 min incubation at 653 654 room temperature. The beats were washed three times with 0.5 x PBS before 50 μ g α -UFD-2 655 antibody was added and incubated for 20 min under constant rotation at room temperature. 656 After repeated washing with 0.5 x PBS, 2.5 mg of corresponding worm protein lysate was 657 added to the beads and incubated over night at 4 °C with rotation. For in vitro 658 immunoprecipitation, antigen (UFD-2) was incubated with antibody-coupled beads for 4 hrs 659 prior to addition of the putative binding partner (CDC-48) for an additional incubation for 4 660 hrs. Both proteins were added in equimolar ratio (1 mM). Elution was performed as described 661 in the manufacturer's manual.

662 **Microscopy and image acquisition.** Immunostained germlines were imaged with 663 AxioImager.M1/Z1 microscope with Apoptome equipped with an AxioCam MRm camera 664 (Carl Zeiss). To allow direct comparison of signal intensities, images were recorded under 665 identical conditions. Processing of selected pictures was done in ZEN2011 and ImageJ.

In vitro ubiquitylation assay. UFD-2b::His, UFD-2b^{C448Y}::GST, and UFD-2b^{P951A}::His 666 667 fusion proteins were expressed in BL21-AI E. coli strain and lysed in buffer A [50 mM Tris 668 pH 7.5, 250 mM NaCl, 5 mM DTT, 1% Triton X-100, 2 mM PMSF and protease inhibitor 669 mix; Roche]. 10 µg of the aforementioned bacterial lysate was mixed with E1 (25 ng), E2 670 (Let-70; 400 ng), 2 µg of FLAG::ubiquitin, energy regenerating solution (Boston 671 Biochemicals) and ubiquitin conjugation reaction Buffer (Enzo Life Sciences). Samples were 672 incubated at 30 °C for 1.5 hrs, terminated by boiling for 5 min with SDS-sample buffer, and 673 resolved by SDS-PAGE followed by immunoblotting using anti-UFD-2 antibodies to monitor 674 ubiquitylation of UFD-2.

Persistence of RAD-51 foci after IR. Synchronized worms were grown until L4 larvae stage and irradiated with 0 and 20 Gy. 1 to 48 hrs later, germlines were isolated and immunostained. Z-stacks were taken of late pachytene cells of the germline. Two focal planes covering the upper and lower part of the germline were subjected to analysis by scoring each plane for RAD-51 positive cells in the last 25 nuclei of pachytene germ cells prior entering diakinesis.

681 **RNA isolation and real-time PCR.** Total RNA was isolated using TRIzol (Invitrogen) and 682 Qiagen RNeasy kit. Briefly, worms were washed off the plates using M9 buffer [3 g/l KH₂P0₄, 6 g/l Na₂ HPO₄, 5 g/l NaCl, 1 mM Mg S0₄ (added after sterilization)] and 600 µl 683 684 TRIzol, and silica beads (1 mm diameter) were added to the samples and homogenized by 685 Precellys tissue homogeniser. Chloroform was added and samples were vortexed vigorously 686 before phase separation through centrifugation. The aqueous phase was transferred on the 687 Qiagen RNeasy Mini spin column and RNA was isolated according to manufacturer's 688 instructions. cDNA was synthesized using 200 ng total RNA and the High-Capacity cDNA 689 Reverse Transcription Kit (Applied Biosystems). Gene expression levels were determined by 690 real time PCR using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent 691 Technologies) and Biorad CFX96 Real-Time PCR Detection System. Relative gene 692 expressions were normalized to tbg-1 (F58A4.8) mRNA levels. In the experiment three 693 biological and three technical replicate samples were analyzed. The primer sequences used in 694 the RT–PCR reactions are the following: *tbg-1* forward:

- 695 5'-GTACACTCCACTGATCTCTGCTGACAAG-3', *tbg-1* reverse:
- 696 5'-CTCTGTACAAGAGGCAAACAGCCATG-3'⁶², *egl-1* forward:
- 697 5'-TACTCCTCGTCTCAGGACTT-3', egl-1 reverse: 5'- CATCGAAGTCATCGCACAT-3'.

698 **Embryonic Survival.** To determine the radiation sensitivity, L4-stage hermaphrodites were 699 irradiated with a single dose of IR as indicated. After 12 hrs, worms were transferred to fresh plates (three worms per plate, five plates in total) and allowed to lay eggs for 5 hrs. After this period, adults were removed and 24 hrs later the number of hatched and unhatched embryos was scored (number of hatched larvae normalized to results after mock-treatment). As a control for embryonic survival, a heterozygous deletion mutant lacking *rad-51* on one chromosome was used.

B02 treatment. Synchronized L1 larvae were grown as liquid culture in S Medium with heat
inactivated (3 x 5 min at 65 °C, vortexing inbetween) *E. coli* strain OP50 ⁶³, containing 200
mM B02.

Mitotic germ cell cycle arrest upon IR. Worms were irradiated with 0 and 60 Gy at the late L4 larval stage as described previously ¹⁰. 16 hours post-irradiation, worms were mounted on 3% agar pads and paralyzed with 60 nM NaN₃ for DIC microscopy and the distal region of the germline was scored for number of nuclei in all focal planes within a defined area of 2 μ m x 6 μ m.

Statistical analysis. Statistical analysis was performed using Excel (Microsoft). Statistical significance was calculated with two-tailed paired Student's t-test. Box plots were generated using BoxPlotR ⁶⁴. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. The notches are defined as +/-1.58*IQR/sqrt(n) and represent the 95% confidence interval for each median. Nonoverlapping notches give roughly 95% confidence that two medians differ.

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Figure 1



Figure 2



60 Gy



Figure 4 a

RPA-1::GFP





α-RAD-51





