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A conserved ubiquitination pathway determines longevity in response to diet restriction

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Abstract

Dietary Restriction (DR) extends longevity in diverse species suggesting that there is a conserved mechanism for nutrient regulation and prosurvival responses[1]. We have discovered a role for the HECT E3 ubiquitin ligase WWP-1 as a positive regulator of lifespan in *C. elegans* in response to diet restriction. We find that overexpression of *wwp-1* in worms extends lifespan up to 20% under conditions of *ad libitum* feeding. This extension is dependent upon the FoxA transcription factor *pha-4*, and independent of the FoxO transcription factor, *daf-16*. Reduction of *wwp-1* completely suppresses the extended longevity of diet-restricted animals. However, loss of *wwp-1* does not affect the long lifespan of animals with compromised mitochondrial function or reduced insulin/IGF-1 signaling. Overexpression of a mutant form of WWP-1 lacking catalytic activity suppresses the increased lifespan of diet-restricted animals, indicating that WWP-1 ubiquitin ligase activity is essential for longevity. Additionally, we find that the E2 ubiquitin conjugating enzyme, UBC-18, is essential and specific for DR induced longevity. UBC-18 interacts with WWP-1 and is required for the ubiquitin ligase activity of WWP-1 and the extended longevity of worms overexpressing *wwp-1*. Taken together, our results indicate that WWP-1 and UBC-18 function to ubiquitinate substrates that regulate DR induced longevity.

HECT (homologous to E6AP C-terminus) E3 ligases promote the ubiquitination of proteins that are essential in a variety of cellular events. The mammalian WWP1, WWP2 and Itch family of WW domain HECT ligases (WWP ligases) were initially identified in a search for novel proteins containing WW domains, which are modular protein interaction domains recognizing short proline motifs in their partners[2]. WWP ligases have an N-terminal C2 domain, a phospholipid membrane interaction motif, followed by four WW domains. To identify cellular pathways in which WWP E3 ligases are required, we have taken advantage of *C. elegans* as a model organism, which contains a single HECT WWP E3 ligase orthologue, *wwp-1* (Y65B4BR.4). Disruption of *wwp-1* using RNA interference (RNAi) yields a lethal phenotype late in embryogenesis characterized by abnormal embryogenesis despite normal cell proliferation[3]. The *wwp-1(ok1102)* mutant allele has a partially penetrant embryonic lethal phenotype[4]. Independent of the early developmental function of *wwp-1*, we found that loss of *wwp-1* decreased stress resistance during adulthood (Supplementary Fig. 1 a-b, e), leading us to investigate a possible role in longevity. Loss of *wwp-1* function by RNAi or mutation reduced lifespan at 25°C (Supplementary Fig. 2 a-b), but not at 20°C (Supplementary Fig. 3 a-b),

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consistent with a role for *wwp-1* in stress resistance. To investigate if increased expression of *wwp-1* extended longevity in N2 (WT) worms, we created stable transgenic lines which express an N-terminal GFP-WWP-1 fusion protein, under the control of the endogenous *wwp-1* promoter in which expression of *wwp-1* mRNA is increased by approximately 50% (Supplementary Fig. 4). Overexpressing *wwp-1* transgenic lines (GFP::WWP-1) lived up to 20% longer than controls expressing *gfp* under the same promoter (Fig. 1a), indicating that *wwp-1* is a positive regulator of lifespan.

When diet is restricted lifespan is extended in diverse species suggesting that there is a conserved mechanism for nutrient regulation of aging. DR in worms can be reproduced genetically using *eat-2(ad1116)* mutant worms[5,6]. Reduced levels of *wwp-1* completely suppressed the extended longevity of *eat-2* mutant animals (Fig. 1b). Suppression of DR extended lifespan by *wwp-1* depletion is unlikely to be due to increased food intake, since no difference in pharyngeal pumping rates with loss or knockdown of *wwp-1* in N2 or *eat-2 (ad1116)* worms was observed (Supplementary Table 4).

We tested whether loss of *wwp-1* suppressed the extended longevity of animals subjected to DR by reduced food intake imposed by bacterial dilution in liquid culture. N2 animals exhibited a bell-shaped curve for lifespan in response to varying bacterial concentrations (Fig. 1c)[7-9]. The lifespan of N2 animals grown under DR conditions was more than double that of animals fed *ad libitum* (AL) (Fig. 1c-d). In contrast, the lifespan of *wwp-1(ok1102)* mutant worms across the entire food concentration range did not change noticeably (Fig. 1c-d), indicating that WWP-1 plays an essential role in regulating the response to nutrient intake and longevity. Similar results were seen using an additional method of DR, solid plate DR[10] (Supplementary Fig. 5). To determine if DR could affect *wwp-1* expression we used qPCR to quantify *wwp-1* mRNA and found no difference in *wwp-1* expression in animals grown at DR and AL conditions (Supplementary Fig. 6). Finally, expression of a GFP::WWP-1 transgene partially rescued the suppression of DR longevity in *wwp-1(ok1102)* mutants (Supplementary Figs. 4 and 7). Since loss of *wwp-1* prevented the extension of lifespan of animals grown using three different DR methods, we conclude that *wwp-1* is essential for the increased longevity response to DR.

The Foxa transcription factor PHA-4 is required to specifically mediate DR induced longevity in *C. elegans*[8]. RNAi reduction of *pha-4* suppressed the increased longevity of worms overexpressing *wwp-1* (Fig. 1e), but not when these worms were fed bacteria expressing dsRNA against *daf-16*, the forkhead transcription factor required for the increased longevity due to reduced insulin/IGF1 signaling[11,12] (Fig. 1f). Mutations in the iron sulfur component of complex III, *isp-1*, increase longevity by reducing mitochondrial function[13-15]. RNAi of *wwp-1* did not suppress the extended lifespan of *isp-1(qm150)* mutant animals (Fig. 1g), and had only minor suppressive effects on lifespan extension of another mitochondrial mutant, *clk-1(qm30)*, and in *cyc-1* RNAi-treated worms (Supplementary Fig. 8). Partial loss of function mutations in the insulin/IGF-1 receptor homolog, DAF-2, increase lifespan in a *daf-16* dependent, *pha-4* independent manner[8,16]. RNAi depletion of *wwp-1* had no effect on the long lifespan of *daf-2* mutant animals (Fig. 1h and Supplementary Fig. 9a). Our results indicate that loss of *wwp-1* does not make animals sick, but rather specifically regulates the response to DR that results in extended longevity.

Ubiquitination by HECT ligases requires the intermolecular transfer of ubiquitin from an associated E2 to the E3 ligase prior to transfer to a lysine in the target protein[17]. These transfers depend on the formation of a thioester bond between ubiquitin and a conserved cysteine in the HECT domain. Mutation of this cysteine renders HECT ligases catalytically inactive and the mutants act as dominant negatives *in vivo*[18]. We established an *in vitro* ubiquitination assay for WWP-1 ligase activity using *C. elegans* embryo extract as a source of substrates. In the presence of extract, bacterially-expressed GST-WWP-1 had very robust ligase activity, which

was abolished by mutation of the catalytic cysteine (C762A) of WWP-1 (Fig. 2a). We then compared the longevity of *eat-2(ad1116)* transgenic animals that overexpress a GFP-WWP-1 (C762A) fusion protein driven by the *wwp-1* promoter to a control line expressing *gfp* under the same promoter. Two independent *eat-2(ad1116)* transgenic lines expressing the dominant negative *wwp-1* had a significantly shorter lifespan, comparable to WT animals (Fig. 2b). Therefore, the ubiquitin ligase activity of WWP-1 is essential for DR-induced longevity.

UBC-18 is a putative E2 that regulates pharyngeal morphogenesis during early embryonic development[19-21]. UBC-18 is homologous to human UbcH7, and similar to *S. cerevisiae* Ubc5p and Ubc4p[21]. Recently a two-hybrid screen using UBC-18 as a bait identified WWP-1 and the RING finger E3 ligases, ARI-1 and F56D2.2 as UBC-18 interactors[19]. Unlike *ubc-18* and *ari-1* dsRNA, inactivation of *wwp-1* by dsRNA treatment failed to produce a pharynx unattached phenotype in *pha-1(e2123)* animals, suggesting that WWP-1 may function with UBC-18 to ubiquitinate targets not involved in pharyngeal development[19]. Consistent with this, RNAi of either *ari-1* or F56D2.2 did not affect the long lifespan of *eat-2* mutant animals (Supplementary Fig. 10).

We found that UBC-18 is indeed a functional E2. UBC-18 formed a thiol ester bond with ubiquitin (Fig. 3a), and recombinant WWP-1 ubiquitin ligase activity required UBC-18 and E1 *in vitro* (Fig. 3b). Extracts prepared from worms mutant for *ubc-18*, *ubc-18(ku354)*[21], greatly reduced WWP-1-dependent ubiquitin ligase activity, which was restored by the addition of recombinant UBC-18 (Fig. 3b). Finally, we confirmed that UBC-18 and WWP-1 associate *in vitro* (Fig. 3c).

We tested whether *ubc-18* was essential for DR-induced longevity. Like *wwp-1*, *ubc-18* plays a role in stress resistance in *C. elegans* (Supplementary Fig. 1c-e). However, we found that overexpression of *ubc-18* was unable to extend lifespan in *C. elegans* (Supplementary Fig. 11). Possibly, UBC-18 is not limiting for WWP-1 function in lifespan. Loss of *ubc-18* function reduced lifespan at 25°C (Supplementary Fig. 2c), but only slightly at 20°C (Supplementary Fig. 3c-d). However, RNAi depletion of *ubc-18* completely suppressed the increased longevity of *eat-2* mutants (Fig. 4a). This decreased lifespan is unlikely to be due to impaired pharynx function, since RNAi was initiated at the L1 stage when the pharynx is completely developed, and *ubc-18* RNAi initiated at the first day of adulthood, also suppressed the increased longevity of *eat-2(ad1116)* animals. Like *wwp-1* depletion, we did not see a difference in pharyngeal pumping rates with loss of *ubc-18* (Supplementary Table 4), and RNAi depletion of *ubc-18* had no effect on the long lifespan of *isp-1(qm150)* (Fig. 4b) or *daf-2* mutant animals (Fig. 4c and Supplementary Fig. 9b). In addition, epistasis analysis of *wwp-1* and *ubc-18* indicated that combined knockdown of both genes by RNAi in *eat-2(ad1116)* animals did not shorten lifespan any further than RNAi of either single gene (Fig. 4d). Finally, knockdown of *ubc-18* suppressed the extended lifespan of *wwp-1* overexpressing animals (Fig. 4e).

In summary, the UBC-18/WWP-1 complex functions to specify the longevity response of DR animals. Because E2s often function with multiple E3s, it is surprising to find that *ubc-18* was not only essential, but also specific for the response to DR. M7.1 (UBC-2/LET-70) is most homologous to UbcH5, a mammalian E2 that associates with HECT ubiquitin ligases. Unlike *ubc-18*, loss of *ubc-2* did not specifically suppress the extended longevity of *eat-2* mutants and resulted in general sickness of animals (Supplementary Fig. 12). The other E3s that interact with UBC-18 may be dedicated instead to the developmental function of UBC-18, as is the case for ARI-1[19]. It is interesting that WWP-1 and UBC-18 expression is observed in several neurons localized in the head and tail of adult animals (Supplementary Fig. 13), since many recent studies in *C. elegans* and *Drosophila* suggest that signals derived from the nervous system can control longevity[22-25]. Although it is intriguing to speculate that a few key neuronal cells in the nervous system are the site of action of WWP-1/UBC-18 to regulate longevity,

expression is not confined to a few neurons, as is the case for the DR regulator, SKN-1B[7]. Furthermore, expression of WWP-1/UBC-18 is found in intestinal cells, another site where longevity cues are expressed in the worm[26].

Since several transcription factors have been identified as targets for the mammalian orthologues of *wwp-1*[17], we investigated whether WWP-1 may target one of the two transcription factors essential for DR longevity in the worm: PHA-4 and SKN-1B[7,8]. The genetic epistasis analysis of *ubc-18/wwp-1* suggested that PHA-4 may be a target for ubiquitination. Using our *in vitro* ubiquitination assay for WWP-1, we were unable to detect ubiquitinated conjugates for either PHA-4 or SKN-1B in a purified system (Supplementary Fig. 14). Recently it has been shown that *pha-4* and the CeTor pathway antagonize one another to regulate longevity in adults[27]. Our results might suggest that *wwp-1* may feed into the CeTor pathway as well. The identification of the targets of UBC-18/WWP-1 is needed to allow precise placement of this complex in the DR pathway.

Our study uncovers for the first time a role of the ubiquitin pathway in longevity in response to dietary restriction. Given the strong conservation of *wwp-1* with mouse and human WWP1, an attractive hypothesis is that the mammalian orthologue will also be critical for DR induced longevity. A detailed understanding of the pathways that mediate the benefits of DR may lead to novel therapies for age-related diseases.

Methods Summary

C. elegans *methods*

The *wwp-1* mutant strain was generated by backcrossing RB1178 [*wwp-1(ok1102)*] to N2 three times (Supplementary Fig. 15b). Nematodes were handled using standard methods [28].

Lifespan analysis

Lifespan analyses were performed as described [29]. Bacterial dilution DR lifespans were performed as described[8] with the following modifications: synchronized populations of eggs were hatched and grown at 20°C on NG agar plates containing OP50 *E. coli* until the L4 larval stage when they were transferred to plates of OP50 containing 100 μg/ml FUDR. At day 1 adulthood, worms were transferred into liquid culture. All lifespans were performed at 20°C unless noted.

Protein extraction for *in vitro* ubiquitination assay

C. elegans embryos were isolated using an alkaline hypochlorite solution from gravid N2 worms grown at 20°C [30]. The embryos were resuspended in lysis buffer [50 mM Tris/HCl (pH 7.5), 0.75 mM EDTA, 1.5 mM DTT, 2.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A] and homogenized with 30 strokes in a Dounce homogenizer. The extract was centrifuged 15,000 × g at 4°C and stored at −70°C.

In vitro ubiquitination assay

The ubiquitination assay was carried out by incubating 1 μg Flag-tagged ubiquitin, 0.5 μg GST-WWP-1 (WT or C762A mutant), 0.1 μg UBC-18 and 15-20 μg embryo extract in 30 μl reaction buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 5 mM ATP) for 1 hour at 30° C. The reaction was stopped with sample buffer and run on denaturing protein gels. Ubiquitinated substrates were identified by anti-Flag (M2, Sigma) immunoblotting. For *in vitro* ubiquitination assays using purified components, similar conditions were administered except 0.5 μg UBC-18 and 1 μg E1 were used. To measure UBC-18 ubiquitin conjugation, a similar reaction was performed and the reaction was stopped with sample buffer lacking β-mercaptoethanol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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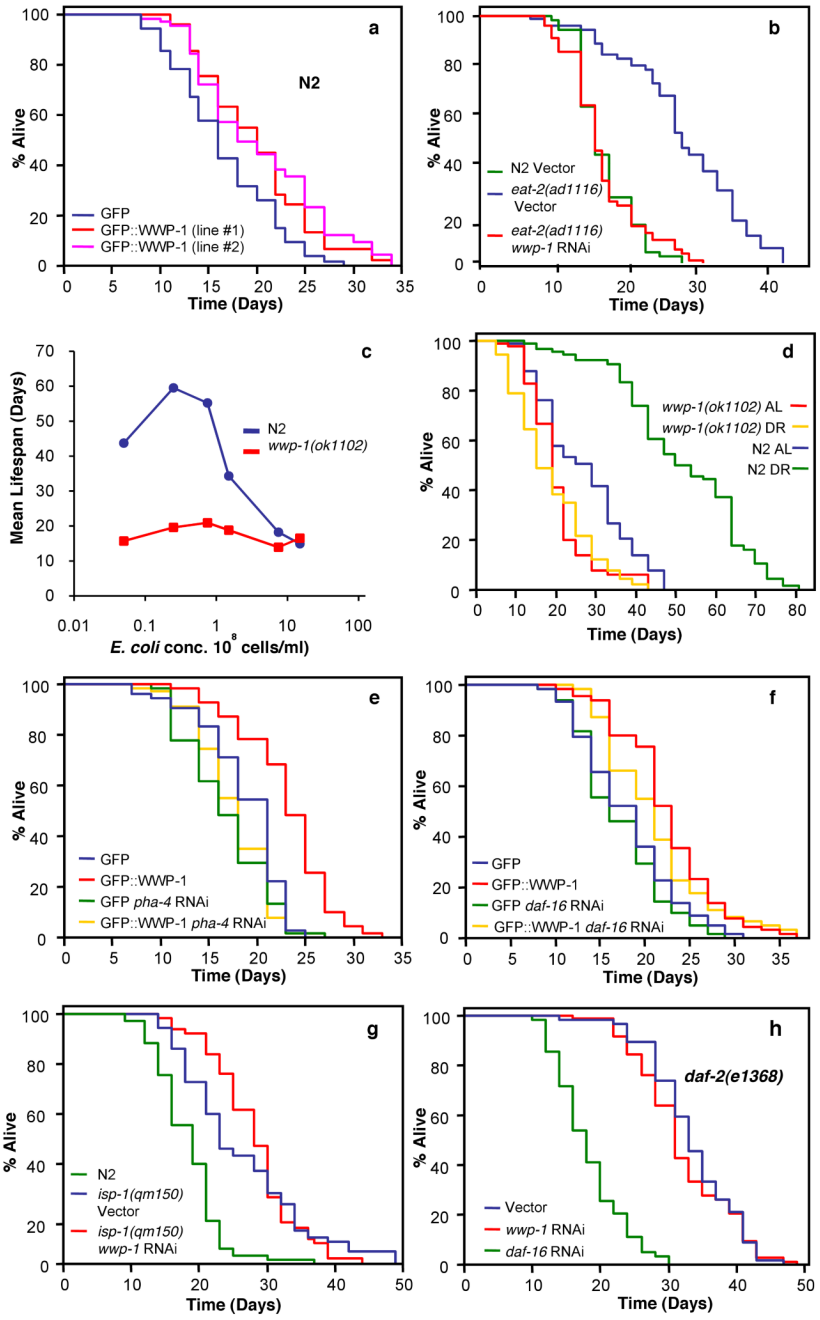

Figure 1. *wwp-1* is required and specific for the extension of lifespan by dietary restriction (DR)

Lifespan values are given in Supplementary Tables 1 and 2. Two-way ANOVA analysis is presented in Supplementary Table 7. Supplementary Fig. 15a,c show data confirming specific knockdown of *wwp-1* expression by RNAi. **a**, Two independent *wwp-1* overexpressing strains (GFP::WWP-1) can extend longevity compared to control worms expressing *gfp*. **b**, Lifespan analysis of *eat-2(ad1116)* mutant animals fed bacteria expressing *wwp-1* dsRNA or control vector. **c**, Lifespans of N2 and *wwp-1(ok1102)* mutant worms grown in S basal buffer with different *E. coli* concentrations. **d**, Lifespan analysis of N2 and *wwp-1(ok1102)* mutant worms grown in DR or AL (*ad libitum*) *E. coli* concentrations. **e**,**f**, Lifespan analysis of *wwp-1*

overexpressing worms (GFP::WWP-1) or a control line fed bacteria expressing *pha-4* dsRNA (**e**), or *daf-16* dsRNA (**f**). **g**,**h**, Lifespan analysis of *isp-1(qm150)* (**g**) and *daf-2(e1368)* (**h**) fed bacteria expressing *wwp-1* dsRNA or control vector.

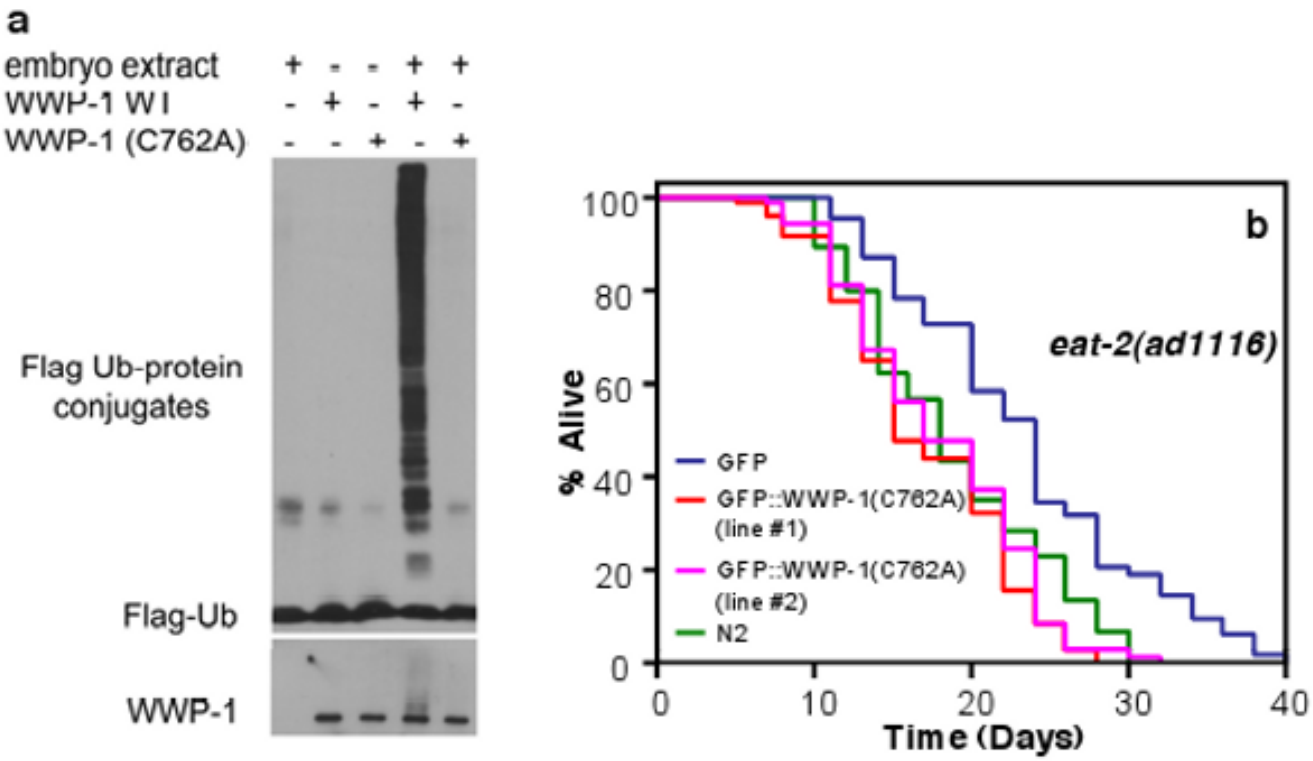

Figure 2. WWP-1 ubiquitin ligase activity is essential for DR induced longevity

a, Mutation of the conserved catalytic cysteine of WWP-1 abolishes ubiquitin ligase activity. *In vitro* ubiquitination assay of recombinant WT WWP-1 or mutant WWP-1 (C762A) using *C. elegans* embryo extract. **b**, *eat-2(ad116)* mutant worms expressing a dominant negative *wwp-1*(C762A) have significantly shorter lifespans than control worms expressing GFP. Lifespan values are given in Supplementary Table 1.

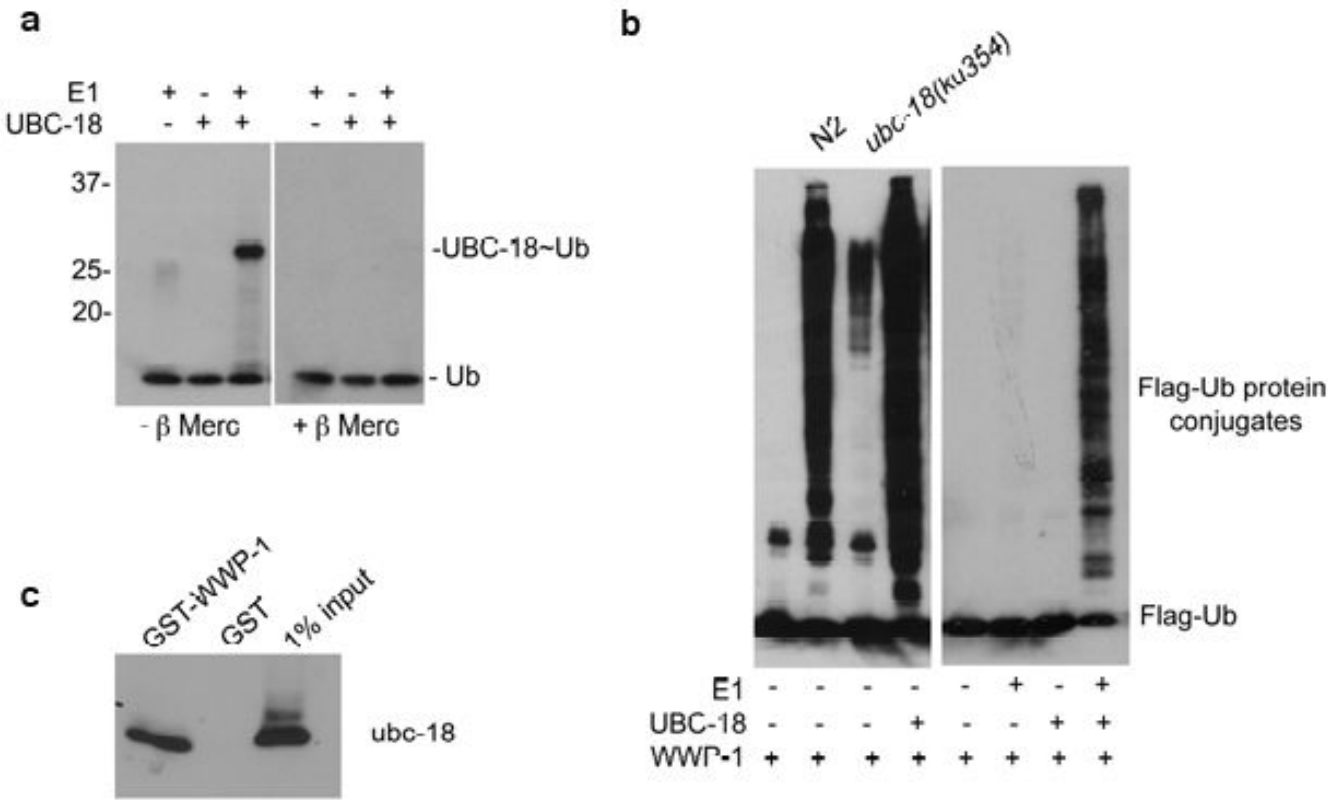

Figure 3. WWP-1 exhibits ubiquitin ligase activity in a UBC-18 dependent manner *in vitro*

a, UBC-18 forms thiol sensitive adducts with ubiquitin. *In vitro* ubiquitin conjugation reaction in which samples were subjected to SDS/PAGE with or without β-mercaptoethanol (β-mercap). **b**, UBC-18 is essential for ubiquitin ligase activity *in vitro*. Left panel: *In vitro* ubiquitination assay of WT WWP-1 using N2 or *ubc-18* mutant [*ubc-18(ku354)*] embryo extract. Recombinant UBC-18 was added in the last lane. Right panel: *In vitro* ubiquitination assay using purified components. **c**, GST pulldown assay in which GST WWP-1 (or GST alone) bound to glutathione-agarose beads was incubated with cell lysates expressing *ubc-18*.

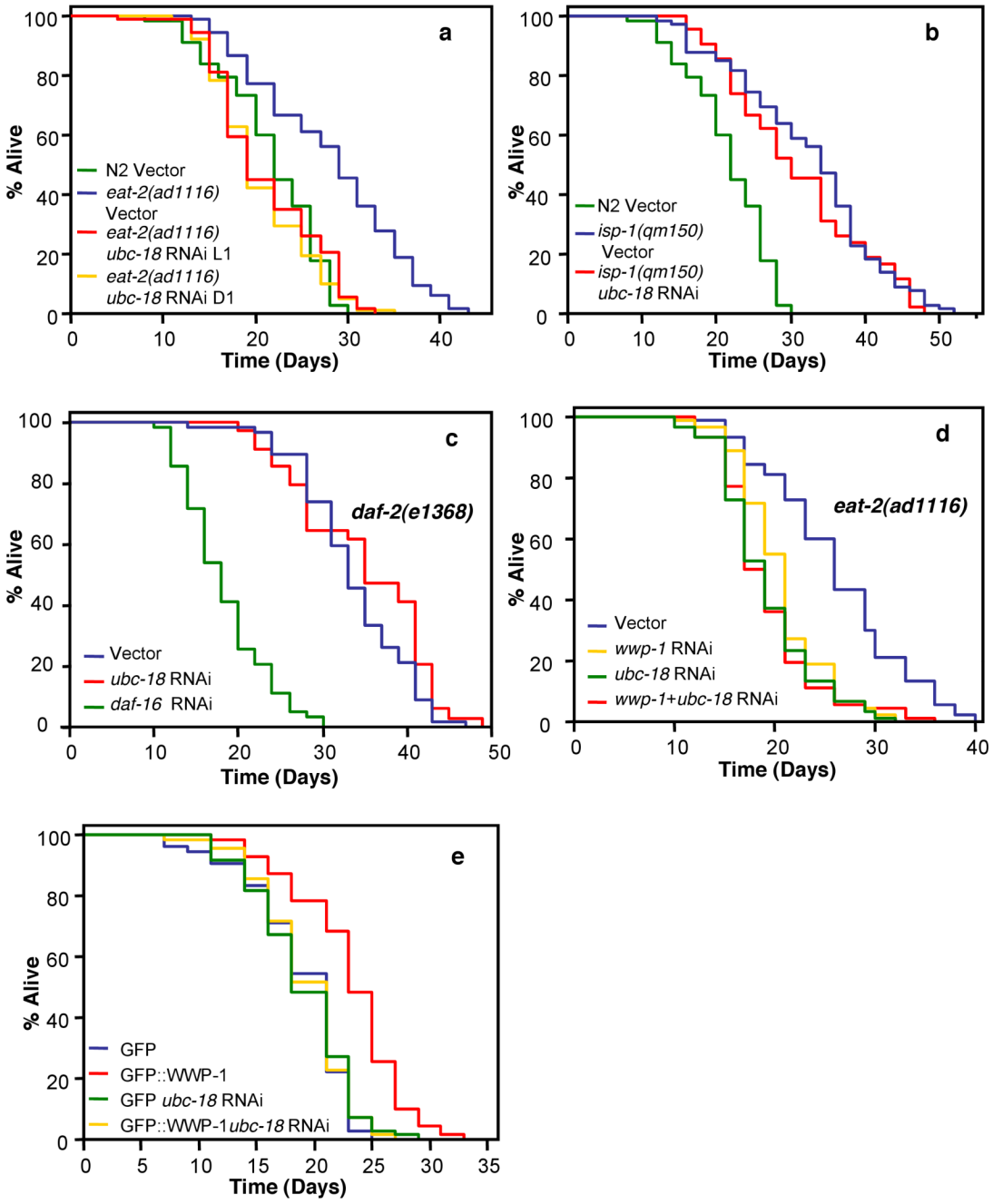

Figure 4. WWP-1 and UBC-18 function together to regulate DR induced longevity

Lifespan values are given in Supplementary Table 1. Two-way ANOVA analysis is presented in Supplementary Table 7. Knockdown of *ubc-18* expression by RNAi is shown in Supplementary Fig. 15d. **a**, Lifespan analysis of *eat-2(ad1116)* mutant worms fed bacteria expressing *ubc-18* dsRNA or control vector initiated upon hatching of eggs (L1) or day 1 adults (D1). **b**,**c**, Lifespan analysis of *isp-1(qm150)* (**b**) and *daf-2(e1368)* (**c**) fed bacteria expressing *wwp-1* dsRNA or control vector. **d**, Lifespan analysis of *eat-2(ad1116)* mutant animals fed bacteria expressing both *wwp-1* dsRNA and vector (*wwp-1* RNAi), *ubc-18* dsRNA and vector (*ubc-18* RNAi) or *wwp-1* and *ubc-18* dsRNA (*wwp-1* + *ubc-18* RNAi). **e**, Lifespan analysis of

wwp-1 overexpressing worms (GFP::WWP-1) or control worms (GFP) fed bacteria expressing *ubc-18* dsRNA or control vector.