A heterotypic assembly mechanism regulates CHIP E3 ligase activity

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Abstract

CHIP (C-terminus of Hsc70-interacting protein) and its worm ortholog CHN-1 are E3 ubiquitin ligases that link the chaperone system with the ubiquitin-proteasome system (UPS). CHN-1 can cooperate with UFD-2, another E3 ligase, to accelerate ubiquitin chain formation; however, the basis for the high processivity of this E3s set has remained obscure. Here, we studied the molecular mechanism and function of the CHN-1–UFD-2 complex in Caenorhabditis elegans. Our data show that UFD-2 binding promotes the cooperation between CHN-1 and ubiquitin-conjugating E2 enzymes by stabilizing the CHN-1 U-box dimer. However, HSP70/HSP-1 chaperone outcompetes UFD-2 for CHN-1 binding, thereby promoting a shift to the autoinhibited CHN-1 state by acting on a conserved residue in its U-box domain. The interaction with UFD-2 enables CHN-1 to efficiently ubiquitylate and regulate S-adenosylhomocysteinease (AHCY-1), a key enzyme in the S-adenosylmethionine (SAM) regeneration cycle, which is essential for SAM-dependent methylation. Our results define the molecular mechanism underlying the synergistic cooperation of CHN-1 and UFD-2 in substrate ubiquitylation.

Keywords C. elegans; CHIP/STUB1/CHN-1; metabolism; ubiquitin ligase; UFD-2

Introduction

The ubiquitin-proteasome system (UPS) comprises a well-studied enzymatic cascade that transfers the small protein ubiquitin (Ub) onto a protein substrate (Kerscher et al., 2006). The last step in the UPS enzymatic cascade is mediated by ubiquitin ligases (E3s), the largest and most diverse group of proteins within the UPS, which are responsible for substrate selection and specificity (Komander, 2009; Buetow & Huang, 2016). Mechanistically, two classes of E3 enzymes are commonly found. HECT (homologous to E6AP C-terminus) E3s form an intermediate thioester bond with ubiquitin before catalyzing substrate ubiquitylation. By contrast, RING (Really Interesting New Gene)/U-box E3s form molecular scaffolds that bring E2-Ub and the target protein into proximity, thereby facilitating direct Ub transfer to the latter (Wenzel et al., 2011; Riley et al., 2013; Buetow & Huang, 2016). In some instances, other proteins, called ubiquitin chain elongation factors, or E4s, may be required to achieve efficient poly-ubiquitylation of substrates. The first E4 described was yeast Ufd2p (Richly et al., 2005), a U-box domain-containing protein that engages Ub via its N-terminal region, thus enhancing Ub chain elongation on a pre-ubiquitylated substrate (Koegl et al., 1999; Hatakeyama et al., 2001; Buetow & Huang, 2016). Although higher eukaryotes, including humans, have an ortholog of yeast Ufd2p, its Ub-interacting motif has little sequence homology (Hänzelmann et al., 2010; Liu et al., 2017), suggesting that the function of UFD-2 as an E4 is not evolutionarily conserved.

Early Caenorhabditis elegans studies showed that UFD-2 interacts directly with CHN-1 (the nematode ortholog of mammalian CHIP) to form an E3-E4 complex that can efficiently oligo-ubiquitylate the myosin chaperone UNC-45 (Hoppe et al., 2004). CHIP (C-terminus of Hsc70-interacting protein), initially identified as a tetratricopeptide repeat (TPR) protein that interacts with heat shock proteins (Ballinger et al., 1999), is a U-box E3 ubiquitin ligase that mediates ubiquitylation of chaperone client proteins, promoting their degradation (Murata et al., 2001; Paul & Ghosh, 2014; Joshi et al., 2016). In contrast to the model proposed based on these early findings,
more recent studies have revealed that UFD-2 acts as a true E3 ligase that poly-ubiquitylates UNC-45 independent of CHN-1, suggesting that both UFD-2 and CHN-1 act as E3s in the same or an overlapping substrate space (Hellerschmidt et al., 2018). A recent study aimed at identifying substrates of human CHIP and the human UFD-2 ortholog UBE4B supports the possibility of shared substrate scope (Bhuripanyo et al., 2018). Nevertheless, despite the key role of CHIP/CHN-1 in protein quality control networks, little is known about the regulation of its activity and its interaction with UFD-2, as well as the functional role of this E3 pair.

To address these questions, we combined in vitro and in vivo assays with computational approaches and lipidomic and proteomic studies in C. elegans to uncover the mechanism that controls CHN-1 activity. The crystal structure of murine CHIP bound to the C-terminal decapetide of the HSP90 chaperone via the TPR domain revealed an asymmetric dimerization in which the two CHIP protomers adopt a “closed” conformation that restricts E2 access to one of the U-box domains, and thus E3 activity (Zhang et al., 2005). Subsequent molecular modeling of mouse CHIP indicates dynamics between symmetric and asymmetric autoinhibited dimers, which can be regulated by binding proteins (Ye et al., 2017). Our results show that although metazoan UFD-2 lacks E4 activity, it acts as a pre-conditioning factor to influence the conformational flexibility of CHN-1, thus boosting its processivity. Mechanistically, UFD-2 binding to the TPR domain stabilizes the open conformation of CHN-1, allowing the U-box dimer to discharge more Ub-conjugating enzymes (E2) in a single ubiquitylation cycle. We also demonstrated that the heat-shock protein HSP70/HSP-1 interacts with the TPR and U-box domains of CHN-1 to stabilize the closed/auto-inhibitory state of the CHN-1 dimer, thus limiting its interaction with E2 and UFD-2. Furthermore, we identified potential substrates for the CHN-1–UFD-2 pair, including S-adenosylhomocysteinase (AHCY-1), a metabolic enzyme previously not known to be a client of heat-shock proteins. However, the UFD-2-dependent increase in CHN-1 processivity and consequent CHN-1 auto-ubiquitylation (auto-Ub) also lead to CHN-1 turnover, thereby reducing organismal proteostasis capacity. Collectively, our results indicate an interplay between chaperones and UFD-2 in modulating CHN-1 activity. This processivity-switching behavior of CHN-1 has important implications for its roles in regulating proteostasis, metabolism, and potentially other cellular processes.

Results

UFD-2 promotes CHN-1 processivity and its cooperation with E2s

Binding between CHN-1 and UFD-2 was previously demonstrated via yeast two-hybrid and in vitro pull-down assays (Hoppe et al., 2004). Beyond the physical interaction, the molecular regulation of the potent ubiquitylation activity of the CHN-1–UFD-2 complex has not yet been studied in detail. A quantitative assessment of an E3 ligase activity is generally performed by examining its auto-ubiquitylation (auto-Ub) ability. For this purpose, an in vitro ubiquitylation assay with recombinant E1, E2, Ub, and E3 proteins with an ATP source can be used. The activity of the E3 enzyme can then be determined via Western blot analysis using antibodies against ubiquitin ligase or Ub itself. First, we chose E2 conjugating enzymes with which CHN-1 and UFD-2 are known to cooperate in the auto-Ub reaction. Mammalian CHIP can interact with various E2s, particularly from the UbeH5/UBE2D family (UbeH5a/UBE2D1, -b/2, and -c/3) (Jiang et al., 2001; Soss et al., 2011). Similarly, CHN-1 cooperates with UBE2D2 to mono-ubiquitylate (mono-Ub) C. elegans DAF-2, the nematode insulin/insulin-like growth factor 1 (IGF-1) receptor (Tawo et al., 2017). To study the activity of CHN-1 and UFD-2, we compared their abilities to auto-Ub in the presence of each of the UBE2D-family proteins. We observed that CHN-1 interacted most efficiently with UBE2D1 and least efficiently with UBE2D3, whereas UFD-2 interacts similarly with UBE2D1-3 (Fig EV1A and B). When we performed an auto-Ub reaction with both E3s, we observed a significant increase in CHN-1 poly-ubiquitylation (poly-Ub) activity, even when the E2 used in the reaction was UBE2D2 or UBE2D3, with which CHN-1 alone inefficiently cooperates (Figs 1A and EV1A). The presence of UFD-2 in the reaction also potentiated CHN-1 auto-Ub with LET-70, the C. elegans ortholog of UBE2D proteins (Fig EV1C). Furthermore, the presence of UFD-2 increased CHN-1 activity with the UBE2N-Uev1a E2 complex (Fig EV1D), which catalyzes the formation of free Ub chains that are then transferred to substrate proteins (Soss et al., 2011). We also concluded that the induction of E3 ligase activity is unidirectional as we did not detect any significant changes in the auto-Ub of UFD-2 under the same conditions (Fig EV1E). Interestingly, UFD-2 did not interact with UBE2N-Uev1a, indicating specificity between E2s with U-box domain-containing E3s (Fig EV1D and E). We also ruled out the possibility that it was UFD-2 that modified CHN-1 because it was unable to ubiquitylate inactive CHN-1H218Q, which probably lost its affinity toward its cognate E2 (Tawo et al., 2017) (Fig EV1F). However, we noted that CHN-1H218Q was modified specifically in the presence of an inactive, recombinant UFD-2 mutant with a P951A substitution (Ackermann et al., 2016) (bands marked with an asterisk), which might suggest recovery of CHN-1H218Q minimal activity, reflecting possible structural changes in the CHN-1 U-box domain during an interaction with UFD-2.

To gain insight into CHN-1 processivity, we performed time-dependent auto-Ub experiments (with reaction times of 10, 20, 40, 80, and 120 min). We observed that the presence of UFD-2 increased both mono- and poly-Ub of CHN-1 from the 40-min time point (Fig 1A). When we included the UBE2D2 or UBE2D3 E2s in the reaction, which CHN-1 does not efficiently utilize (Fig EV1A), we also observed a significant increase in CHN-1 auto-Ub in the presence of UFD-2, even at the earliest time point (30 min) (Fig EV1G and H). Next, we aimed to determine what molar ratio of the two E3 triggers high CHN-1 processivity. To this end, we performed UFD-2 titrations (0.3, 0.65, and 1.3 μM) with a fixed concentration of CHN-1 (1.3 μM). We observed an almost twofold increase in CHN-1 auto-Ub in the presence of 0.65 μM UFD-2, which roughly translates to one CHN-1 dimer per one UFD-2 monomer (Fig 1B). At higher UFD-2 concentrations, we did not observe any further increase in CHN-1 ubiquitylation; however, this effect could also be related to a Ub shortage in the reaction buffer, as UFD-2 robustly consumes the available Ub for its auto-Ub (Fig EV1B). Additionally, by deleting the CHN-1 TPR domain and generating CHN-1Δ110aa), we confirmed the involvement of the TPR domain in UFD-2 binding (Hoppe et al., 2004), as we did not observe an increase of CHN-1Δ110aa activity by UFD-2 (Fig EV1I). Therefore, we wanted to test whether UFD-2 can regulate the processivity of
**Figure 1. UFD-2 activates CHN-1.**

A. Time-dependent (0, 10, 20, 40, 80, and 120 min) CHN-1 auto-Ub was performed as indicated using UbWT and UBE2D1 E2. Protein samples were resolved via SDS-PAGE and immunoblotted with anti-CHN-1 antibodies. Below, a graph representing the nmol of ubiquitylated CHN-1 vs. time for CHN-1 alone (black) or CHN-1 + UFD-2 (cyan). Plotted data are the mean of three technical replicates. Error bars represent the standard error of measurement (SEM); statistical significance was determined using Pearson’s correlation coefficients which define the statistical relation between two continuous variables [CHN-1 vs. time, CHN-1 + UFD-2 vs. time, and CHN-1 vs. CHN-1 + UFD-2 with increasing time] (*P < 0.05; **P < 0.01; ***P < 0.001).

B. CHN-1 auto-Ub was performed in the presence of UFD-2 with the increasing molar concentration as indicated. Protein samples were resolved via SDS-PAGE and immunoblotted with anti-CHN-1 antibodies. Right, signal quantification of the unmodified CHN-1 (yellow), ubiquitylated fraction <70 kDa (cyan) and >70 kDa (magenta), plotted as a percentage of different CHN-1 species present in the indicated condition. Plotted data are the mean from the three technical replicates. Error bars represent the SEM; statistical significance was determined using a two-way ANOVA test (**P < 0.01).

C. Auto-Ub was performed as indicated using recombinant CHN-1 and UFD-2P951A, UBE2D1 E2, UbWT, UbKTR, or Ub with substitutions of lysines 29, 48, and 63 to arginines (UbKTR). Protein samples were resolved via SDS-PAGE and immunoblotted with anti-CHN-1 antibodies.

D. Surface plasmon resonance (SPR) sensorgrams of the interaction between linear di-Ub (M1 - linear from UbiQ) and *C. elegans* UFD-2 (magenta) or *S. cerevisiae* Ufd2p (cyan). Y-axis: Response unit (RU) value. X-axis: nmolar (nM) concentration of linear di-Ub.

E. CHN-1 auto-Ub was performed as indicated in the presence of recombinant *C. elegans* UFD-2 or *S. cerevisiae* Ufd2p and UBE2D1 E2. Protein samples were resolved via SDS-PAGE and immunoblotted with anti-CHN-1 antibodies.

Data information: Representative immunoblots for at least three independent experiments are shown in the panels.
CHN-1 independent of its E3 activity. First, we found that the defect in UFD-2<sup>2951A</sup> activity is due to its inability to bind an E2 enzyme (Fig EV1J). Next, we performed a CHN-1 ubiquitination reaction in the presence of UFD-2<sup>2951A</sup>. We detected substantial enhancement of both the mono- (using lysine-less Ub (UbK0)) and the poly-Ub activity of CHN-1, regardless of the type of Ub chain (wild-type Ub or variants with substitutions of lysines 29, 48, 63 to arginines (UbK3TR)) (Fig 1C). To rule out the possibility that UFD-2<sup>2951A</sup> retained residual activity, we also used a UFD-2 variant (1–910 aa) lacking the entire U-box domain (909–984 aa). We confirmed that this UFD-2 deletion mutant could still stimulate CHN-1 activity (Fig EV1K). Our results suggest that UFD-2 binding to CHN-1 via its TPR domain enhances the cooperation between CHN-1 and E2s, thus resulting in more efficient auto-Ub.

Budding yeast protein Ufd2p can operate as a Ub chain elongation factor by interacting directly with Ub through its N-terminal region (Liu et al., 2017). Although higher eukaryotes have an ortholog of yeast Ufd2p, the Ub-interacting motif has little sequence homology (Hänzelmann et al., 2010; Liu et al., 2017), suggesting that the function of UFD-2 as an E4 is not evolutionarily conserved. To investigate whether the increased activity of the CHN–UFD-2 complex might stem from the elongation function of UFD-2, we tested whether UFD-2 retained its ability to interact with Ub using surface plasmon resonance (SPR) experiments. By contrast to Ufd2p, full-length UFD-2 did not bind linear Ub chains (Fig 1D).

Unlike yeast Ufd2p, and perhaps to compensate for Ub binding loss, UFD-2 can induce proteosynthesis of its partner CHN-1 (Fig 1A and E). This observation suggests that UFD-2 lost its ability to directly elongate Ub chains during evolution.

**UFD-2 induces a structural gain of function in CHN-1**

The different conformations afforded by dynamic and flexible motifs and oligomerization are important for the functionality of various E3 ligases (Liu & Nussinov, 2011; Kamadurai et al., 2013; Narayan et al., 2015; Kolipoulos et al., 2016; Faull et al., 2019). Therefore, we decided to analyze CHN-1 for oligomerization and conformational flexibility after binding to UFD-2. We observed a tendency of CHN-1 to form oligomers, which can be seen in the size-exclusion chromatography (SEC) as a prominent peak corresponding to its oligomeric distribution followed by a peak corresponding to the CHN-1 dimer (Fig EV2A). When we mixed CHN-1 and UFD-2 in equal molar ratios and performed SEC separation, we obtained peaks corresponding to the respective proteins without the CHN-1 oligomerization signal, suggesting a shift toward CHN-1 dimer stabilization by UFD-2 (Fig EV2A). Unfortunately, we did not detect a stable CHN-1–UFD-2 complex upon SEC separation, highlighting the dynamic and transient nature of this interaction.

To gain mechanistic insight into the role of UFD-2 binding to CHN-1, we performed hydrogen-deuterium exchange mass spectrometry (HDX–MS) of the dimerization process of both CHN-1 alone and CHN-1 in the presence of UFD-2. We detected 99 peptides with 84.2% sequence coverage of CHN-1. Some discrepancies in the profile of detected peptides between the two conditions (CHN-1 alone and CHN-1 + UFD-2) (Fig EV2B and Table EV1). In this table, peptides with missing "%Diff U%" values are CHN-1 peptides that were not detected when CHN-1 was complexed with UFD-2. However, most of these were redundant with other detected peptides, except for the "NNLKMRT" peptide, which extends from the 52<sup>nd</sup> to 57<sup>th</sup> residue on CHN-1 and is a linker between the pair of antiparallel alpha helices of the 2<sup>nd</sup> TPR in CHN-1. This may indicate that CHN-1 interacts with UFD-2 through this region. However, this peptide was not detected over the entire time range of the HDX–MS experiment, and therefore, its role in the CHN-1–UFD-2 complex cannot be determined. Figures 2A and EV2B depict the following model of our HDX–MS data, which detects at least three dynamic events at 10 s and 60 s. Namely: (i) the turn in the coil-coil motif (aa 145–159) is stabilized early on upon dimerization of the coiled-coil domains, (ii) the TPR domain is stabilized upon recognition by UFD-2, leaving the distal helices (aa 21–40 and 92–112) exposed to the solvent. At later times, the TPR domain stabilizes against the long helix of the coil-coil domain, (iii) the U-box domain (aa 198–
Figure 2.
transitions from a weak interaction with its coil-coil domain to a stable dimer on a longer time scale. Available crystal structures of CHIP homologs support our HDX-MS analysis without a chaperone (Nikolay et al., 2004) and with HSP90 (Zhang et al., 2005). In the absence of a TPR-binding chaperone, only the dimer domains are revealed by the crystal structure, with no resolution of either the turn in the coil-coil domain or the TPR domain. Notably, the TPR domain has only been resolved by NMR, whereas it stabilizes into its crystal form in the presence of an HSP substrate (Zhang et al., 2005). Furthermore, structural analysis of murine CHIP shows that the bound TPR domain is further stabilized against the long helix of its coiled-coil domain in one of its monomers (Zhang et al., 2005; Ye et al., 2017). Our computational model noted that this interaction is much weaker in CHN-1, suggesting more structural dynamics in worms. Based on prediction with AlphaFold-Multimer (preprint; Evans et al., 2022), we argue that, unlike CHIP (Zhang et al., 2005; Ye et al., 2017), CHN-1 folds into a symmetric structure. It has been shown that HSP90, which regulates CHIP activity (Narayan et al., 2015), presumably by blocking the E2 binding site of one of the protomers (Zhang et al., 2005). Thus, our finding that UFD-2 promotes CHN-1 processivity is consistent with a symmetric structure for CHN-1—in such a system, upon binding, UFD-2 stabilizes the U-box dimer with both E2 sites (Fig EV2C).

To investigate this possibility, we titrated UBE2D1 (0.5–4 μM) at a fixed concentration of CHN-1 (1.3 μM) or CHN-1 complexed with UFD-2P951A, which cannot interact with UBE2D1, and conducted an auto-Ub assay. We observed that at a constant Ub concentration, increasing the E2 concentration led to increased CHN-1 activity. However, even at the highest E2 concentration (4 μM), CHN-1 processivity did not reach the same level as in the presence of inactive UFD-2P951A and approximately eightfold lower E2 concentration (0.5 μM) (Fig 2B). Thus, the increased CHN-1 activity of the CHN-1–UFD-2 complex was not due to an increased local E2 concentration but rather to the enhanced processivity of the E2 enzyme bound to the CHN-1 U-box domains. To verify this hypothesis, we performed an E2-discharging assay in the presence of CHN-1 alone or after mixing with UFD-2P951A to track the use of charged E2 by CHN-1 only. We observed that in the presence of UFD-2P951A, CHN-1 could discharge at least twice as much UBE2D1-Ub compared with CHN-1 alone, which becomes prominent when used 2 μM concentration of UBE2D1-Ub, which was revealed by the increasing accumulation of uncharged UBE2D1 (Figs 2C and EV2D). These results suggest that the binding of inactive UFD-2P951A can stabilize the U-box dimer CHN-1 and that both domains can bind and unload E2. To further verify this possibility, we performed another auto-Ub assay with the Ube2W conjugating enzyme, which is known to maintain a strict 1:1 stoichiometry with a substrate (Christensen et al., 2007; Tatham et al., 2013; Vittal et al., 2013) and to catalyze mono-Ub of CHIP (Scaglione et al., 2011). Considering these characteristics, we added CHN-1 or CHN-1/UBE2D1P951A to Ube2W-charged Ube2W and Ube2WFLAG-charged Ube2W and followed the ubiquitylation profile. When both charged E2 species are included in the reaction, we should observe mono-Ub of CHN-1 by Ub and Ube2WFLAG (due to the FLAG tag, Ub molecules migrate slower on SDS–PAGE, allowing differentiation of mono-Ub from mono-UbFLAG on a single immunoblot) and a twofold increase in the level of CHN-1 ubiquitylation for the complex with UFD-2P951A compared with that of CHN-1 alone. Indeed, we noted the predicted increase in CHN-1 mono-Ub with the two Ub variants (Fig 2D), which confirms double increase in E2 enzyme capacity of CHN-1 in the presence of UFD-2. It has been previously shown that HSP70 chaperone can reduce CHIP-dependent ubiquitylation of folded substrates (Wang et al., 2011; Narayan et al., 2015; Kim et al., 2017). To verify whether the worm ortholog of HSP70, HSP-1, affects the interaction of CHN-1 with E2, we performed another auto-Ub experiment with charged Ube2W. Indeed, the presence of HSP-1 in the reaction reduced the Ub conjugation to CHN-1 and altered the ratio of Ub to UbFLAG (Fig EV2E). The increase in the stoichiometric ratio of ubiquitylated CHN-1 in the presence of UFD-2P951A suggests the existence of dimeric CHN-1 with two available U-box domains, while its HSP-1-induced decrease might suggest that HSP-1 promotes a CHN-1 conformation that limits E2 access to the U-box domains.

HSP-1 and UFD-2 modulate CHN-1 processivity by stabilizing its inactive and active conformation, respectively

The three TPR domains in CHIP act as a binding platform for C-terminal peptides of the HSP70 and HSP90 chaperones, which contain a conserved EEVD motif (Zhang et al., 2005; Paul & Ghosh, 2014). As CHN-1 also binds UFD-2 via the TPR domain, we investigated whether HSP-1 or DAF-21 (the nematode HSP90 ortholog) could interfere with the interaction between CHN-1 and UFD-2. We first examined protein-protein interactions between CHN-1 and UFD-2, HSP-1, or DAF-21 using enzyme-linked immunosorbent assays (ELISAs). CHN-1 showed a higher affinity for HSP-1 and DAF-21 compared with UFD-2 (Fig EV3A). Next, we tested whether the chaperones could compete with UFD-2 for CHN-1 binding. We performed an ELISA-based titration assay to determine the dissociation of CHN-1 from immobilized UFD-2 induced by the presence of HSP-1 or DAF-21. As the concentration of chaperones increased, the CHN-1 signal decreased (increased dissociation from the complex with UFD-2), indicating that chaperones compete with UFD-2 for the CHN-1 (Fig EV3B and C). To verify the influence of HSP-1 and DAF-21 on the activity of the CHN-1–UFD-2 pair, we performed auto-Ub reactions in the presence of the chaperones. HSP-1 significantly reduced the auto-Ub activity of CHN-1 and blocked the stimulatory capacity of UFD-2 in this process (Figs 3A and EV3D). Removal of the C-terminal EEVD motif mitigated the inhibitory effect of HSP-1. By contrast, DAF-21 did not affect the UFD-2-dependent enhancement of CHN-1 activity (Fig 3A).

Next, we performed peptide mapping on peptide microarrays to pinpoint the interaction interface between the two ligases. For this, we used purified CHN-1 tagged with His::SUMO and His::SUMO alone (as a control). These proteins were incubated on two UFD-2 peptide microarrays consisting of 7- and 13-aa peptides. Signal enrichment analysis suggested that the two UFD-2 consensus sequences, EAAKAELEE and EEDYDVP, were the predominant interactor motifs. HSP70/90 uses a similar acidic C-terminal peptide with the EEVD sequence to bind to the TPR domain of target proteins (Scheufler et al., 2000; Gazda et al., 2013), and the HSP-1 C-terminal EEVD peptide affected CHN-1 activity (Fig 3B). Therefore, we examined whether the identified UFD-2 peptides could also regulate CHN-1. To this end, we performed CHN-1 auto-Ub reactions in the presence of the UFD-2-derived peptides identified in the peptide microarray data. We found that only the KEYEAKAELEEYEDV peptide from UFD-2 significantly stimulated CHN-1 auto-Ub...
Figure 3.
An EEYD sequence is present in this peptide, suggesting that UFD-2 can utilize an EEYD-like motif for CHN-1 binding. Furthermore, multiple sequence alignment revealed that in the EEVD motif, the tyrosine (Y) is evolutionarily conserved among higher eukaryotes (Fig EV3E). To define the functional role of the UFD-2 EEYD motif, we generated a chimeric recombinant HSP-1 protein carrying an N-terminal EEYD instead of EEVD and UFD-2 with the opposite change (EEVD to EEYD). Notably, we observed stimulation of CHN-1 auto-Ub by HSP-1EEVD, opposite to the inhibitory effect of wild-type HSP-1 (Figs 3D and EV3F). By contrast, UFD-2EEVD no longer stimulated CHN-1 activity and even abrogated the CHN-1 poly-auto-Ub activity. These data indicate that independent of ubiquitylation activity, UFD-2 promotes CHN-1 turnover, presumably by not being involved in the modulation of CHN-1 processivity. By contrast, removing the subsequent eight residues (Δ95 variant) abrogated the CHN-1 poly-auto-Ub activity. Interestingly, the stimulating effect of UFD-2 was still observed, indicated by an increase in mono-Ub CHN-1Δ95 (Fig EV3G). CHN-1Δ95 has residues that might be involved in an interaction with UFD-2, including D110 and subsequent coils and helices; thus, the CHN-1D110 mutant (lacking D110) does not show any gain of activity in the presence of UFD-2 (Fig EV1LI). It is known that a position homologous to D110 in mouse CHIP (D135) is involved in HSP90 binding (Fig EV3H), suggesting that this residue is also important for the interaction with UFD-2 (Fig EV3I).

To understand why HSP-1 and UFD-2 peptides exhibit distinct effects on CHN-1 activity, we looked closely at the mechanism by which increased HSP90 or HSP70 concentrations reduce CHIP activity (Narayan et al., 2015). HSP90 stabilizes an auto-inhibited monomer in murine CHIP (Zhang et al., 2005). This state involves a salt bridge between HSP90 D501 and CHIP R273, latching the U-box and TPR domains (Fig EV3H). This observation suggests that chaperone binding can directly restrain the U-box from participating in Ub processivity. To show that a similar mechanism is at play in inhibiting ubiquitylation by HSP-1, we mutated R230 (homologous position to R273 in CHIP) to alanine to weaken the CHN-1 U-box interaction with the HSP-1 peptide, thus abrogating its inhibitory effect. Indeed, we observed reduced inhibition of the CHN-1R230A/UFD-2 complex by HSP-1 (Fig 3F). This finding is consistent with the model that HSP-1 stabilizes the autoinhibited state of CHN-1 by interacting with the TPR and U-box domains, thereby affecting its interaction with E2 enzymes (Fig 3G). On the other hand, UFD-2 can avoid interacting with R230 by, for example, forming a helix that cannot extend toward the U-box, thus inducing uncorrelated mobility of the TPR domains with respect to the U-box domains that promotes a steady-state open conformation of CHN-1 (Fig 3G), which explains maintaining its boosting effect on CHN-1R230A.

**UFD-2 promotes CHN-1 turnover independent of E3 activity**

Our *in vitro* studies indicate that interaction with UFD-2 enhances CHN-1 auto-Ub; therefore, *in vivo* interaction with UFD-2 could promote CHN-1 turnover. To verify this hypothesis, we performed Western blot analysis of CHN-1 and UFD-2 protein levels in young adult worms. As expected, CHN-1 abundance was significantly increased in *ufd-2(tm1380)* null allele worms. Next, we used CRISPR/Cas9 editing to generate animals expressing the catalytically inactive UFD-2P951A. Consistent with our prediction, in worms expressing UFD-2P951A, the CHN-1 protein level was significantly lower than in *ufd-2(tm1380)* animals and comparable to wild-type worms (Fig 4A). Furthermore, proteasome inhibition by MG132 stabilized CHN-1 in wild-type worms to levels similar to those in *ufd-2(tm1380)* worms (Fig 4B). Finally, in *ufd-2(tm1380)* animals, MG132 treatment did not increase the CHN-1 level further (Fig 4B). These data indicate that independent of ubiquitylation activity, UFD-2 is involved in CHN-1 turnover, presumably by promoting CHN-1 auto-Ub, which might lead to its proteasomal degradation.
This outcome might have negative consequences for proteostasis, for example, during proteotoxic stress, as it would decrease the level of CHN-1, which is an important quality control E3 ligase. Therefore, we hypothesized that ufd-2 deletion might enhance the proteostasis capacity of the organism by reducing CHN-1 turnover. To test this possibility, we examined the worm proteome that was sensitive to trichloroacetic acid (TCA) precipitation, an established method to assess the fraction of unfolded and aggregation-prone proteins (Cortese et al., 2005; Rajalingam et al., 2009; Depuydt et al., 2016). Indeed, we observed an increased fraction of stable proteins (insensitive to TCA) in the proteome of ufd-2(tm1380) worms compared with wild-type, and this effect was suppressed by chn-1 deletion (chn-1(by155); ufd-2(tm1380)). In contrast to the ufd-2(tm1380) worms, animals expressing the UFD-2P951A variant showed a decrease in stable protein fraction.
displayed similar levels of unstable proteins compared with wild-type worms (Fig 4C). To further explore the functional importance of CHN-1 and UFD-2 cooperation for organellar proteostasis, we measured the motility recovery rate of synchronized worms after heat stress. The results showed improved recovery in ufd-2 (tm1380) nematodes compared with controls. As expected, this effect was suppressed in the chn-1(by155); ufd-2(tm1380) double mutant, while worms expressing UFD-2P951A showed similar recovery to that of control animals (Fig 4D). In summary, our results suggest that in an E3-independent manner, stimulation of CHN-1 processivity by UFD-2 can simultaneously potentiate CHN-1 auto-Ub. One possible consequence of this interaction could be a limiting effect on the proteostasis network induced by CHN-1 turnover.

The CHN-1/UFD-2 pair regulates phosphatidylcholine synthesis via AHCY-1

Based on our results, we hypothesized that CHN-1, when functioning unaided, would exhibit low poly-Ub activity, mainly catalyzing mono-Ub of its substrates. Indeed, earlier, we showed that CHN-1, when functioned alone mono-Ub the DAF-2 insulin receptor in worms (Tawo et al., 2017). We further assumed that interaction with UFD-2 would stimulate the poly-Ub activity of CHN-1, consequently leading to efficient degradation of its specific substrates. Thus, to delineate the role of CHN-1 and UFD-2 in vivo, we decided to identify such substrates. We searched for proteins whose levels increased after chn-1 deletion (substrate ubiquitylation by CHN-1 would be affected directly) or ufd-2 (CHN-1 would not be stimulated to efficiently poly-Ub its substrates). To unbiasedly define the consequences of chn-1 and ufd-2 deletion on the C. elegans proteome and to detect proteins that accumulate in the deletion mutants, we performed label-free mass spectrometry (LC-MS/MS)-based proteomics experiment. We analyzed chn-1(by155), ufd-2(tm1380), and chn-1(by155); ufd-2(tm1380) double-mutant worms via single-shot LC-MS/MS gradients with five biological replicates. To obtain a view on the global structure of the data, we performed dimensional reduction using principal component analysis (PCA). We observed that the proteomes of the chn-1(by155), ufd-2(tm1380) and chn-1(by155); ufd-2(tm1380) mutants clustered closer together with respect to the proteomes from wild-type animals (N2 strain) (Fig EV4A). We hypothesized that potential substrates should accumulate in all mutants; therefore, we filtered the set of significantly altered proteins requiring a two-fold enrichment in all mutants versus the N2 control strain. We obtained 65 potential substrate candidates, which we visualized via hierarchical clustering (Fig EV4B and C). These potential substrates were enriched in metabolic processes, including lipid biosynthesis, as shown in Gene Ontology over-representation analysis (Fig EV4D). Among them, we identified the AHCY-1 enzyme (Figs 5A and EV4C). AHCY-1 catalyzes the reversible hydrolysis of S-adenosylhomocysteine (SAH) to homocysteine and adenosine (Palmer & Abeles, 1976, 1979). Despite the fundamental role of AHCY-1 in metabolism, its regulatory mechanisms are still enigmatic. In a yeast two-hybrid screen using a C. elegans cDNA library, we identified AHCY-1 as the prominent interactor of CHN-1 (Fig EV4E). We confirmed the interaction between the two proteins in worms via co-immunoprecipitation (Fig EV4F). Next, we tested whether AHCY-1 is a CHN-1 substrate by performing in vitro ubiquitylation assays. We confirmed that recombinant AHCY-1 is a specific substrate of CHN-1 that UFD-2 does not ubiquitylate (Fig EV4G and H). Furthermore, in the absence of UFD-2, CHN-1 poly-Ub AHCY-1 more effectively, and the level of this modification was reduced by UFD-2(Δ) or HSP-1 (Figs 5B, EV4G and I). The cooperation between CHN-1 and UFD-2 is also consistent with the detection of a similar increase in the AHCY-1 level in chn-1(by155), ufd-2(tm1380), and double-mutant worms in our proteomic analysis (Fig 5A). To further validate this observation, we monitored the endogenous AHCY-1 level via

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**Figure 5. The CHN-1/UFD-2 pair regulates lipid metabolism via AHCY-1.**

A Boxplot analysis showing the Z-score of normalized intensities of the 50 LC-MS/MS-identified peptides from AHCY-1 detected in N2 (wild-type), chn-1(by155), ufd-2(tm1380), and chn-2(by155); ufd-2(tm1380) mutant worms. The central band of each box is the median value, and the box defines the 25th (lower) and 75th (higher) quantile. The whiskers represent the minimum and maximum values in the data, excluding outliers. A data point is considered an outlier if the distance to the median is greater than 1.5 times the interquantile range distance to the median.

B Ubiquitylation of recombinant AHCY-1 was performed as indicated. Protein samples were resolved via SDS-PAGE and immunoblotted with anti-AHCY-1 antibodies. Right, quantification of the AHCY-1 modifications (unmodified, oligo-monoubiquitylated, poly-ubiquitylated) when CHN-1 alone (black), CHN-1–UFD-2 (magenta), or CHN-1–UFD-2Δ (cyan). Plotted data are the mean of three technical replicates. Error bars represent SEM; statistical significance was determined using a two-way ANOVA test (P < 0.05).

C Protein level of endogenous AHCY-1 in N2 (wild-type), chn-1(by155), CHN-1 OE, and ufd-2(tm1380) young adult worms treated with the proteasome inhibitor (MG-132, 10 μM) and DUB inhibitor (NEM, 100 mM). Protein samples were resolved via SDS-PAGE and immunoblotted with anti-AHCY-1 antibodies. Tubulin served as a loading control. Right, quantification of the modified AHCY-1 signals plotted as Ub-modified AHCY-1 species normalized to unmodified endogenous AHCY-1 signal and plotted for N2 (wild-type; black), chn-1(by155) (magenta), CHN-1 OE (yellow), and ufd-2(tm1380) (cyan). Plotted data are the mean of three biological replicates. Error bars represent SEM; statistical significance was determined using an unpaired t-test (P < 0.01).

D Representative images of GFP:AHCY-1 fluorescence in chn-1(by155), ufd-2(tm1380), and CHN-1 OE background. Scale bar = 200 μm. Below, quantification of the AHCY-1 GFP signal plotted as fluorescence intensity for GFP:AHCY-1 expressing worms (control; black), chn-1(by155) (magenta), ufd-2(tm1380) (cyan) or CHN-1 OE (yellow). Plotted data are the mean of three biological replicates. Error bars represent SEM; statistical significance was determined using a one-way ANOVA test (**P < 0.0001).

E Total lipid content in N2 (wild-type), chn-1(by155), ufd-2(tm1380), and CHN-1 OE young adult worms grown on control (plain) and ahcy-1(tm1045) (lined) feeding plates. Higher fluorescence intensity indicates increased lipid levels. Plotted data are the mean of three biological replicates. Error bars indicate SEM; statistical significance was determined using a one-way ANOVA test (**P < 0.0001).

F Schematic diagram representing the core function of AHCY-1. AHCY-1 catalyzes the reversible hydrolysis of SAH (S-adenosylhomocysteine) to Hcy (homocysteine). SAH accumulation inhibits PC (phosphatidylcholine) synthesis from PE (phosphatidylethanolamine). Right, ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) in N2 (wild-type; black), chn-1(by155) (magenta), and ufd-2(tm1380) (cyan) young adult worms. Plotted data are the mean of three biological replicates. Error bars indicate SEM; statistical significance was determined using a one-way ANOVA test (**P < 0.001).
Figure 5.
Western blotting of total lysates of wild-type worms, chn-1(by155), and ufd-2(tm1380) mutant worms, as well as worms overexpressing chn-1 (CHN-1 OE), treated with the proteasome (MG132) and deubiquitinating enzyme (DUB) (N-methylmaleimide, NEM) inhibitors. We did not observe any significant changes in the AHCY-1 level, which, according to our other observations, is a stable and abundant protein in C. elegans. However, immunoblotting with anti-AHCY-1 antibodies detected higher molecular weight smeared bands when chn-1 was overexpressed, likely corresponding to poly-Ub AHCY-1 species (Fig 5C). Furthermore, these bands were reduced in chn-1(by155) and ufd-2(tm1380) mutant worms compared with the ACHY-1 status in wild-type animals (Fig 5C). As we did not observe a change in the stability of unmodified AHCY-1 in worm lysates, which could be related to the tendency of AHCY-1 to precipitate during sample preparation (Fig EV4K), we generated a CRISPR/Cas9 knock-in GFP-based worm line to label AHCY-1 to track its localization and abundance without compromising the integrity of the worms while maintaining its natural expression level. The GFP tag did not affect AHCY-1 functionality, as its knock-out is lethal to worms (WormBase and our observations). Next, we crossed chn-1(by155), ufd-2(tm1380), and CHN-1 OE worms with animals expressing GFP::AHCY-1. Microscopic analysis of GFP::AHCY-1 showed no statistically significant changes in the AHCY-1 transcript levels (Fig EV4I), suggesting that the increase in AHCY-1 levels is posttranslationally regulated by CHN-1 and UFD-2.

Elevated homocysteine levels are linked to the deregulation of lipid metabolism and increased fat accumulation, apparent after RNA interference (RNAi) depletion of AHCY-1 in worms (Vrablik et al., 2015; Visram et al., 2018). Using the lipophilic fluorophore RediStain WormDye Lipid Green to stain and quantify the fat content of C. elegans, we confirmed that AHCY-1 depletion increases the abundance of lipids in wild-type worms by almost 60%. Overexpression of chn-1 caused an increase in total lipid content to a level similar to that detected in ahcy-1 RNAi-treated worms, and this effect was not further enhanced by AHCY-1 depletion (Fig 5E). Interestingly, mutations in chn-1 and ufd-2 caused a reduction in the overall lipid levels and uncoupled the stimulation of lipid biogenesis induced by ahcy-1 RNAi (Fig 5E). Synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE) via the de novo phospholipid methylation pathway requires a significant amount of S-adenosylmethionine (SAM) and is particularly sensitive to SAH levels (Tehlivets, 2011). Consistent with our assumption that deletion of either chn-1 or ufd-2 would positively affect AHCY-1 stability, leading to intensification of SAM-dependent methylation and PE to PC conversion, we noted that the ratio of PC to PE increased in chn-1(by155) and ufd-2(tm1380) worms (Fig 5F). In conclusion, our data suggest a functional role for the CHN-1–UFD-2 complex in AHCY-1-dependent lipid metabolism regulation.

Discussion

The crystal structure of murine CHIP E3 bound to an HSP70 decapeptide containing the EEVD motif revealed an asymmetric dimerization in which the two CHIP protomers adopt different conformations. Given the limited conformational accessibility to E2 enzymes, we consider this a “closed” state, where only one of the U-box domains in the dimer is accessible for E2 binding, and the TPR domain blocks the other (Zhang et al., 2005). In agreement with a computational model of human CHIP (Ye et al., 2017), our homology modeling of the CHN-1 dimer suggested that it can take the form of both a metastable symmetric dimer (“open” state, representing unrestricted conformational accessibility to E2), in which both U-box domains can simultaneously bind E2 enzymes and an asymmetric dimer with low ubiquitylation activity. We showed that the interaction of UFD-2 with the CHN-1 TPR domain reduces its dynamics, thus liberating its U-box domains. In this steady-state open conformation, CHN-1 achieves high poly-Ub activity due to the full functionality of the U-box domains. Consistently, we observed a twofold increase in the utilization of charged E2 by the CHN-1–UFD-2 complex compared with that of CHN-1 alone. We also showed that not only poly-Ub but also CHN-1 mono-Ub, which is the rate-limiting step of ubiquitylation, is also enhanced upon UFD-2 binding (Petroski & Deshaies, 2005). We also found that UFD-2 is unaffected, unlike CHN-1, in the complex and that CHN-1 is not a substrate of UFD-2. We believe that CHN-1 undergoes different conformational flexibility upon binding to interaction partners, affecting its activity and providing a functional regulation layer.

The N-terminal TPR domain of CHIP has been shown to interact specifically with the C-terminal EEVD motif of HSP70 and HSP90 (Zhang et al., 2005; Xu et al., 2006; Graf et al., 2010). We discovered that UFD-2 uses a slightly modified motif, that is, EEVD, to engage the CHN-1 TPR domain. Furthermore, we demonstrated that only the presence of a UFD-2 peptide containing the EEVD sequence was sufficient to promote CHN-1 activity. By contrast, the C. elegans HSP70 homolog, HSP-1, negatively regulates CHN-1 and CHN-1/UFD-2 complex activity by promoting its “closed” state and preventing E2 discharge. The GHFDVPTR sequence in the U-box domain is evolutionarily conserved in CHIP homologs from different species, but its role was not previously known. Here, we showed that CHN-1 activity is negatively regulated by the interaction between positions associated with the EEVD motif of HSP-1 and the conserved R230 position in the GHFDVPTR sequence. Through direct interactions with the CHN-1 TPR and U-box domains, HSP-1 brings both regions into proximity, thereby impairing the U-box dimer. This effect depends only on the local interaction of the HSP-1 C-terminus with the U-box and not on steric hindrance induced by the entire chaperone that could limit E2 access to U-box domains. In a co-crystal structure with CHIP, HSP90 also forms hydrogen bonds (H-bonds) between T and S in its C-terminal peptide (TSMREEVD) and the CHIP TPR domain (Zhang et al., 2005). The existence of these H-bonds between the HSP-1 peptide (GPTIEEVD) and CHN-1 is not apparent. However, the HSP-1 C-terminal sequence is rich in glycines that could more efficiently tailor the binding by forming H-bonds with the CHN-1 backbone, possibly leading to a very close interaction. It has been shown that HSP70 can interact with the CHIP TPR domain through the conserved EEVCPNIKTLQYSSAGMP sequence (in addition to the EEVD motif) (Zhang et al., 2015). However, we did not find a similar bipartite interaction between UFD-2 and CHN-1. After many attempts, we were unable to obtain a co-crystal of CHN-1 with UFD-2, and, thus, detailed insight into the organization of the complex remains enigmatic.
We observed that worm DAF-21/HSP90 has a lower affinity for CHN-1 and does not affect CHN-1 activity, unlike HSP-1/HSP70. Consistent with this observation, the C-terminal HSP70 peptide blocks CHIP activity markedly greater than the HSP90 peptide, which binds to the CHIP TPR domain weaker than the HSP70 peptide (Narayan et al., 2015). The K30A missense mutation in CHIP, which likely mimics HSP70 binding, also reduces CHIP activity. Furthermore, HSP70 inhibits CHIP-dependent ubiquitylation of folded substrates such as Smad1/5 (Wang et al., 2011), PPARγ2 (Kim et al., 2017), p53, or IRF-1 (Narayan et al., 2015). It is noteworthy that HSP70 can stimulate BAG-1 cochaperone ubiquitylation under experimental conditions that inhibit p53 and IRF-1 modification; however, the increase in BAG-1 modification was not accompanied by an increase in CHIP auto-Ub, an indicator of its activity. Moreover, stimulation of BAG-1 ubiquitylation was suppressed by the C-terminal peptide of HSP70 (64–GPTIEEVD58). Thus, the authors suggest that HSP70, through its direct interaction with BAG-1, may facilitate its modification by CHIP. HSP70 can also exert different effects on CHIP-dependent ubiquitylation of TP63 isoforms (a homolog of the p53 tumor suppressor), that is, it potentiates ubiquitylation of the TAp63 isoform and reduces modification of the ANP63 isoform (Wu et al., 2021). However, the authors did not present results on CHIP auto-Ub or on the competition between TP63 isoforms and HSP70 for CHIP binding, which would allow a precise determination of the effect of HSP70 on CHIP processivity in their experimental system. Two different heat-shock cognate protein 70 (HSC70, a member of the heat-shock protein 70 family) cochaperones, BAG-2 and HspBP1, limit CHIP activity (Alberti et al., 2004; Arndt et al., 2005; Dai et al., 2005). BAG-2-mediated inhibition is associated with reduced E2 accessibility, which is likely related to a shift favoring the CHIP “closed” state and stimulation of cochaperone-assisted CFTR maturation. Moreover, CHIP can enhance the ubiquitylation of Pael-R (Parkin-associated endothelin receptor-like receptor) by Parkin E3 ligase, and this modification was inhibited by HSP70 (Imai et al., 2002). The C. elegans Parkin ortholog, PDR-1, also interacts with CHN-1, and both are expressed in neurons and body wall muscles. However, their mechanism of action and the substrates modified by the CHN-1–PDR-1 complex are obscure. In concert with our data, the above examples indicate an evolutionarily conserved role for HSP70/HSP-1 as a negative regulator of CHIP/CHN-1. We cannot exclude the possibility that posttranslational modifications of CHN-1 or the presence of specific factors that would limit the interaction of HSP-1 with the U-box domains could sustain the ability of CHN-1 to ubiquitylate chaperone-bound substrates.

To propose a non-quality-control role for the CHN-1–UFD-2 pair, we wanted to determine the protein(s) regulated by these E3s. We identified AHCY-1 as a novel substrate that undergoes ubiquitylation-dependent turnover driven by the CHN-1–UFD-2 complex. AHCY-1 is the only eukaryotic enzyme capable of hydrolyzing SAH, which is essential for SAM-dependent methylation (Cantoni, 1975). According to the results of the yeast two-hybrid screen (conducted by Hybrigenics), AHCY-1 uses its substrate-binding domain (amino acid residues 1–156) to interact with CHN-1. This finding might suggest that CHN-1 binding alone can regulate SAH processing by AHCY-1, and we intend to investigate this possibility. PC synthesis from PE via phospholipid methylation involves a significant amount of SAM and is sensitive to SAH levels; thus, maintenance of this process requires AHCY-1 (Tehlivets, 2011). Consistent with the regulatory effect of the CHN-1–UFD-2 pair on AHCY-1, we observed an increase in PCs in worms lacking CHN-1 or UFD-2. To further understand the effect of CHN-1–UFD-2 on the SAM cycle, analysis of the levels of bound metabolites such as methionine, homocysteine, SAM, SAH, MTA, or GSH would be required. This metabolomic analysis might also help to explain the cause of the sensitivity of chn-1(by155) worms to oxidative stress (Tawo et al., 2017). This sensitivity might be related to the deregulation of homocysteine formation due to the impaired control of AHCY-1 as approximately 50% of the cysteine in glutathione, which plays a crucial role in cellular defence against reactive oxygen species, is derived from homocysteine (Vitvitsky et al., 2003). RNAi depletion of AHCY-1 increases fat accumulation in worms (Vrblík et al., 2015; Visram et al., 2018 and our results). Interestingly, we found that chn-1 or ufd-2 knockout inhibits the lipid biogenesis induced by acey-1 RNAi. Perhaps, this effect is related to AHCY-1 stabilization in tissues that are less sensitive to RNAi, meaning that its depletion was incomplete. Recent findings support the importance of CHIP in regulating the methylation status of the cellular proteome by mediating proteasomal turnover of the SAM-dependent methyltransferases PRMT1, PRMT5, and EZH2 (Zhang et al., 2016; Bhuripanyo et al., 2018). However, further studies are necessary to delineate the involvement of the CHN-1–UFD-2 pair in modulating the cellular methylation potential. Additional CHN-1–UFD-2 substrates are likely to be present in our proteomic data set, although their confirmation requires detailed kinetic analyses.

CHN-1–UFD-2 assembly may be desirable by cells in certain contexts, such as efficiently loading a substrate for the proteasome. We observed that in vitro, the CHN-1–UFD-2 pair generates long Ub chains. Long Ub chains linked via Lys48 are resistant to multiple DUBs, which might promote proteasomal signaling of substrates by providing efficient targeting for degradation (Schaefer & Morgan, 2011). Furthermore, we showed that through interactions with UFD-2, CHN-1 could cooperate with various classes of E2 enzymes. We cannot exclude the possibility that in the complex, each ligase might be able to recruit different E2s, which could, in turn, enhance the generation of a mixed or branched type of Ub chains. In addition, Kuhlbrodt and coworkers suggested a link between the DUB enzyme ataxin-3 and the CHN-1–UFD-2 complex, which could allow modulation of Ub chain signaling with various functional consequences for their targets (Kuhlbrodt et al., 2005). While our analyses do not provide a comprehensive view of the functions of the CHN-1–UFD-2 pair in vivo, they establish a starting point for elucidating the details of its regulation and cellular functions.

Based on our in vitro data, we hypothesized that interaction of CHN-1 with UFD-2 would simultaneously increase ubiquitylation of specific CHN-1 targets and turnover of CHN-1 in vivo, resulting from its increased auto-Ub. Indeed, deletion of ufd-2 in worms led to increased CHN-1 stability and protection against proteasomal degradation. Concerning the protein quality control role of CHN-1/CHIP in C. elegans, this stabilization would increase the proteostasis capacity. We believe that a reduction in CHN-1 processivity is probably desirable for HSP-1 as it would not lead to an imbalance between chaperone-mediated folding/maturation and degradation, inducing the latter. In summary, our data provide mechanistic insight into the distinct regulation of CHN-1 activity by HSP-1 and UFD-2.

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## Materials and Methods

### Reagents and Tools table

| Reagent/Resource | Source           | Identifier |
|------------------|------------------|------------|
| **Chemicals, peptides, and Recombinant proteins** | | |
| UBE1             | Boston Biochem   | Cat. # E-304 |
| UBE2D1           | Boston Biochem   | Cat. # E2-616 |
| GST UbcH5a/UBE2D1| Boston Biochem   | Cat. # E2-615 |
| UBE2D2           | Boston Biochem   | Cat. # E2-622 |
| UBE2D3           | Boston Biochem   | Cat. # E2-627 |
| UBE2N/Uev1a      | Boston Biochem   | Cat. # E2-664 |
| UBE2W            | Boston Biochem   | Cat. # E2-740 |
| 10X E3 Ligase Reaction Buffer | Boston Biochem | Cat. # B-71 |
| 10X Ubiquitin conjugation Reaction Buffer | Boston Biochem | Cat. # B-70 |
| 10X Energy Regeneration Solution | Boston Biochem | Cat. # B-10 |
| Ubiquitin        | Boston Biochem   | Cat. # U-100H |
| FLAG (DYKDDDDK)-Ubiquitin | Boston Biochem | Cat. # U-120 |
| UbNoK            | Boston Biochem   | Cat. # UM-NOK |
| UBSKTR           | Boston Biochem   | Cat. # UM-3KTR |
| M1linked- linear ubiquitin | UbiQ | Cat. # UbiQ-L01 |
| UBE2D1 ubiquitin charged | Boston Biochem | Cat. # E2-800 |
| N-Ethylmaleimide (NEM) | Sigma-Aldrich | Cat. # E3876 |
| cOmplete™, EDTA-free Protease Inhibitor Cocktail | Roche | Cat. # 11873580001 |
| MG132            | Selleckchem      | Cat. # 52619 |
| Apyrase          | Sigma-Aldrich    | Cat. # A2230 |
| Alkaline Phosphatase Yellow (pNPP) Liquid Substrate | Sigma-Aldrich | Cat. # P7998 |
| Q5 Site-Directed Mutagenesis Kit | NEB | Cat. # E05525 |
| 4x Laemml Sample Buffer | Bio-Rad | Cat. # 1610747 |
| β-Mercaptoethanol | Sigma-Aldrich    | Cat. # M6250 |
| Dynabeads™ Co-Immunoprecipitation Kit | Invitrogen | Cat. # 14321D |
| Pierce™ Anti-DYKDDDDK Magnetic Agarose | Invitrogen | Cat. # A36797 |
| RedStain™ WormDye Lipid Green | NemaMetrix | Cat. # DYE9439 |
| Rapid Gold BCA Protein Assay Kit | Thermo Fisher Scientific | Cat. # A53225 |
| IMPACT™ Kit      | NEB              | Cat. # E69015 |
| AHCY-1:6xHis     | This paper       |            |
| CHN-1            | This paper       |            |
| 6xHis-SUMO:CHN-1 | This paper       |            |
| CHN-1<sup>Δ110</sup> | This paper    |            |
| CHN-1<sup>ΔM7</sup> | This paper    |            |
| CHN-1<sup>Δ95</sup> | This paper    |            |
| CHN-1<sup>Δ229A</sup> | This paper |            |
| UFD-2            | This paper       |            |
| UFD-2<sup>Δ140</sup> | This paper    |            |
| 6xHis:UFD-2      | This paper       |            |
| 6xHis:UFD-2<sup>PP11A</sup> | Ackermann et al (2016) | |
| 6xHis:UFD-2<sup>ΔLubox</sup> | This paper |            |
### Reagents and Tools table (continued)

| Reagent/Resource | Source | Identifier |
|------------------|--------|------------|
| 6xHis::UfdZp     | This paper |           |
| HSP-1::6xHis     | This paper |           |
| 6xHis::DAF-21    | This paper |           |
| HSP-1<sup>EEVD</sup>6xHis | This paper |           |
| 6xHis::DAF-21<sup>EEVD</sup> | This paper |           |
| HSP-1<sup>EEVD</sup>6xHis | This paper |           |

### Antibodies

| Antibody          | Source                          | Identifier |
|-------------------|---------------------------------|------------|
| Anti-CHN-1 antibody | Tawo et al (2017)               |            |
| Anti-UFD-2 antibody | Ackermann et al (2016)          |            |
| Anti-AHCY-1 antibody | This study                     |            |
| Anti-Histidine antibody | Santa Cruz Biotechnology, Inc.  | Cat. # SC-53073 |
| Anti-GST antibody  | Sigma-Aldrich                  | Cat. # G1160 |
| Anti-UBE2D1 antibody | Biorad                         | Cat. # VPA00296 |

### Bacterial Strains

| Strain                        | Source                          | Identifier |
|-------------------------------|---------------------------------|------------|
| *E. coli* RNAi feeding strain | Caenorhabditis Genetics Center  | HT115(DE3) |
| *E. coli* feeding strain      | Caenorhabditis Genetics Center  | OP50       |
| Ahringer RNAi library         | Source BioScience               | C. elegans RNAi Collection (Ahringer) |
| Rosetta™ 2 (DE3)              | Novagen                         | Cat. # 71400 |
| BL21 Star™ (DE3)              | Thermo Fisher Scientific        | Cat. # C601003 |
| Top10                          | Thermo Fisher Scientific        | Cat. # C4040 |

### Oligonucleotides

| Oligonucleotide | Source | Identifier |
|----------------|--------|------------|
| pTYB21-MBP::Intein-UFD-2 | This paper |            |
| Forward: GGTGCTGGCTTTCAACAATGATTGAAGACGAGAAAGCAGG |            |            |
| Reverse: GGTGGTCTGCAGTCATTATTTCTTTGAATTTCTTT   |            |            |
| pET-6xHis::SUMO-CHN-1<sup>AE110</sup> | This paper |            |
| Forward: ATTAGAAGCCCTCCTAAAC |            |            |
| Reverse: GCTAGCTAGACCAACAAATC |            |            |
| pET-6xHis::SUMO-CHN-1<sup>AB7</sup> | This paper |            |
| Forward: TACAGTGAACAAATAGCTG |            |            |
| Reverse: GCTAGCTACAGCACAAATC |            |            |
| pET-6xHis::SUMO-CHN-1<sup>AG</sup> | This paper |            |
| Forward: TCCAACAGCGCTTACCAT |            |            |
| Reverse: GCTAGCTAGACCAAATC |            |            |
| pET-21a-VSV-HSP-1<sup>AEVO</sup>6xHis | This paper |            |
| Forward: GGCGGCCGCACTCGAG |            |            |
| Reverse: TCTCTCCGCCGCTCTCCTC  |            |            |
| pET-21a-6xHis::DAF-21<sup>EEVD</sup> | This paper |            |
| Forward: TAATGAGCATCGACATTCGAG |            |            |
| Reverse: CTCAGCTCCCTCAATCTT   |            |            |
| pET-6xHis::SUMO-CHN-1<sup>R230A</sup> | This paper |            |
| Forward: TCCACTCAGACCAACACCTAC |            |            |
| Reverse: TCGAATGCGCCAAATCTC   |            |            |
| pTYB21-MBP::Intein-UFD-2<sup>YM99</sup> | This paper |            |
| Forward: GAAGAAGAGGCTGATGATCACA |            |            |
| Reverse: TGCTCATACCATCAACCTTC   |            |            |
| pET-21a-VSV-HSP-1<sup>EEVD</sup> | This paper |            |
| Forward: ATCCAGGACTACGACCGGCCC |            |            |
### Reagents and Tools table (continued)

| Reagent/Resource | Source | Identifier |
|------------------|--------|------------|
| Reverse: GGCCGCGTCGTACTCCTCGAT | | |

### Recombiant DNA

| Reagent/Resource | Source | Identifier |
|------------------|--------|------------|
| pET28a-6xHis:Ufd2p | Liu et al (2017) | |
| pET-6xHis:SUMO-CHN-1 | | |
| pET28a-6xHis:UFD-2 | This paper | |
| pLATE31-AHCY-1-6xHis | This paper | |
| pET21a-VSV-HSP-1-6xHis | This paper | |
| pET21a-6xHis:DAF-21 | This paper | |
| pYB21-MBP:Intein-UFD-2 | This paper | |
| pET-6xHis:SUMO-CHN-1A110 | This paper | |
| pET-6xHis:SUMO-CHN-1A87 | This paper | |
| pET-6xHis:SUMO-CHN-1A95 | This paper | |
| pET21a-VSV-HSP-1EEVO-6xHis | This paper | |
| pET21a-VSV-HSP-1EEVO-6xHis | This paper | |
| pET-6xHis:SUMO-CHN-1D260A | This paper | |
| pYB21-MBP:Intein-UFD-2VEBO| This paper | |
| pET-21a-VSV-HSP-1VEBO| This paper | |

### Experimental models: Organisms/Strains

| C. elegans: Bristol (N2) strain as wild-type | CC | N/A |
| C. elegans: chn-1(by155) | CC | WormBase ID: WBVar00000641 |
| C. elegans: unc-119(ed4)II; hhIs136[unc-119(+); chn-1p:chn-1::FLAG] | Tawo et al (2017) | N/A |
| C. elegans: ufd-2(tm1380) II | CC | WormBase ID: WBVar00250374 |
| C. elegans: chn-1(by155); ufd-2(tm1380) II | This paper | N/A |
| C. elegans: chn-1(by155); ahcy-1(syb646[ahcy-1::GFP]) | This paper | N/A |
| C. elegans: ahcy-1(syb646[ahcy-1::GFP]); ufd-2(tm1380)II | This paper | N/A |
| C. elegans: unc-119(ed4)III; hhIs136[unc-119(+); chn-1p:chn-1::FLAG]; ahcy-1(syb646[ahcy-1::GFP]) | This paper | N/A |

### Software and Algorithms

| Software | Graph Pad Software, Inc. | www.graphpad.com |
|----------|--------------------------|------------------|
| ImageLab™ Version 6.0.0 build 25 | Bio-Rad Laboratories, Inc. | www.bio-rad.com/de-de/product/image-lab-software?id=KRE6P5E8Z |
| ImageJ 1.53c | Wayne Rasband, NIH, USA | www.imagej.nih.gov/ij |

### Materials

| Material | Supplier | Cat. # |
|----------|----------|--------|
| Nunc MaxiSorp™ flat-bottom | Thermo Fisher Scientific | 44-2404 |
| Hiload 16/600 Superdex S200 | GE Healthcare | GE28-9893-3S |

### Methods and Protocols

**C. elegans strains**

Worms were maintained on nematode growth medium (NGM) plates seeded with OP50 Escherichia coli bacteria at 20°C unless otherwise stated (Brenner, 1974). The following strains were used in this study: Bristol (N2), as wild-type strain, chn-1(by155), ufd-2(tm1380)II, chn-1(by155); ufd-2(tm1380)II, unc-119(ed4)III; hhIs136[unc-119(+); chn-1p:chn-1::FLAG] (Tawo et al, 2017). These strains were at least six times outcrossed against the wild-type strain to provide isogenic conditions. Strain generated in this study—PHX646 (ahcy-1(syb646[ahcy-1::GFP])) and PHX792 (ufd-2(syb792[ufd-2P951A]II) were generated by SunyBiotech using CRISPR services (http://www.sunybiotech.com). PHX646 was outcrossed 2X to N2 to generate strain WOP122. PHX792 was outcrossed 2X to N2 to generate strain WOP160.
**Generation of recombinant proteins**

All recombinant proteins were produced using a bacterial expression system. CHN-1 and the CHN-1 variants were expressed and purified from Rosetta™ 2 (DE3) cells. UFD-2, HSP-1, DAF-21, and their variants were expressed and purified from BL21 Star™ (DE3) cells. Truncations and point mutations in the protein constructs were introduced using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs; NEB). Protein over-expression was induced using 0.4 mM IPTG at 22°C for 16 h. Respective induced cell pellets were harvested via centrifugation at 4,000 rpm for 20 min at 4°C. Cells were lysed in a lysis buffer (20 mM HEPES pH 8, NaCl 300 mM, 2 mM BME, protease inhibitor, and DNase) by sonication. After sonication, the supernatant and pellet fractions were separated via high-speed centrifugation at 14,000 rpm for 1 h at 4°C. Tagged proteins were purified from the soluble fraction of the cell lysates using appropriate Ni-NTA or GST-Trap columns or chitin beads (NEB). After removing the affinity tags, affinity-purified protein fractions were subjected to gel filtration chromatography (Hiload 16/600 Superdex 200, GE Healthcare) to obtain more than 95% pure protein fractions for use in subsequent biological and biochemical experiments. For the in vitro ubiquitylation reactions, we first generated a pTYB21-UFD-2 expression vector and purified tagless UFD-2 fraction using the intein cleavage site as per the manufacturer’s protocol (NEB). The lysis buffer used for purifying this variant contained HEPES 20 mM, TritonX 0.1%, 5% glycerol, 500 mM NaCl, pH 8.0. To generate tagless CHN-1 and His-tagged CHN-1, we affinity-purified the proteins using Ni-NTA columns. Furthermore, His-SUMO tag were cleaved using SUMO protease treatment (16 h) at 4°C, and untagged CHN-1 dimeric fraction was purified via SEC.

**Peptide microarray for protein-peptide interaction studies**

This assay was performed by PEPperPRINT GmbH (https://www.pepperprint.com/). Briefly, the UFD-2 sequences were elongated with neutral GSQSGSQ linkers on the C- and N-termini to avoid truncated peptides and translated into 7 and 13 amino acid peptides with overlaps of 6 and 12 amino acids. The resulting UFD-2 peptide microarrays (containing 1,986 different peptides) were printed in duplicate and incubated with recombinant 6xHis::SUMO::CHN-1 for 16 h at 4°C. After washing, the microarrays were incubated with mouse anti-6xHis Epitope Tag DyLight680 secondary antibodies to detect bound 6xHis::SUMO::CHN-1.

**Ubiquitylation assays**

In vitro assays were performed according to an earlier protocol (Hellerschmied et al., 2018). The reactions were run at 30°C for 90 min using 60 μM ubiquitin and its variants (Boston Biochem) in the presence of 100 nM E1 (UBE1, Boston Biochem), 0.6 μM E2 (Boston Biochem), E3 ligase (CHN-1 and variants or UFD-2 and variants), E3 ligase reaction buffer (Boston Biochem), and Energy Regeneration Solution (Boston Biochem). For performing the in vitro reaction in the presence of both the CHN-1 and UFD-2 or His-tagged UFD-2 P951A, proteins were first preincubated at 16°C for 30 min in the presence of E3 ligase reaction buffer. After that, the remaining reagents were added for the ubiquitylation reaction and incubated at 30°C for the indicated time. For substrate ubiquitylation, C. elegans AHCY-1 was added as the substrate along with the other reagents and mixed with preincubated CHN-1 or pre-incubated CHN-1/UFD-2 and incubated at 30°C for 90 min. For performing the in vitro reaction in the presence of a chaperone, C. elegans 1 μM His-tagged HSP-1, His-tagged DAF-21, or other variants were preincubated with CHN-1 or CHN-1/UFD-2 at 16°C for 30 min in the presence of 1× E3 ligase reaction buffer. After that, the remaining reagents were added for the reaction and incubated at 30°C for 90 min. Reaction stopped by adding Laemmli sample buffer (Bio-Rad), including β-mercaptoethanol (Sigma-Aldrich), and incubated at 95°C for 5 min. Samples were run in 12% SDS–PAGE gels and blotted with an antibody against the protein of interest.

**E2 discharging assays**

E2 discharging experimental protocol was designed based on a modified method from Page et al., 2012. Discharging of increasing molar concentration of charged UBE2D1 (UBE2D1-Ub, Boston Biochem) was performed at 30°C for 40 min in ubiquitin conjugation reaction buffer (Boston Biochem, Cat#:B-70). Similarly, a time-dependent assay was performed using 3.3 μM UBE2D1-Ub at 30°C for different time points (5, 10, 20, 30 min) with equimolar concentrations (1 μM) of CHN-1, His-tagged UFD-2 P951A and His-tagged HSP-1. The reaction was stopped by the addition of Laemmli sample buffer (Bio-Rad). As a control experiment to check the total UBE2D1 used in the reaction, the charged UBE2D1 were treated with 50 mM DTT and boiled the sample at 90°C for 5 min to obtain the total uncharged E2. Samples were run in a 15% SDS–PAGE gel. For detecting the available UBE2D1-Ub in each condition, Western blotting was performed using an anti-ubiquitin or anti-UBE2D1 antibody. Normalized chemiluminescence intensity was obtained after maximum background subtraction from each lane. The discharging capacity at a specific condition was analyzed by detecting the band intensity of the uncharged fraction of UBE2D1 while probing with an anti-UBE2D1 antibody or available charged UBE2D1 while probing with an anti-ubiquitin antibody at the end of the reaction.

**Generation of ubiquitin-charged GST-tagged UbcH5a/UBE2D1**

1.25 μM E2 GST::Ubch5a/UBE2D1 (Boston Biochem) was charged using 58 μM ubiquitin (Boston Biochem), 0.25 μM E1 (UBE1, Boston Biochem), ATP and ubiquitin-conjugating buffer (Boston Biochem), and incubated at 30°C for 30 min. Charging reaction was stopped by adding 5 mM Ayrase (Sigma-Aldrich). The reaction mix was used as a source of UBE2D1 GST::Ubch5a/UBE2D1.

**E3-ubiquitin stoichiometry analysis using UBE2W ubiquitylation assay**

0.6 μM E2 UBE2W (Boston Biochem) was charged using 60 μM ubiquitin (Boston Biochem) or 60 μM FLAG-Ubiquitin (Boston Biochem) in the presence of ATP, ubiquitin-conjugating buffer (Boston Biochem) and incubated at 30°C for 30 min. After that, the conjugation reaction was stopped by adding 5 mM Ayrase (Sigma-Aldrich). Then, reaction mix containing UBE2W-Ub or UBE2W-UbFLAG was used in a discharging assay bin at 30°C for 40 min. The reaction was stopped by the addition of Laemmli sample buffer (Bio-Rad) including β-mercaptoethanol (Sigma-Aldrich), and boiled.
for 5 min. Samples were run in 12% SDS–PAGE gels and blotted with an antibody against the protein of interest.

**Western blotting and quantification**

Protein samples in SDS-loading dye (reducing/non-reducing) were run in 12 or 15% acrylamide gels using running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) at 120 volts (constant). The wet transfer was made at a constant 200 mA for 2 h at room temperature using transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol, pH 8.3). Blots were blocked with 5% skimmed milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature and incubated overnight with primary antibody prepared in 5% skimmed milk in TBST at 4°C. The blots were then washed three times with TBST for 10 min. Finally, the blots were incubated with secondary antibodies prepared in 5% skimmed milk in TBST for 1 h at room temperature. Imaging was performed using a ChemiDoc™ Imaging System (Bio-Rad). All antibodies used in this study are listed in the resource table. Image Lab™ (version 6.0.0 build 25) software was used for quantification. After probing with a particular antibody, first, the lane marked the bands that appeared were marked in high sensitivity mode and quantified. Normalized chemiluminescence intensities were determined after maximum background subtraction from each lane. The graphs were plotted using GraphPad Prism 9.

**Enzyme-linked immunosorbent assay (ELISA)**

2 μg/ml of UFD-2, His-tagged DAF-21, and His-tagged HSP-1 in coating buffer (100 mM NaHCO₃, 32 mM Na₂CO₃, pH 9.2) were immobilized on Nunc-Immuno plates for ELISA (Thermo Fisher Scientific) overnight at 4°C. Blocking was performed with 2% BSA for 1 h at 25°C, followed by washing with TBST (0.1% Tween 20). After incubation with increasing CHN-1 concentrations for 1 h at 16°C, unbound CHN-1 was washed away by subsequent TBST washing steps. Interacting proteins were detected using an antibody against CHN-1 (1:5,000 dilution, overnight 4°C), followed by TBST washing and the addition of an HRP-conjugated secondary antibody. After the final wash, 100 μl of pnPP substrate (Alkaline Phosphatase Yellow, Sigma-Aldrich) was added in the dark. After 15 min, the reaction was stopped by adding 50 μl of 3 M NaOH, and the absorbance was measured at 450 nm.

**Microscopy of worms**

Day 0 adult worms maintained at 20°C were immobilized with tetramisole and immediately imaged using Nikon SMZ25 microscope. Data analysis: Image processing was performed with ImageJ (Fiji) using Binary Mask and Particle Analysis Procedure with background signal subtraction.

**qPCR**

RNA extraction, genomic DNA digestion at the young adult (adult day 0-1) stage was performed as described in Ly *et al.*, 2015, with the exception that min. A total of 100 worms were used instead of a single worm per sample as in the publication, cDNA synthesis was performed using the Maxima H Minus cDNA synthesis kit (Thermo Fisher). RT-PCR was performed with SG qPCR Master Mix (EURx), according to the manufacturer’s protocol. Equipment used—Light Cycler 98. Data analyzed as the ratio between CT candidate gene (ahcy-1) averaged from 3 replicates/CT ref (actin coding gene act-1 as a reference).

**Modeling and molecular dynamics**

CHN-1 model was generated by homology modeling using the SWISS-MODEL web server (Waterhouse *et al.*, 2018) with PDB ID 2F42 and 2C2L as the templates. The primary sequence of peptides used for docking on the CHN-1 dimer model was 628–640 HSP-1 (P09446) and 894–911 UFD-2 (Q09349). The protein and peptide complex structures were subjected to an energy minimization strategy using pmem.cuda (Goetz *et al.*, 2012; Salomon-Ferrer *et al.*, 2013) from the AMBER18 package (Case *et al.*, 2018). tLeap binary (part of AMBER18) was used for solvating the structures in an octahedral TIP3P water box with 15 Å distance from the structure surface to the box edges; closeness parameter was set to 0.75 Å. The system was neutralized and solvated in a solution of 150 mM NaCl. Simulations were carried out by equilibrating the system for 1 ns (NPT) at 1 atm in 300K, followed by 10 ns NPT for nonbonded interaction using the AMBER ff14SB force field (Maier *et al.*, 2015). The particle mesh Ewald (PME) method was used to treat the long-range electrostatic interactions; hydrogen bonds were constrained using SHAKE algorithm and integration time-step at 2 fs. (Ryckaert *et al.*, 1977).

**Hydrogen deuterium exchange mass spectrometry (HDX-MS)**

Prior to HDX-MS reactions, a complex of CHN-1 (3 mg/ml) and His-tagged UFD-2 (2 mg/ml) was formed by mixing the proteins in a 1:1 molar ratio followed by incubation at 25°C for 30 min. HDX-MS of CHN-1 and CHN-1 in complex with UFD-2 were performed at five time points during the incubation with deuterium (10 s, 1 min, 5 min, 25 min, and 2 h) in triplicate. 5 μl aliquots of proteins were added to 45 μl of deuterated buffer (10 mM HEPES, 150 mM NaCl in 99.99% D₂O, pH = 8.0) at room temperature. The exchange reaction was quenched by moving the exchange aliquots to precooled tubes (on ice) containing 10 μl of quenching buffer (2 M glycine, 4 M guanidine hydrochloride, 100 mM TCEP in 99.99% D₂O, pH 2.3). After quenching, samples were frozen immediately in liquid nitrogen and kept at -80°C until mass spectrometry measurement. Samples were thawed directly before measurement and injected manually onto the nano ACQUITY UPLC system equipped with HDX-MS Manager (Waters). Proteins were digested on 2.1 mm × 20 mm columns with immobilized Nephenthesis-2 (AffiPro), for 1.5 min at 20°C and eluted with 0.07% formic acid in water at a flow rate of 200 μl/min. Digested peptides were passed directly to the ACQUITY BEH C18 VanGuard pre-column from which they were eluted onto the reversed-phase ACQUITY UPLC BEH C18 column (Waters) using a 6–40% gradient of acetonitrile in 0.01% of formic acid at a flow rate of 90 μl/min at 0.5°C. Samples were measured on the SYNAPT G2 HDX-MS instrument (Waters) in IMS mode. The instrument parameters for MS detection were as follows: ESI—positive mode; capillary voltage—3 kV; sampling cone voltage—35 V; extraction cone voltage—3 V; source temperature—80°C; desolvation temperature—175°C; and desolvation gas flow 800 l/h. The CHN-1 peptide list was obtained using non-deuterated protein samples, processed as described above for HDX experiments, and measured in Mse mode. Peptides were identified using ProteinLynx Global Server Software (Waters). The HDX-MS experiment was analyzed using DynamX 3.0 (Waters) software. The PLGS peptide list was filtered by minimum intensity criteria—3000 and minimal product per amino acid—0.3. All MS spectra were inspected manually. Final data analysis was carried out using the
Surface plasmon resonance (SPR)
SPR-based interaction analysis was carried out at 25°C on a Biacore S200 instrument (GE Healthcare). Recombinant purified His-tagged UFD-2 and His-tagged Ufd2p proteins were immobilized on NTA Biacore sensor Chips (Series S) at 20 µg/ml. Single-cycle kinetics studies were performed by passing increasing concentrations (0, 100, 200, 500, 1,000, and 2,000 nM) of analyte M1 diUb conjugates (UbiQ) in SPR buffer (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20, 0.1% BSA, 50 µM EDTA, pH 8.0). The runs for both proteins were carried out under identical conditions. All injections were compiled in the same sensorgram with the response unit (RU) on Y-axis versus time (sec) on the X-axis.

Preparation of C. elegans lysates and co-immunoprecipitation
Worms were grown at 20°C. For protein extraction, worms were collected in M9 buffer and lysed using a lysis buffer (50 mM KCl, 10 mM Tris-HCl pH 8.2, 2.5 mM MgCl₂, 0.07% NP-40, 0.7% Tween-20, 0.1% gelatine) with protease inhibitor (Roche) and in the presence of DUB inhibitor, NEM (Sigma-Aldrich). First, worms in lysis buffer were snap-frozen in liquid nitrogen. Next, the frozen samples were sonicated (40% amplitude, 5 cycles of 30 s pulses at 30 s intervals, Vibra-Cell™) on ice. Samples were centrifuged at 13,000 rpm for 15 min and the supernatants were collected. For co-immunoprecipitation, anti-DYKDDDDK (FLAG tag) magnetic beads (Anti-DYKDDDDK Magnetic Agarose, Pierce) were used. 50 µl of anti-DYKDDDDK magnetic beads slurry were used for 200 µl of worm lysate. Lysate of CHN-1::FLAG-expressing worms was used as the experimental sample and wild-type (N2) worms were used as a negative control. Worm lysates were incubated with equilibrated magnetic beads at 4°C for 1 and 2 h for UFD-2 and AHCY-1 pull down, respectively. After the desired incubations, the beads were washed three times using washing buffer (PBS with 100 mM NaCl). Samples were eluted via the addition of Laemmli sample buffer (Bio-Rad) containing β-mercaptoethanol (Sigma-Aldrich), and boiled for 5 min.

RNA interference (RNAi)
RNAi was performed using the standard RNAi feeding method and RNAi clones (Kamath & Ahringer, 2003). NGM plates supplemented with 1 mM IPTG and 25 µg/ml carbenicillin were seeded with HT115 E. coli expressing double-stranded RNA (dsRNA) against the gene of interest or, as a control, bacteria with the empty vector were used. Worms were placed on freshly prepared RNAi plates as age-synchronized L1 larvae.

TCA precipitation
Approximately 1,000 young adult worms were broken by sonication in 50% Tris-sodium dodecyl sulfate buffer (25 mM Tris, 250 mM NaCl, 5% sodium dodecyl sulfate, pH 7.4), and the debris was pelleted by centrifugation for 5 min at 20,000 rcf. To precipitate proteins in the supernatant, trichloroacetic acid (TCA, final concentration 9.3%) was added to the supernatant and incubated at room temperature for 1 h. The supernatant was removed, leaving the protein pellet intact. Pellet was washed twice with 200 µl of cold acetone and centrifuged at 14,000 rpm for 5 min. Next, the pellet was dried by placing the tube in a 95°C heat block for 5–10 min to evaporate the acetone. The protein precipitate (TCA-insoluble fraction) was dissolved in 150 µl of 350 mM NaOH for 1 h at room temperature. Total protein concentration was determined using the Rapid Gold BCA Protein Assay (Thermo Scientific).

Heat stress recovery
Approximately 50 young adult worms were washed from NGM plates and rinsed three times with M9 buffer. The worms were further suspended in a 1 mL M9 buffer. The worms to be heat stressed were incubated at 33°C for 60 min, whereas the control animals were incubated at 20°C for 60 min. Next, worm movement was recorded for 2 min using the WormLab system (MBF Bioscience). The frame rate, exposure time, and gain were set to 7.5 frames per second, 0.0031 s, and 1, respectively. The distance the worms travelled before and after heat stress was analyzed using the WormLab software (MBF Bioscience).

Proteomics
For proteomic analysis, the following young adult strains were utilized: N2, ufd-2(tm1380), chin-1(by155) and ufd-2(tm1380); chin-1 (by155). Protein digestion: 4% SDS in 100 mM HEPES pH = 8.5 was used for lysis, and the protein concentrations were determined. 50 µg of protein was subjected to tryptic digestion. Proteins were reduced (10 mM TCEP) and alkylated (20 mM CAA) in the dark for 45 min at 45°C. Samples were subjected to SP3-based digestion (Hughes et al., 2014). Washed SP3 beads (SP3 beads (Sera-Magcan) Magnetic Carboxylate ModifiedParticles (Hydrophobic), and Sera-Can (TM) Magnetic Carboxylate Modified Particles (Hydrophilic)) were mixed equally, and 3 µl of beads were added to each sample. Acetonitrile was added to a final concentration of 50%, and the samples were washed twice using 70% ethanol (200 µl) on an in-house-made magnet. After an additional acetonitrile wash (200 µl), 5 µl of digestion solution (10 mM HEPES pH 8.5 containing 0.5 µg Trypsin (Sigma-Aldrich) and 0.5 µg LysC (Wako) was added to each sample and incubated overnight at 37°C. Peptides were cleaned on a C18 column using 2 × 200 µl acetonitrile washes and eluted in 10 µl of 5% DMSO in an ultrasonic bath for 10 min. Formic acid and acetonitrile were added to final concentrations of 2.5% and 2%, respectively. Samples were frozen until LC-MS/MS analysis. Liquid chromatography and mass spectrometry: LC-MS/MS instrumentation consisted of a nLC 1200 coupled to a nanoelectrospray source to a QExactive HF-x (Thermo Fisher Scientific) mass spectrometer. Peptide separation was performed on an in-house-packed column (75 µm inner diameter, 360 µm outer diameter), and the column temperature was maintained at 50°C using a column oven (PRSO-V2). The LC buffer system consisted of 0.1% formic acid (A) and 0.1% formic acid in 80% acetonitrile (B). Peptides were separated using a 90 min gradient applying a linear gradient for 70 min from 7 to 29 % B and then ramped to 65% B within 10 min, followed by a linear increase to 95% B within 5 min. 95% B was held for 5 min. Before each run, the column was re-equilibrated to
0% B. The mass spectrometer operated in a data-dependent acquisition mode targeting the top 22 peaks for collision-induced fragmentation and MS2 spectra acquisition. MS1 spectra were acquired in a scan range from 350 to 1,650 m/z allowing a maximum injection time of 20 ms for an AGC target of 3e6. Spectra were acquired at a resolution of 60,000 (at 200 m/z). Ions were isolated in an isolation window of 1.3 m/z using an AGC target of 1e6 and a canaximum injection time of 22 ms. Spectra were acquired at a resolution of 15,000. The scan range for the MS2 spectra was set to 200–2,000 m/z. The normalized collision energy was 28. Dynamic exclusion was set to 20 s. Data analysis: Acquired raw files were correlated to the Uniprot reference C. elegans proteome using MaxQuant (1.5.3.8) (Cox & Mann, 2008) and the implemented Andromeda search engine (Cox et al., 2011). Label-free quantification and matching between runs were enabled using default settings. Carbamidomethylation of cysteine residues was set as a fixed modification. Oxidation of methionine residues and acetylation of protein N-termini were defined as variable modifications. The false discovery rate (FDR) was controlled using the implemented revert algorithm to 1% at the protein and the peptide-spectrum match (PSM). To identify significantly changed proteins, we performed a one-way analysis of variance (ANOVA), correcting for multiple testing using a permutation-corrected FDR method (Lencz et al., 2011).<ref>Score) was attributed to each interaction as previously described (Formstecher et al., 2005). The PBS relies on two different levels of analysis. First, a local score considers the redundancy and independence of prey fragments and the distribution of reading frames and stop codons in overlapping fragments. Second, a global score considers the interactions found in all of the screens performed by</ref>
the Hybrigenics Services using the same library. This global score represents the probability of interaction being non-specific. The scores were divided into four categories for practical use, from A (highest confidence) to D (lowest confidence). A fifth category (E) flags explicit interactions involving highly connected prey domains previously found several times in screens performed on libraries derived from the same organism.

**Statistical analysis**

For normal distribution with one independent variable experiments, unpaired t-test or one-way ANOVA analysis was used, while two-way ANOVA was used for the data with more than one independent variable. For nonlinear data, the correlation between the data sets computed using Pearson’s correlation coefficients to determine the extent of statistical relationship using P-value (Two-tailed test). Level of significance was represented in the form of P-value (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001). All plots, unless stated otherwise, were plotted in the GraphPad Prism 9.

**Data availability**

Computational models, HDX-MS data, plasmids, antibodies and worms generated by the authors will be distributed upon request to other researchers. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD028023 (Perez-Riverol et al., 2019), and are accessible at http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD028023

**Expanded View for this article is available online.**

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**Author contributions**

Aniruddha Das: Conceptualization; data curation; formal analysis; validation; investigation; visualization; methodology. Pankaj Thapa: Conceptualization; data curation; formal analysis; investigation; visualization; methodology. Ulises Santiago: Formal analysis; visualization. Nilesh Shanmugam: Formal analysis; investigation. Katarzyna Banasiak: Formal analysis; investigation; visualization. Katarzyna Dobrowska: Formal analysis; investigation. Hendrik Nolte: Formal analysis; investigation; visualization. Natalia A Szulc: Formal analysis; visualization. Rose M Gathanugu: Investigation. Dominik Cysowski: Formal analysis. Marcus Kruger: Resources. Michał Dadlez: Resources. Marcin Nowotny: Resources; supervision. Carlos J Camacho: Conceptualization; formal analysis. Thorsten Hopper: Resources. Wojciech Pokrzywa: Conceptualization; resources; data curation; formal analysis; supervision; funding acquisition; validation; visualization; project administration.

**Disclosure and competing interests statement**

The authors declare that they have no conflict of interest.

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