Ubiquitination is a reversible post-translational modification that has emerged as a critical regulator of synapse development and function. However, the mechanisms that regulate the deubiquitinating enzymes (DUBs) responsible for the removal of ubiquitin from target proteins are poorly understood. We have previously shown that the DUB ubiquitin-specific protease 46 (USP-46) removes ubiquitin from the glutamate receptor GLR-1 and regulates its trafficking and degradation in Caenorhabditis elegans. We found that the WD40-repeat proteins WDR-20 and WDR-48 bind and stimulate the catalytic activity of USP-46. Here, we identified another mechanism by which WDR-48 regulates USP-46. We found that increased expression of WDR-48, but not WDR-20, promotes USP-46 abundance in mammalian cells in culture and in vivo. Inhibition of the proteasome increased USP-46 abundance, and this effect was nonadditive with increased WDR-48 expression. We found that USP-46 is ubiquitinated and that expression of WDR-48 reduces the levels of ubiquitin–USP-46 conjugates and increases the t1/2 of USP-46. A point-mutated WDR-48 variant that disrupts binding to USP-46 was unable to promote USP-46 abundance in vivo. Finally, siRNA-mediated knockdown of wdr48 destabilizes USP46 in mammalian cells. Together, these results support a model in which WDR-48 binds and stabilizes USP-46 protein levels by preventing the ubiquitination and degradation of USP-46 in the proteasome. Given that a large number of USPs interact with WDR proteins, we propose that stabilization of DUBs by their interacting WDR proteins may be a conserved and widely used mechanism that controls DUB availability and function.

Ubiquitination is a widely used post-translational modification that regulates a large variety of neuronal processes including synapse development and function (1–3). The covalent attachment of ubiquitin to lysine residues on substrates by ubiquitin E3 ligases has many consequences for target proteins including degradation in the proteasome, changes in protein trafficking, or altered function. Ubiquitination is a highly regulated and reversible process, in which the removal of ubiquitin is achieved by a family of proteases called deubiquitinating enzymes (DUBs). Although there are ~600 ubiquitin ligases encoded by the human genome, there are only ~95 DUBs, suggesting that DUB function is tightly regulated (4, 5). Indeed, increasing evidence shows that DUBs can be highly selective for substrates and appear to have very specific cellular functions (5–7). Additionally, because DUBs are intracellular proteases and the ubiquitination of proteins affects their function with profound cellular consequences, tight regulation of DUBs is needed to prevent indiscriminate proteolytic cleavage of ubiquitin conjugates (8).

The ubiquitin-specific protease (USP) family represents the largest subfamily of DUBs and is comprised of 56 members that regulate diverse cellular functions (4, 7, 9). Three related DUBs, USP-46, USP-46, and USP-1, have received particular attention because of their roles in regulating neuronal function, cell growth and division, and DNA damage (10, 11). We previously showed that USP-46 regulates glutamate receptor levels in neurons to control glutamatergic behavior in Caenorhabditis elegans (12). USP-46 deubiquitinates the glutamate receptor GLR-1 and protects it from degradation in the lysosome. Similarly, in mammalian neurons, USP-46 can promote glutamate receptor subunit stability by deubiquitinating the glutamate receptor subunits GluA1 and GluA2 (13), indicating that this mechanism is conserved. Characterization of Usp46 mutant mice suggests that the DUB can also regulate GABA signaling and depression-like behaviors (14, 15). In nonneuronal cells, USP-46 and USP-12 regulate a variety of cellular processes including cell proliferation and tumorigenesis (16–19). For example, USP-12 and USP-46 promote the stability of the Akt phosphatase PHLPP1, resulting in decreased cell proliferation and tumorigenesis in colon cancer cells (17, 18). PHLPP1 is a tumor suppressor that has been implicated in several cancers including glioblastoma, colon cancer, and breast cancer (20–22). In contrast, overexpression of USP-12 promotes the stability and function of androgen receptors, resulting in increased proliferation and survival of prostate cancer cells (16). Lastly, USP-1 is a critical mediator of two major DNA damage response pathways: the Fanconi anemia and DNA translesion synthesis pathways (23, 24). The critical roles of these USPs in nervous system function, cell proliferation, and cancer progression underscore the need to understand how these DUBs are regulated.

Recent work shows that DUBs can be regulated by a variety of mechanisms including transcription, post-translational modi-
fication, and interaction with other proteins (8, 9). Large-scale proteomic studies revealed that the vast majority of DUBs interact with multiple proteins (25, 26) that can regulate their subcellular localization, substrate recognition, and catalytic activity (8, 9). Interestingly, 35% of USP enzymes interact with one class of proteins, WD40-repeat (WDR) proteins, and 45% of those interact with multiple WDR proteins (10, 25). The WDR domain forms a rigid β-propeller structure that provides a stable binding surface for protein–protein interactions (27, 28).

In this study, we investigated the regulation of USP-46 protein levels by the WDR proteins WDR-48 (also known as USP-1-associated factor 1, or UAF1) and WDR-20. USP-46 and its homolog USP-12 have low intrinsic catalytic activity (29–31). Biochemical studies show that WDR-48 interacts with USP-46, USP-12, and USP-1 and stimulates their catalytic activity (25, 29, 31–33). WDR-20 forms a ternary complex with WDR-48 and either USP-46 or USP-12, but not USP-1 (25,30), and further enhances their catalytic activity in vitro (30, 31). The mechanisms underlying the ability of the WDR proteins to activate USP-12 and USP-46 were recently revealed by their crystal structures. The crystal structures of both DUBs were solved in complex with WDR-48 (34–36), and USP-12 was additionally solved in a ternary complex with WDR-48 and WDR-20 (35). Together, these structural studies revealed that the WDR proteins interact at a site distal to the active site, suggesting that an allosteric mechanism underlies the ability of the WDR proteins to stimulate the catalytic activity of the DUBs.

Here we identify another mechanism by which WDR proteins regulate DUBs. We found that USP-46 is ubiquitinated and degraded in the proteasome. We show that WDR-48 promotes USP-46 protein abundance by binding to the DUB and inhibiting its ubiquitination. We propose that binding of WDR proteins to DUBs may provide a general mechanism to regulate the stability and thus the availability of DUBs to carry out their various cellular functions.

Results

WDR-48 promotes USP-46 protein abundance in vivo

We previously showed that the C. elegans WD40-repeat proteins WDR-48 and WDR-20 bind to the deubiquitinating enzyme USP-46 and increase its catalytic activity (37). We noticed that expression of WDR-48 but not WDR-20 increases USP-46 protein levels in HEK293T cells. To test whether this effect of WDR-48 occurs in neurons in vivo, we generated transgenic worms (pzls40) containing an integrated multicopy array that overexpresses GFP-tagged USP-46 (USP-46::GFP) under control of the nmr-1 promoter (38) in ventral cord interneurons where the WDR proteins and USP-46 are known to act (12, 37). GFP-tagged USP-46 is functional because this transgene rescues specific glutamatergic behavioral defects observed in usp-46(ok2232) null mutants (Fig. S1; also “Experimental procedures”). We first tested whether expression of the WDR proteins in ventral cord interneurons altered total USP-46::GFP levels by immunoblotting total worm lysates with anti-GFP antibodies. Consistent with our previous findings in HEK293T cells (37), we found that overexpression of WDR-48 alone (wdr-48(xs)) or WDR-48 and WDR-20 together (wdr-48(xs);wdr-20(xs)) in GLR-1–expressing interneurons increased USP-46::GFP protein levels. In contrast, overexpression of WDR-20 alone (wdr-20(xs)) had no effect on USP-46::GFP protein levels (Fig. 1, A and B). Next, we directly measured the levels of USP-46::GFP fluorescence in glr-1–expressing neurons in the ventral nerve cord (VNC) of C. elegans. In WT animals, USP-46::GFP is localized in a diffuse pattern throughout the cell bodies and ventral nerve cord processes (Fig. 1C). We estimated the levels of USP-46::GFP protein in these neurons by measuring the average fluorescence of USP-46::GFP in a defined anterior region of the VNC or in the cell body of the VNC neuron PVC (see “Experimental procedures”). We found that overexpression of WDR-48 and WDR-20 in these interneurons increases USP-46::GFP abundance by 2.6-fold (Fig. 1, C and D). This effect can be largely attributed to WDR-48, because overexpression of WDR-48 alone results in a 2.2-fold increase in USP-46::GFP levels, whereas overexpression of WDR-20 alone results in a smaller increase of 1.4-fold in USP-46::GFP levels (Fig. 1, C and D). We observed similar effects in the soma of VNC neurons (see Fig. 3C), suggesting that the WDR proteins increase USP-46::GFP levels throughout the neuron. We confirmed our findings by generating a single-copy integrant of USP-46::GFP (under control of the nmr-1 promoter (pzSi1) using the Mos1 transposon-mediated single-copy insertion (MosSCI) technique (39, 40). pzSi1 was subsequently crossed into a usp-46(ok2232) null mutant background. Single-copy expression of USP-46::GFP (pzSi1 in the background of the usp-46 (ok2232) loss-of-function mutation) resulted in low but detectable levels of USP-46::GFP in the VNC. Similar to our findings with overexpressed USP-46::GFP (pzls40), we found that overexpression of WDR-48 and WDR-20 (wdr-48(xs);wdr-20(xs)) increases single copy–expressed USP-46::GFP levels by ∼2.6-fold (Fig. 1, E and F).

Because our USP-46::GFP fluorescence reporters are expressed under the control of the nmr-1 promoter, the effects of the WDR proteins on USP-46 protein levels could be indirectly due to increased nmr-1 promoter activity. We performed two experiments to test this possibility. First, we analyzed the effects of the WDR proteins on a second USP-46::GFP reporter transgene (pzls37) under control of an independent promotor, the glr-1 promoter, which is also expressed in the ventral cord interneurons (41–43). We found that expression of WDR-20 and WDR-48 (wdr-48(xs);wdr-20(xs)) resulted in a similar 3-fold increase in USP-46::GFP fluorescence in the VNC using this independent transgene (Fig. 1G). Second, we explicitly measured the activity of the nmr-1 promoter using a transcriptional reporter (Pnmr-1::GFP). We found that co-expression of WDR-48 and WDR-20 had no effect on GFP fluorescence (Fig. 1H).

These data, together with the fact that we previously observed similar effects of the WDR proteins on USP-46 protein levels in HEK293T cells (where USP-46 was expressed using the mammalian expression vector pMT3) (37), suggest that the WDR proteins increase the abundance of USP-46 protein.

USP-46 is regulated by the proteasome

We next sought to determine the mechanism by which WDR-20 and WDR-48 regulate USP-46 protein abundance by
WDR-48 promotes USP-46 stability

A. USP-46::GFP in Whole Worm Lysates

B. USP-46::GFP in Whole Worm Lysates

C. USP-46::GFP in ventral cord neurons

D. USP-46::GFP in ventral cord neurons

E. Single copy USP-46::GFP in ventral cord neurons of usp-46 null mutants

F. Single copy USP-46::GFP in ventral cord neurons of usp-46 null mutants

G. Pglr-1::USP-46::GFP in ventral cord neurons

H. Pnrr-1::GFP in ventral cord neurons
WDR-48 promotes USP-46 stability

Figure 2. USP-46 is regulated by the proteasome. A, representative immunoblot analysis of HEK293T cells transiently transfected with FLAG–USP-46 and either left untreated (UT, lane 1) or treated for the indicated times with CHX (100 μg/mL) alone (lanes 2–6) or CHX together with BTZ (1 μM) (lanes 7–11). Cell lysates were immunoblotted for FLAG–USP-46 or proliferating cell nuclear antigen (PCNA, as a loading control). B, quantification of the levels of FLAG–USP-46 from three independent experiments as described in A (normalized) are shown (means ± S.E.). C, representative immunoblot analysis of HEK293T cells treated either with CHX (100 μg/mL) alone (lanes 1–4) or CHX together with BTZ (5 μM) (lanes 5–8) for the indicated times. Cell lysates were immunoblotted for endogenous (endo.) USP46, WDR48, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as a loading control). D, quantification of USP-46::GFP fluorescence intensities (normalized) in the soma of PVC ventral cord neurons of L4 larval animals harboring a USP-46::GFP transgene expressed under control of the nmr-1 promoter (pzSi40) treated with vehicle alone (DMSO) (n = 12) or 50 μM BTZ (n = 26, means ± S.E.) for 6 h. E, quantification of USP-46::GFP fluorescence intensities (normalized) in the ventral nerve cord of animals harboring a single-copy USP-46::GFP transgene expressed under the control of the nmr-1 promoter (pzSi51) in the background of usp-46(ok2232) loss-of-function mutants treated with vehicle alone (DMSO) (n = 17) or 50 μM BTZ (n = 10, means ± S.E.) for 6 h. Values that differ significantly from the control (Student’s t test) are indicated as follows: **, p ≤ 0.001.

testing the hypothesis that the WDR proteins promote the stability of the DUB. We first measured the t1/2 of transiently transfected FLAG-tagged C. elegans USP-46 (FLAG–USP-46) in HEK293T cells. We blocked protein synthesis with the translational inhibitor cycloheximide (CHX) and measured the levels of FLAG-tagged USP-46 over time by Western blotting with an anti-FLAG antibody. We found that FLAG–USP-46 is relatively unstable and is degraded over time with a t1/2 of ~3–4 h (Figs. 2, A and B, and 3, A and B). We next tested whether USP-46 is degraded by the proteasome by co-incubating CHX-treated cells with the proteasome inhibitor bortezomib (BTZ). Compared with cells treated with CHX alone, we found that
We confirmed these results. We found that single copy expression of USP-46::GFP expressed at single-copy (1 μg/ml) alone (lanes 1–4 and 9–12) or together with BTZ (5 μM) (lanes 5–8 and 13–16). Cell lysates were immobilized for endogenous (endo.) USP46, WDR48, total ubiquitin, and GAPDH (as a loading control). F, quantification of endogenous USP46 levels from at least three independent experiments similar to the immunoblot shown in E are represented (means ± S.E.). Effects of a second independent siRNA targeting WDR48 (siWDR48#2) on endogenous USP46 are also shown. Statistical significance (p < 0.03) was calculated using one-way ANOVA with Tukey's multiple comparison test.

Figure 3. WDR-48 increases USP-46 protein stability. A, representative immunoblot analysis of HEK293T cells transiently transfected with FLAG–USP-46 and either left untreated (UT, lane 1) or treated with CHX (100 μg/ml) for the indicated times (lanes 2–21). In addition, the cells were either treated with BTZ (1 μM) (lanes 6–9) or transiently co-transfected with HA–WDR-20 (lanes 10–13), Myc–WDR-48 (lanes 14–17), or both HA–WDR-20 and Myc–WDR-48 together (lanes 18–21). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Whole cell lysates were immobilized for the various epitope-tagged proteins or GAPDH, as indicated. B, quantification of FLAG-USP46 levels from at least three independent experiments similar to the immunoblot shown in A. Normalized values are represented (means ± S.E.). Statistical significance (p < 0.05) was calculated using one-way ANOVA with Tukey's multiple comparison test. C, quantification of USP-46::GFP fluorescence intensities (normalized) in the soma of PVC ventral cord neurons of L4 larval animals harboring a USP-46::GFP transgene expressed under the control of the nmr-1 promoter (pzIs40) with (black bars) or without (white bars) 6-h exposure to BTZ. Shown are the USP-46::GFP fluorescence intensities for WT animals treated with DMSO vehicle control (n = 21) or BTZ (n = 10), wdr-48(xs);wdr-20(xs) animals in DMSO (n = 10) or BTZ (n = 8), and wdr-48(xs) animals in DMSO (n = 10) or BTZ (n = 8). D, quantification of USP-46::GFP fluorescence intensities (normalized) in the VNC of L4 larval animals harboring a single-copy USP-46::GFP transgene expressed under the control of the nmr-1 promoter (pzSli1) in a usp-46(ok2232) null mutant background (control) with (black bars) or without (white bars) 6-h exposure to BTZ. Shown are the USP-46::GFP fluorescence intensities for control animals treated with DMSO vehicle control (n = 17) or BTZ (n = 10) and wdr-48(xs);wdr-20(xs) animals in DMSO (n = 10) or BTZ (n = 10). BTZ treatment values that differ significantly from DMSO control within genotypes (ANOVA and Dunnett's multiple comparison tests) are indicated as follows: **, p < 0.01; n.s., p > 0.05. E, representative immunoblot of HEK293T cells transfected with either siRNA control (siControl (20 nM)) or siRNA targeting WDR48 (siWDR48#1 (20 nM)). In addition, the cells were treated either with CHX (100 μg/ml) alone (lanes 1–4 and 9–12) or together with BTZ (5 μM) (lanes 5–8 and 13–16). Cell lysates were immobilized for endogenous (endo.) USP46, WDR48, total ubiquitin, and GAPDH (as a loading control). F, quantification of endogenous USP46 levels from at least three independent experiments similar to the immunoblot shown in E are represented (means ± S.E.). Effects of a second independent siRNA targeting WDR48 (siWDR48#2) on endogenous USP46 are also shown. Statistical significance (p < 0.03) was calculated using one-way ANOVA with Tukey's multiple comparison test.

**WDR-48 promotes USP-46 stability**

FLAG–USP-46 was degraded more slowly over time and had an increased t1/2 (>8 h) in the presence of CHX and BTZ (Fig. 2, A and B). In contrast, we found that endogenous human USP46 was relatively stable in HEK293T cells treated with CHX (Fig. 2C), suggesting that USP46 becomes unstable when USP-46 is overexpressed. To test whether USP-46 is also regulated by the proteasome in *C. elegans* neurons in vivo, we treated USP-46::GFP–overexpressing worms (pzIs40) with BTZ for 6 h and measured USP-46::GFP fluorescence in the soma of the VNC neuron PVC. We found that USP-46::GFP levels increase ~3-fold in the presence of BTZ (Fig. 2D). Analysis of USP-46::GFP expressed at single-copy (pzSli1) levels confirmed these results. We found that single copy–expressed USP-46::GFP levels increased ~2.7-fold in the presence of BTZ in VNC neurons (Fig. 2E). These data suggest that in *C. elegans* neurons in vivo USP-46 protein is unstable and continuously degraded regardless of whether it is expressed at single or multicopy levels. However, in HEK293T cells, endogenous human USP46 appears relatively stable but USP46 becomes unstable and is degraded by the proteasome when USP46 is overexpressed.
We measured protein levels of transiently transfected FLAG-tagged USP-46 over time in HEK293T cells treated with CHX in the presence or absence of HA–WDR-20 alone, Myc–WDR-48 alone, or HA–WDR-20 and Myc–WDR-48 together (Fig. 3, A and B). Similar to the effect of BTZ on USP-46 protein levels (Fig. 3A, lanes 6–9), we found that expression of Myc–WDR-48 alone (lanes 14–17) or HA–WDR-20 together with Myc–WDR-48 (lanes 18–21) increased the stability of FLAG–USP-46 compared with CHX treatment alone (lanes 2–5). In contrast, expression of HA–WDR-20 alone (lanes 10–13) did not stabilize FLAG–USP-46, resulting in a similar decline in USP-46 levels as observed with CHX treatment alone (quantified in Fig. 3B). Because USP-46 is degraded in the proteasome, we wanted to determine whether the promotion of USP-46 abundance via WDR-48 is acting through the same mechanism in vivo. As described above, treatment of transgenic animals expressing USP-46::GFP in VNC interneurons with BTZ results in a 3-fold increase in USP-46::GFP fluorescence (Fig. 3C). Co-expression of WDR-48 alone (wdr-48(xs)) or WDR-48 and WDR-20 together (wdr-48(xs);wdr-20(xs)) results in similar increases in USP-46::GFP fluorescence levels, and in the presence of the proteasome inhibitor BTZ, expression of WDR-48 does not confer any additional stability (Fig. 3C). Similarly, co-expression of WDR-48 and WDR-20 (wdr-48(xs);wdr-20(xs)) combined with BTZ treatment results in a nonadditive increase in single copy–expressed USP-46::GFP (pzIs40) (Fig. 3D). Together, these data suggest that increased expression of WDR-48 promotes USP-46 protein levels by reducing its degradation in the proteasome.

We next tested whether loss of function of wdr-48 results in decreased protein levels of overexpressed or endogenous USP-46. Interestingly, siRNA knockdown of WDR-48 had no effect on the stability of overexpressed FLAG–USP-46 in HEK293T cells (Fig. S2, A and B). Similarly, the abundance of overexpressed USP-46::GFP (pzIs40) was not altered in wdr-48 (tm4575) or wdr-20 (gk547140) loss-of-function mutants (Fig. S2C). In contrast, treatment of HEK293T cells with two independent siRNAs against WDR48, which efficiently knocked down WDR48 expression, resulted in decreased protein levels of endogenous human USP46 (Fig. 3, E and F). Furthermore, this reduction in USP46 was partially blocked by BTZ (Fig. 3, E and F), suggesting that loss of WDR48 leads to degradation of endogenous USP46 via the proteasome. Unfortunately, we were unable to test whether wdr-48 loss-of-function mutants affected single copy–expressed USP-46::GFP in C. elegans because the low fluorescence signal of this transgene precludes our ability to detect further decreases in fluorescence. Collectively, these data are consistent with the idea that USP-46 becomes unstable in the absence of WDR-48, which occurs when either USP-46 is overexpressed alone or when endogenous WDR-48 is knocked down.

**WDR-48 inhibits ubiquitination of USP-46**

If USP-46 is degraded by the proteasome, we would expect the DUB to be covalently modified by ubiquitin. We tested this idea using a HEK293T cell line stably expressing FLAG-tagged human USP-46. We treated these cells with BTZ for 6 h to block degradation by the proteasome, immunoprecipitated FLAG–USP-46 under denaturing conditions, and used anti-Ubiquitin antibodies to probe for Ubiquitin–USP-46 conjugates. We found a low level of Ubiquitin–USP-46 conjugates in the absence of BTZ (Fig. 4A, lane 1); however, treatment with BTZ results in readily detectable high-molecular-weight ubiquitin–USP-46 conjugates (Fig. 4A, lane 2) consistent with polyubiquitination of USP-46. Interestingly, expression of Myc–WDR-48 alone (Fig. 4A, lane 4) or Myc–WDR-48 together with HA–WDR-20 (Fig. 4A, lane 5) in the presence of BTZ results in decreased levels of Ubiquitin–USP-46 conjugates compared with cells treated with BTZ alone (Fig. 4A, lane 2). In contrast, expression of HA–WDR-20 by itself (Fig. 4A, lane 3) did not reduce the levels of Ubiquitin–USP-46 conjugates, suggesting that WDR-48 specifically blocks ubiquitination of USP-46. Together, our data are consistent with the idea that USP-46 is ubiquitinated and degraded in the proteasome and that WDR-48 inhibits USP-46 ubiquitination to promote its availability in the cell.

We and others (29–31, 37) previously showed that WDR-48 and WDR-20 bind to USP-46 and increase its catalytic activity. Several USPs, such as USP4 and USP37, have been shown to autodeubiquitinate in trans (44, 45). Thus, we tested whether the ability of WDR-48 and WDR-20 to increase USP-46::GFP levels in vivo was due to increased autodeubiquitination of USP-46. We mutated the active-site cysteine of USP-46, which is known to eliminate the catalytic activity of the DUB (37), and expressed catalytically inactive USP-46 (C→A):GFP in an usp-46(ok2232) null mutant background. We found that co-expression of WDR-48 and WDR-20 (wdr-48(xs);wdr-20(xs)) was still able to increase the abundance of USP-46 (C→A):GFP in the VNC by 3-fold (Fig. 4B), which is similar in magnitude to the effect we observed on WT USP-46::GFP (Fig. 1D). These results suggest that the WDR proteins increase USP-46 levels independent of USP46 catalytic activity.

**WDR-48 binding to USP-46 is required to stabilize the DUB**

WDR-48, WDR-20, and USP-46 form a complex in vitro (25, 29, 30, 37), and the crystal structures of mammalian WDR-48/USP-46, WDR-48/USP-12, and WDR-48/WDR-20/USP-12 were recently solved (34–36). C. elegans USP-46 is the sole homolog of both mammalian USP-46 and USP-12, which share 90% sequence similarity with each other (12). The crystal structures defined the critical interaction interface between WDR-48 and USP-46/USP-12, revealing several key amino acid residues that are required for the interaction. Yin et al. (36) showed that mutation of three key residues in WDR-48 (K214E, W256A, and R272D) at the interface with USP-46 disrupted binding between the two proteins. These three residues are conserved in C. elegans WDR-48 as either identical or similar amino acids (Fig. 5A). We mutated these three residues in C. elegans WDR-48 (R224E, W266A, and K282D, hereafter referred to as WDR-48(3Xmut)) to test whether they would disrupt binding between WDR-48 and USP-46, as predicted by the high homology of these residues. We co-transfected HEK293T cells with FLAG–USP-46 and either WT Myc–WDR-48 or Myc–WDR-48(3Xmut). As we showed previously...
**WDR-48 promotes USP-46 stability**

(A) FLAG-USP46 Stable HEK293T Cell Line

|                | BTZ (1mM, 6hr) | HA-WDR-20 | Myc-WDR-48 |
|----------------|----------------|-----------|-------------|
| Ub             | -              | +         | +           |
| 25 kDa         |                |           |             |
| 75 kDa         |                |           |             |
| IP: FLAG       | Ub             |           |             |

(B) USP-46(C>A)::GFP fluorescence in ventral cord neurons

![Graph showing fluorescence intensity](image)

**Figure 4. WDR-48 blocks ubiquitination of USP-46.** Representative immunoblot comparing the levels of ubiquitin–USP-46 conjugates in HEK293T cells under various conditions. HEK293T cells stably expressing HA/FLAG-tagged human USP46 (HA/FLAG-USP46) were either left untreated (lane 1) or treated with BTZ (1 μM) for 6 h (lanes 2–5). Some cells were also transiently transfected with either HA–WDR-20 (lane 3) alone, Myc–WDR-48 (lane 4) alone, or Myc–WDR-48 and HA–WDR-20 together (lane 5). Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibody under denaturating conditions followed by immunoblot analysis with anti-ubiquitin antibodies or anti-HA/FLAG antibodies, as indicated. Whole cell lysates (WCL) were immunoblotted for the various epitope-tagged proteins and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as indicated. Similar results were obtained in three independent experiments. B, quantification of GFP fluorescence intensity (means ± S.E.) in the soma of PVC ventral cord neurons of L4 larval usp-46(ok2232) null mutants harboring a USP-46(C38A)::GFP transgene expressed under the control of the glr-1 promoter (pzEx378) either without (n = 19) or with co-expression of wdr-48 and wdr-20 (wdr-48(wdr-20(wdr-48(wdr-20)(kcr))) (n = 20). Values that differ significantly from the WT (Student's t test) are indicated as follows: **, p < 0.001.

**Discussion**

Ubiquitin can be added and removed from target proteins; however, the regulatory mechanisms that control deubiquitinating enzymes are not well-understood. We previously showed that USP-46 regulates glutamate receptor levels in the VNC of *C. elegans* and that two WDR proteins bind and stimulate the catalytic activity of USP-46 (12, 37), consistent with other studies (25, 29, 30). Here, we identify another mechanism by which the WDR proteins regulate DUBs. We find that expression of WDR-48, but not WDR-20, increases the abundance of USP-46 in cultured mammalian cells and *C. elegans* neurons in vivo. Conversely, we find that siRNA knockdown of WDR48 results in decreased stability of endogenous USP46 in mammalian cells. We show that USP-46 is ubiquitinated and degraded in the proteasome. Overexpression of WDR-48 blocks ubiquitination of USP-46, and binding of WDR-48 to USP-46 is required to promote USP-46 protein levels. These data show that WDR-48 stabilizes USP-46 and suggest that controlling WDR-48 expression may provide a novel mechanism to control DUB availability.

What is the mechanism by which WDR-48 decreases ubiquitination of USP-46? Our data show that expression of WDR-48 decreases levels of ubiquitin–USP-46 conjugates (Fig. 4A) and that WDR-48 binding to USP-46 is required for its ability to stabilize the DUB (Fig. 5). Because WDR proteins can stimulate the catalytic activity of USP-1, USP-12, and USP-46 (29–32, 37) and several DUBs such as USP4 and USP37 have been shown to deubiquitinate themselves in *trans* (44, 45), we tested whether USP-46 catalytic activity was required for the ability of WDR-48 to regulate USP-46 levels. We found that expression of WDR-48 increased the levels of catalytically inactive USP-46(C → A)::GFP by ~3-fold (Fig. 4B), which is similar to the magnitude of its effect on WT USP-46::GFP (37), FLAG–USP-46 co-immunoprecipitates with WT Myc–WDR-48 (Fig. 5B, lane 1). In contrast, a much lower amount of Myc–WDR-48(3Xmut) is pulled down with FLAG–USP-46 (Fig. 5B, lane 2), suggesting that the triple-point mutant has a diminished ability to interact with USP-46. We next tested whether the ability of WDR-48 to promote USP-46 protein levels required a direct interaction between the two proteins. We generated transgenic animals expressing WT Myc–WDR-48 or Myc–WDR-48(3Xmut) under control of the glr-1 promoter in the VNC neurons. Consistent with our previous data, we found that overexpression of WT Myc–WDR-48 results in increased levels of USP-46::GFP as determined by Western blotting of whole worm lysates (Fig. 5C) and by measuring USP-46::GFP fluorescence in the VNC (Fig. 5D). In contrast, overexpression of Myc–WDR-48(3Xmut) was unable to increase levels of USP-46::GFP (Fig. 5, C and D). The inability of Myc–WDR-48 (3Xmut) to increase USP-46::GFP levels in vivo is not due to differences in expression levels because the WT Myc–WDR-48 and Myc–WDR-48(3Xmut) transgenes were expressed at comparable levels as assessed by Western blotting whole worm lysates (Fig. 5E). These data suggest that direct binding of WDR-48 to USP-46 is required for the ability of WDR-48 to promote USP-46 protein levels in neurons in vivo.
Figure 5. Direct binding of WDR-48 to USP-46 is required to stabilize the DUB. A, partial protein sequence alignment illustrating similar (gray shading) or identical (black shading) amino acids between Homo sapiens and C. elegans WDR48 in the region that interacts with USP46 (36). Three point mutations in H. sapiens WDR48 (K214E, W256A, and R272D) that disrupt binding to the DUB are marked by arrows. The positions of the corresponding amino acids in C. elegans WDR-48 that were mutated in Myc–WDR-48(3Xmut, K226, K235, and K282D), also referred to as Myc–WDR-48(3Xmut), are marked by asterisks. B, representative immunoblot showing the amount of Myc–WDR-48 and Myc–WDR-48(3Xmut) that was co-immunoprecipitated by FLAG–USP-46 from HEK293T cells. The cells were transiently co-transfected with FLAG–USP-46 together with either WT Myc–WDR-48 (lane 1) or Myc–WDR-48(3Xmut, lane 2). Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibodies followed by immunoblotting with anti-Myc antibodies. Whole cell lysates (WCL) were immunoblotted for the various epitope-tagged proteins, as indicated. Similar results were obtained in three independent experiments. C, representative immunoblot for total USP-46::GFP, as detected with anti-GFP antibodies, in whole worm lysates of L4 larval animals harboring a USP-46::GFP transgene expressed under the control of the glr-1 promoter (pzzIs40, top panel) from WT animals or animals expressing either WT wdr-48 (xs) or wdr-48(3xmut, xs) expressed under control of the glr-1 promoter. Tubulin was also detected in these lysates (bottom panel) as a loading control. D, quantification of USP-46::GFP fluorescence in the soma of PVC ventral cord neurons of L4 larval animals harboring a USP-46::GFP transgene expressed under the control of the nmr-1 promoter (pzzIs40). Shown are USP-46::GFP fluorescence intensities (normalized) for WT (n = 15), wdr-48(xs), n = 16, and wdr-48(3xmut, xs, n = 12) (means ± S.E.). E, representative immunoblots for Myc–WDR-48 and Myc–WDR-48(3Xmut) expression, as detected with anti-Myc antibodies, of lysates from L4 larval animals of WT, wdr-48(xs), and wdr-48(3xmut, xs) animals (top panel). Tubulin was also detected in these lysates (bottom panel) as a loading control. The results from three independent experiments show that the relative abundance of Myc–WDR-48 is similar to that of Myc–WDR-48(3xmut, means ± S.E.). Values that differ significantly from the WT (ANOVA, Dunnett’s multiple comparison tests) are indicated as follows: **, p ≤ 0.001. n.s., p > 0.05.

(Fig. 1D). These data are consistent with the idea that WDR-48 decreases ubiquitination of USP-46 and promotes USP-46 protein levels independent of its own catalytic activity. Although we do not know the precise mechanism by which WDR-48 prevents USP-46 ubiquitination, we propose that WDR-48 binding to USP-46 either prevents the recruitment of an E3 ligase or blocks the ability of an E3 ligase to ubiquitinate key lysine residues on the surface of USP-46. Intriguingly, the crystal structure of WDR-48 bound to USP-46 reveals the presence of two USP-46 surface lysine residues (K^226 and K^235) at the interface of USP-46 and WDR-48 (36). It will be interesting in the future to test whether WDR-48
WDR-48 promotes USP-46 stability

inhibits ubiquitination of USP-46 by blocking access of an E3 ligase to these surface lysines. Interestingly, one or both of these lysine residues are conserved in USP-1 (K503) and USP-12 (K230 and K239), suggesting that WDR-48 may also stabilize these closely related USPs via the same mechanism. The WDR proteins and USP-46/USP-12 are conserved across phylogeny from yeast to humans, where they regulate several important processes including endocytosis, cell polarity, signal transduction, and mitochondrial biogenesis (11, 26, 46, 47). These WDR protein homologs may also regulate the stability of USP-46 homologs in nonneuronal cells in other species. For example, overexpression of WDR-48, but not WDR-20, can increase protein levels of human USP-12, resulting in stabilization of androgen receptors and proliferation of prostate cancer cells (16). In the filamentous fungi Aspergillus nidulans, protein levels of the USP-46 homolog CreB were dramatically increased after expression of the WDR protein CreC (48). Although the precise mechanism controlling DUB levels was not investigated in these studies, our work suggests that these WDR proteins may increase protein levels of their interacting DUB partners by preventing their ubiquitination and degradation in the proteasome. We propose that this mechanism of stabilizing USP-46 and its homologs is likely conserved across phylogeny and may be a widely used mechanism to stabilize DUBs given that over 35% of all USPs interact with WDR proteins (25).

WDR-48 regulates USP-46 related DUBs via multiple mechanisms and is thus emerging as a critical DUB regulatory protein. First, WDR-48, either alone or together with WDR-20, can bind and stimulate the catalytic activity of USP-1, USP-46, and USP-12 (29–32, 37). Second, WDR-48 can function as a substrate adaptor. For example, WDR-48 can bind substrates such as FANCDC2/FANCI heteromers via its SUMO-like domain and recruit them to USP-1 (49). Similarly, the C-terminal region of WDR-48 which includes the SUMO-like domain is sufficient to bind the USP-12 substrate PHLPPI1 (17). Third, WDR-48 can regulate the subcellular localization of its interacting USP. Bun107 and Bun62, the yeast homologs of WDR-48 and WDR-20, respectively, can recruit the USP-46 homolog Ubp9, to specific locations in the cytoplasm (26). The ability of WDR-48 to relocalize USPs is underscored by a study showing that human papilloma viruses have evolved to manipulate WDR-48 to control the subcellular localization of their interacting USPs. Specifically, human papilloma virus protein E1 binds to WDR-48 and recruits USP-1, USP-12, and USP-46 to viral origins of replication in the nucleus (50). These studies, together with our data showing that WDR-48 promotes USP-46 stability, reveal that WDR-48 is a versatile regulator of this group of USPs.

Although our data show that WDR-48 promotes USP-46 stability, we observed one notable difference between HEK293T cells and C. elegans neurons. In HEK293T cells, endogenous USP46 is relatively stable (Fig. 2C) but becomes unstable and is degraded by the proteasome in the absence of endogenous WDR48 (Fig. 3, E and F). Furthermore, when USP-46 is overexpressed alone, without co-expression of WDR-48, the DUB also becomes unstable and is degraded via the proteasome. However, co-expression of WDR-48 together with USP-46 results in stabilization of the DUB (Fig. 3, A and B). These data indicate that USP46 stability depends on WDR48 and suggest that under conditions where there is an increase in “free” USP-46 that is not complexed with WDR-48, USP-46 is ubiquitinated and degraded by the proteasome. In C. elegans neurons, both overexpressed and single copy–expressed USP-46 are unstable and appear to be continuously degraded by the proteasome (Fig. 2, D and E). Increased expression of WDR-48 promotes the stability of both overexpressed and single copy–expressed USP-46 (Figs. 1 and 3). These data show that in C. elegans neurons USP-46 stability is also dependent on WDR-48. However, in contrast to endogenous USP46 in HEK293T cells, there appears to be a higher amount of “free” USP-46 in C. elegans neurons. This “free” USP-46 can be stabilized by inhibition of the proteasome or by overexpression of WDR-48. It will be interesting in the future to test whether neurons have a unique ability to regulate WDR-48 expression as a mechanism to control USP-46 stability and function. We previously showed that overexpression of USP-46 alone does not increase its ability to regulate GLR-1 (37), suggesting that in vivo there are other limiting factors required to regulate USP-46. Although our data show that expression of WDR-48 alone is sufficient to increase the stability of USP-46, co-expression of WDR-48 and WDR-20 are required to fully stimulate the catalytic activity of the DUB and modulate glutamatergic behavior (37). Together, these data suggest that increasing the expression of WDR-48 alone is not sufficient to promote USP-46 function. A signal that increases expression of WDR-48 together with another signal that controls WDR-20 expression could function as an “AND” gate to precisely control USP-46 function. Although the relevant signals that regulate WDR-48 expression in vivo are not yet known, we speculate that WDR-48 expression in neurons may be regulated during development or in response to changes in synaptic activity. Interestingly, another DUB that can also regulate mammalian α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid–type glutamate receptors (AMPARs), USP8, has been shown to be regulated by synaptic activity. In this case, activation of N-methyl-d-aspartate receptors (NMDARs) leads to rapid dephosphorylation and activation of the DUB. Consistent with this idea, when USP46 is overexpressed alone, without co-expression of WDR48, the DUB becomes unstable and is degraded via the proteasome. Further studies will be necessary to identify the upstream signals that control the expression of both WDR-48 and WDR-20 in neurons to regulate USP-46 function.

USP-46 and USP-12 have been implicated in several cancers (including colon cancer, prostate cancer, and glioblastoma) and in regulating both glutamatergic and GABAergic signaling in the nervous system. The fact that DUBs are proteases makes them an attractive drug target; however, blocking the catalytic cysteine residue present in the active sites of most DUBs and many other proteases could lead to nonspecific effects. Our study shows that WDR-48 stabilizes USP-46 and that interaction of WDR-48 with USP-46 is required for this effect. Designing drugs that disrupt the interaction of WDR-48 with USP-46 could be an effective and more specific strategy to inhibit USP-46 function. We propose that targeting the interaction interface between WDR proteins and their DUB partners may be a
promising and more specific approach to destabilize DUBs and thus inhibit their function.

**Experimental procedures**

**Strains**

The following strains were used for experiments described in this manuscript: N2 (Bristol) WT, wzr-48 (tm4575) III, wzr-20 (gk547140) V, pzlsl40 (Pnmr-1::USP-46::GFP) II, pzs11 (MosSCI Pnmr-1::USP-46::GFP II), ljs114 (Pgpa-1::FLPase; Psrs-6::FTT::ChR2::YFP) X (gift from William Schaefer), pzlsl37 (Pglr-1::USP-46::GFP) III, pzx386 (Pnmr-1::USP-46::GFP), usp-46 (ok2223) III, pzlsl25 (Pglr-1::WDR-20; Pglr-1::WDR-48) I, pzx230 (Pglr-1::WDR-20), pzx231 (Pglr-1::WDR-48), pzx378 (Pglr-1::USP-46::GFP; C38A::GFP), pzx456 (Pglr-1::Myc::WDR-48), and pzx457 (Pglr-1::Myc::WDR-48::GFP II), Pmyo-2::NLS-mCherry (50 ng/μl) and co-injection marker Pmyo-2::NLS-mCherry (50 ng/μl) (also referred to as 3Xmut). All strains were maintained at 20 °C as described previously (51).

** Constructs, transgenics, and germline transformation**

pzs125, pzx230, pzx231, and pzx378 were described previously (37). Pnmr-1::USP-46::GFP (FJ#129) was generated by replacing the Pglr-1 promoter in Pglr-1::USP-46::GFP (FJ#109) with Pnmr-1 (~1 kb) from pBM16 (52) using SphI and BamHI enzymes. pzlsl40 was created by injecting FJ#129 (50 ng/μl) with the co-injection marker Ptx-3::GFP (50 ng/μl) followed by integration using a UV Stratalinker. pzlsl40 was backcrossed three times prior to imaging. pzlsl37 was created by injecting FJ#109 (50 ng/μl) with the co-injection marker Ptx-3::GFP (50 ng/μl) followed by integration using a UV Stratalinker. pzlsl37 was backcrossed three times prior to imaging. pzx386 was created by injecting FJ#109 (50 ng/μl) with the co-injection marker Ptx-3::dsRed (50 ng/μl). pzx386 was created by injecting pBM16 (50 ng/μl) with the co-injection marker Ptx-3::GFP (50 ng/μl), pzs11 was created using the MosSCI technique (39, 40). Using Gibson Assembly (NEBuilder), Pnmr-1, USP-46::GFP, and the usp-46 3′-UTR were cloned into the pCF350 MosSCI vector for chromosome II to create pMH35. Pnmr-1 and USP-46::GFP were generated by PCR from pMH10, and the usp-46 3′-UTR was generated by PCR from cosmid R10E11.3 containing genomic usp-46. pMH35 (10 ng/μl) and co-injection markers J86p (5 ng/μl), pDS249 (5 ng/μl), pCF601 (50 ng/μl), pMA122 (10 ng/μl), and pSK (30 ng/μl) were all purified using PureLinkHQ mini plasmid DNA purification kits (Invitrogen, catalog no. K2100-01) prior to injection into the EG6699 MosSCI strain. The animals were then allowed to grow for two generations to allow for the MosSCI event, after which they were heat-shocked to remove array bearing nonintegrants and screened for normal movement and lack of red fluorescent co-injection markers. Resulting pzs11 animals were then backcrossed at least three times prior to imaging.

pMT3–FLAG–USP-46 (FJ#66), pMT3–HA–WDR-20 (FJ#94), and pMT3–Myc–WDR-48 (FJ#96) have been previously described (37). The mammalian expression vector pMT3 was kindly provided by Dr. Larry Feig. Myc–WDR-48 (~2 kb) was subcloned into the *C. elegans* expression vector pV6 for expression under the glr-1 promoter to create Pglr-1::Myc–WDR-48 (FJ#130) by first cloning into pBlueScript using HindIII and KpnI sites and then into FJ#109 using BamHI and KpnI sites. pzEx456 was created by injecting FJ#130 (50 ng/μl) with the co-injection marker *Pmyo-2::NLS-mCherry* (50 ng/μl). pMT3–Myc–WDR-48 (R224E, W266A, K282R) (FJ#132) was referred to as Myc–WDR-48 (3Xmut) (FJ131), was generated by using QuickChange (Invitrogen) to generate point mutations in the parent plasmid pMT3–Myc–WDR-48 (FJ#96) (37). Myc–WDR-48 (R224E, W266A, K282R) (~2 kb) was subcloned into the *C. elegans* expression vector pV6 for expression under the glr-1 promoter to create Pglr-1::Myc–WDR-48 (R224E, W266A, K282R) (FJ#132) as described for FJ#130. pzEx457 was created by injecting FJ#132 (50 ng/μl) with the co-injection marker *Pmyo-2::NLS-mCherry* (50 ng/μl).

**Imaging**

Fluorescence imaging of USP-46::GFP was performed as follows. Briefly, L4 larval-stage animals were immobilized using 30 mg/ml 2,3-butanedione monoxamine (Sigma–Aldrich), and the VNC was imaged in the anterior region of the animals just posterior to the RIG neuronal cell bodies or in the cell body of the VNC neuron PVC. 1 μm (total depth) Z-series stacks were collected using a Carl Zeiss Axioscope M1 microscope with a 100× Plan Apochromat (1.4 numerical aperture) objective equipped with GFP and Cy3.5 filters. Images were collected with an Orca-ER charge-coupled device camera (Hamamatsu) and MetaMorph (version 7.1) software (Molecular Devices). Maximum intensity projections of Z-series stacks were used for quantitative analyses of fluorescence. Exposure settings and gain were adjusted to fill the 12-bit dynamic range without saturation and were identical for all images. The intensity for each worm was determined by averaging the maximum intensity from three separate regions of interest (ROIs). For proteasome inhibitor pretreatment, the animals were placed on NGM plates containing 50 μM bortezomib for 6 h prior to imaging, as previously described (53).

**Cell culture and transfections**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin. The cells were maintained in a humidified, 5% CO2 atmosphere at 37 °C.

For protein synthesis inhibition experiments, HEK293T cells were seeded into a 100-mm dish and transfected with 6 μg of FLAG–USP-46 using polyethyleneimine (PolySciences) for 16–18 h. The cells were trypsinized and plated into a 6-well plate and reverse transfected with Myc–WDR-48 (3 μg of DNA) and/or HA–WDR-20 (2 μg of DNA) using Lipofectamine 2000 (Invitrogen). 24 h post-transfection the cells were again split into 12-well plates at 100,000 cells/well for cycloheximide time course studies. The cells were lysed in mammalian cell lysis buffer (MCLB, 50 mm Tris, pH 7.5, 150 mm NaCl, 0.5% Nonidet P-40, HALT Inhibitors (Pierce)). The cells were incubated at 4 °C for 10 min and then centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatant was removed, and the protein concentration was estimated using the Bradford method (Bio-Rad).

For immunoprecipitation studies, HEK293T cells in 6-well plates were transfected with FLAG–USP-46, Myc–WDR-48,
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Myc–WDR-48(R224E, W266A, K282D), or HA–WDR-20, with a total of 2 \( \mu g \)/well DNA, using Lipofectamine 3000, harvested, and lysed at 24–36 h post-transfection for immunoprecipitations. HEK293T cells stably expressing human FLAG/HA-USP46 was created by lentiviral transduction as described elsewhere (54). Stable clones were selected using 1 \( \mu g/ml \) puromycin.

Immunoprecipitation and immunoblotting

For immunoprecipitation experiments, HEK293T cells were washed once with PBS and lysed after 24 h with MCLB. Lysed cells were centrifuged at 14,000 rpm for 10 min at 4 °C. Cleared lysate was incubated for 4–12 h with anti-FLAG antibody-coupled magnetic beads (Sigma) in the presence of 10 \( \mu l \) HALT protease inhibitors and phosphatase inhibitors (50 \( \mu g/ml \) sodium fluoride, 5 \( \mu g/ml \) sodium orthovanadate). Immunoprecipitated complexes were washed four times with MCLB and resuspended in 2 \( \times \) SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on 10% acrylamide gels and subsequently transferred to a nitrocellulose membrane. The membranes were blocked in Tris-buffered saline with Tween and 5% milk prior to incubation with various primary antibodies.

For denaturing immunoprecipitation studies, FLAG/HA USP46 293T cells were lysed in MCLB containing 1% SDS and vortexed at room temperature for 10 min. Insoluble material was removed by centrifugation (14,000 rpm, 5 min). Protein concentration in the supernatants was determined by BCA assay (Pierce). Equal amounts of protein from each experimental sample were taken and diluted 10-fold in MCLB to acquire a final SDS concentration of 0.1%. Lysates were incubated with anti-FLAG antibody-coupled magnetic beads overnight at 4°C and then washed three times with MCLB. The beads were suspended in 2 \( \times \) SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with Tween and 5% milk prior to incubation with various primary antibodies.

Whole worm lysates were obtained by placing 100 animals in 2 \( \times \) SDS sample buffer and boiling at 95°C for 5 min, vortexing for 1 min, and boiling again at 95°C for 5 min. The samples were subjected to SDS-PAGE and immunoblotting.

The following antibodies were used for immunoblotting: rabbit anti-USP46 (HPA07288, Sigma), rabbit anti-WDR48 (16503-1-AP, Proteintech), mouse anti-GFP (JL-8, Covance), rabbit anti-tubulin (ab4074, Abcam), mouse anti-FLAG (clone M2, F3165, Sigma), mouse anti–c-Myc (clone 9E10, sc-40, Santa Cruz), mouse anti–HA (clone 16B12, MMS-101P, Covance), mouse anti–proliferating cell nuclear antigen (clone PC10, sc-56, Santa Cruz), and mouse anti–GAPDH (clone 4011, sc-47724 Santa Cruz), mouse anti–ubiquitin (clone P4D1, sc-8017, Santa Cruz), goat anti-mouse IgG polyclonal antibody HRP (W4021, Promega), and goat anti-rabbit IgG polyclonal antibody HRP (W4011, Promega).

Behavioral assays

All behavioral assays were performed using at least 10 young adult hermaphrodites over at least 3 independent experiments and by an experimenter who was blinded to the genotypes of the animals being tested. Optogenetic activation of the ASH-dependent nose-touch response was performed similarly to what has been described previously (55). Gentle touch to the nose of the worm (nose touch) (42, 43, 56) or photostimulation of ASH expressing ChR2 (55, 57) result in locomotion reversals away from the stimulus. This nose-touch response depends on presynaptic glutamate and GLR-1 in postsynaptic interneurons (42, 43, 56). We previously showed that mutants with decreased levels of GLR-1 in the VNC exhibit defects in the nose-touch response (12). Animals expressing ChR2 specifically in ASH sensory neurons (ljIs114, Pagna-13::FLPase; Psra6::FT::ChR2:: YFP) (58) were grown for one generation in the dark on NGM agar plates spotted with OP50 and the ChR2 co-factor all-trans-retinal (ATR, 100 \( \mu M \)). The animals were subsequently transferred to an NGM agar plate spotted with OP50 (without ATR) and illuminated with 1-s pulses of blue light (0.47 milliwatt/mm²) from a mercury bulb filtered through a GFP excitation filter (480 nm) under 32 \( \times \) total magnification on a LeicaMZ16F microscope. A locomotor reversal was scored as a positive response if the backward movement was greater than the distance from the nose to the terminal bulb of the pharynx observed during or immediately after blue light illumination.

Data availability

All the data are included in the article.

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Abbreviations—The abbreviations used are: DUB, deubiquitinating enzyme; WDR, WD40-repeat; VNC, ventral nerve cord; USP, ubiqu-
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