The evolutionarily conserved deubiquitinase UBH1/UCH-L1 augments DAF7/TGF-β signaling, inhibits dauer larva formation, and enhances lung tumorigenesis

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Modification of the transforming growth factor β (TGF-β) signaling components by (de)ubiquitination is emerging as a key regulatory mechanism that controls cell signaling responses in health and disease. Here, we show that the deubiquitinating enzyme UBH-1 in Caenorhabditis elegans and its human homolog, ubiquitin C-terminal hydrolase-L1 (UCH-L1), stimulate DAF-7/TGF-β signaling, suggesting that this mode of regulation of TGF-β signaling is conserved across animal species. The dauer larva–constitutive C. elegans phenotype caused by defective DAF-7/TGF-β signaling was enhanced and suppressed, respectively, by ubh-1 deletion and overexpression in the loss-of-function genetic backgrounds of daf7, daf-1/TGF-βRI, and daf4/R-SMAD, but not of daf-8/R-SMAD. This suggests that UBH-1 may stimulate DAF-7/TGF-β signaling via DAF-8/R-SMAD. Therefore, we investigated the effect of UCH-L1 on TGF-β signaling via its intracellular effectors, i.e. SMAD2 and SMAD3, in mammalian cells. Overexpression of UCH-L1, but not of UCH-L3 (the other human homolog of UBH1) or of the catalytic mutant UCH-L1C90A, enhanced TGF-β/SMAD-induced transcriptional activity, indicating that the deubiquitination activity of UCH-L1 is indispensable for enhancing TGF-β/SMAD signaling. We also found that UCH-L1 interacts, deubiquitinates, and stabilizes SMAD2 and SMAD3. Under hypoxia, UCH-L1 expression increased and TGF-β/SMAD signaling was potentiated in the A549 human lung adenocarcinoma cell line. Notably, UCH-L1–deficient A549 cells were impaired in tumorigenesis, and, unlike WT UCH-L1, a UCH-L1 variant lacking deubiquitinating activity was unable to restore tumorigenesis in these cells. These results indicate that UCH-L1 activity supports DAF-7/TGF-β signaling and suggest that UCH-L1’s deubiquitination activity is a potential therapeutic target for managing lung cancer.

The ubiquitin (Ub) system is involved in numerous cellular processes including protein quality control, cell proliferation, apoptosis, signal transduction, and membrane protein internalization (1, 2). The Ub–dependent process is regulated by a ubiquitination process involving the E1, E2, and E3 enzymes and by a reverse process carried out by deubiquitinating enzymes (DUBs). In humans, there are nearly 100 DUB genes, which are classified into cysteine proteases and metalloproteases. The cysteine protease–type DUBs are classified into six families depending on the basis of the mechanisms of catalysis (3, 4). Thus, the DUB superfamily is highly diverse. The specific roles of each DUB, however, are thus far poorly characterized. In this study, we focused on the UCH family by performing functional analysis of its member proteins.

Four genes in the human genome, UCH-L1, UCH-L3, UCH-L5, and BAP1, encode UCH-family proteins. Among the proteins, UCH-L1 and UCH-L3 are closely related in structure, as shown in Fig. S1. UCH-L1 is abundant in neurons and is essential for maintaining axonal integrity, and its dysfunction is involved in neurodegenerative diseases (5). UCH-L1 is also involved in several cancers (6). UCH-L3, which shares 52% amino acid identity with UCH-L1, is expressed in a variety of tissues, being particularly abundant in the testis and thymus. UCH-L3 can cleave not only the C terminus of Ub but also that of a Ub-like protein, NEDD-8, in vitro (7).

The signaling pathways of the nematode Caenorhabditis elegans and mammals are very similar, and the signal transduction pathways are conserved. Thus, this nematode has often been used to delineate developmental signaling pathways of high relevance to cancer initiation and development in mammals. The C. elegans genome contains four UCH-like genes, ubh-1–4. The ubh-1–3 genes code for UCH-L1/L3 orthologs, whereas the ubh-4 gene codes for the UCH-L5 ortholog (Fig. S1). To shed light on novel biological roles of UCH, we here performed functional analyses on the C. elegans UCH-L1/L3 orthologs and found that mutant worms with a deletion in the ubh-1 gene showed various phenotypes that were qualitatively similar to those observed in worms with a loss-of-function mutation in the daf-7 gene encoding a transforming growth factor β (TGF-β) ligand.

TGF-β is a multifunctional cytokine that plays a key role in numbers of cellular processes regulating both embryogenesis and tissue homeostasis of adult tissues (8). Therefore, abnormal TGF-β signaling has also been associated with various diseases including cancer, fibrosis, and vascular malformation (9–11). TGF-β signaling pathway initiated through the heteromeric receptor complexes of types II and I (also termed activin receptor-like kinase-5, or ALK5) serine/threonine kinase receptors. In canonical TGF-β signaling, the activated receptor complex phosphorylates specific receptor-regulated Smads (R-Smads;
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Results

The C. elegans ubh-1 gene encodes ubiquitin C-terminal hydrolase homologous to UCH-L1/L3

Of the four genes that encode UCH-like proteins in the C. elegans genome, three, ubh-1, ubh-2, and ubh-3, are predicted to organize an operon on chromosome V. The enzymatic activities of the recombinant UBH proteins were analyzed. C. elegans Ub, Ub-like NEDD8, and SUMO-1 are 98, 88, and 58% identical to their human counterparts, respectively. These recombinant proteins fused to the HSV–His6 tag at the C terminus were prepared by means of an Escherichia coli expression system (Fig. 1A) and used in the assay as substrates. All the recombinant UBH proteins cleaved Ub–HSV–His6 and NEDD8–HSV–His6 into two fragments (Fig. 1B) but did not digest SUMO–HSV–His6 (Fig. 1C). Thus, UBH-1, UBH-2, and UBH-3 have C-terminal hydrolase activity toward both Ub and NEDD8, analogously to human UCH-L1/L3 (14). These UBHs also showed C-terminal hydrolase activity toward Ub–7-amido-4-methylcoumarin, a fluorogenic substrate, whose kinetic parameters \( k_{\text{on}} \) and \( k_{\text{cat}} \) were determined to be 0.026 to 0.043 \( \mu \text{M}^{-1} \text{s}^{-1} \) and 6.6 to 43.6 \( \text{s}^{-1} \), respectively (Table S1). These kinetic parameters suggested that UBHs were closer to those of human UCH-L3 rather than to those of human UCH-L1.

To examine the expression pattern of ubh genes, their translational fusion genes with GFP, i.e. \( \text{ubh-1::gfp} \), \( \text{ubh-2::gfp} \), and \( \text{ubh-3::gfp} \), were constructed (Fig. 1D) and introduced into WT N2 worms. In the descendants harboring the \( \text{ubh-1::gfp} \) transgene, intense GFP fluorescence was observed in neuronal cells throughout the late-embryonic and postembryonic stages (Fig. 1F). In addition, the GFP signal was observed in intestinal cells. The \( \text{ubh-2::gfp} \) and \( \text{ubh-3::gfp} \) transgenes also showed expression patterns similar to \( \text{ubh-1::gfp} \) (data not shown). In terms of high expression in neurons, these UBHs resemble human UCH-L1 rather than ubiquitously expressed UCH-L3. The neuronal cells expressing UBH-1::GFP included several amphid ciliated neurons including ASI, ASK, ADL, and ASI neurons. Fluorescence images of Dil (left panel) and GFP (middle panel) and their merged image (right panel) are shown. The regions containing ASI, ASK, and ADL are shown. Scale bar, 5 \( \mu \text{m} \).

Figure 1. Expression and enzymatic profiles of C. elegans UCHs. A–C, recombinant UBH-1, UBH-2, and UBH-3 proteins with a His6 tag at the C-terminal toward recombinant substrates Ub–HSV–His6 (A), NEDD8–HSV–His6 (B), and SUMO–HSV–His6 (C). After incubation for 2 h, the Ub and NEDD8 substrates were cleaved into two fragments, whereas the SUMO substrates were not. D, diagram of translational fusion of the ubh genes with a GFP reporter. E, expression pattern of the ubh-1::gfp fusion gene (young adult). Scale bar, 100 \( \mu \text{m} \). F, UBH-1::GFP expression was observed in several amphid ciliated neurons including ASI, ASK, and ADL. Scale bar, 5 \( \mu \text{m} \).
the DAF-7/TGF-β ligand (15), and its receptor DAF-1/DAF-4 is expressed in these amphid neurons (16).

Reverse genetic analyses of the ubh genes

The alleles tm526, tm2267, and tm2550 have a deletion in the ubh-1, ubh-2, and ubh-3 genes, respectively (Fig. 2A). Because of the deletions, each protein encoded by these mutant genes lacks a region including at least two or three of the catalytic triad Cys/His/Asp conserved among UCHs. RT-PCR confirmed that in the homozygous tm526, tm2267, and tm2550 worms, no WT transcripts of the respective genes were expressed (Fig. 2B). Therefore, ubh-1(tm526), ubh-2(tm2267), and ubh-3(tm2550) are thought to be loss-of-function mutants of ubh-1, ubh-2, and ubh-3, respectively, although it is possible that each deleted ubh gene is functionally compensated by the other two, at least in part. Among the homozygous mutants, the ubh-2(tm2267) and ubh-3(tm2550) mutants showed no abnormal phenotypes, whereas the ubh-1(tm526) mutant showed abnormal phenotypes as follows: slow growth (Gro) (Fig. 2C), reduced brood size (Fig. S2A), bag of worms (Bag) caused by defective egg-laying (Fig. S2B), reduced rate of pharyngeal pumping (Fig. S2C), increased accumulation of lipid droplets (Fig. S2D), and decreased food-feeding behavior (Fig. S2E). Although these phenotypes were less pronounced, they appeared to be qualitatively common to those described for deletion mutants of daf-7 encoding a TGF-β ligand (17–19). However, the functional relationship between the UBH-1 and DAF-7 signals is unknown, as is that between the UCH and TGF-β signals.

Functional relationships between the ubh-1 and daf-7/TGF-β signaling pathways

One of the most representative functions of the DAF-7/TGF-β signal in C. elegans is to negatively regulate dauer larva formation (15, 19). Although the ubh-1(tm526) mutant showed phenotypes similar to those of daf-7 loss-of-function mutants, it showed no dauer-constitutive (Daf-c) phenotype, the most representative phenotype of the daf-7 loss-of-function mutants, at least up to 25°C (Fig. 2F). To examine whether ubh-1 is functionally related to the DAF-7/TGF-β signal, effects of the ubh-1(tm526) mutation on the Daf-c phenotype were analyzed in the genetic backgrounds of loss-of-function mutants of genes involved in the DAF-7/TGF-β signal. When we analyzed the effects of the ubh-1(tm526) mutation in the daf-7(e1372) genetic background, population ratios at each growth stage were compared at 15°C 120 h after hatching (Fig. 2C). Both the ubh-1(tm526) and the daf-7(e1372) mutant worms grew slower than the WT worms, and further delay in growth was observed in the ubh-1(tm526);daf-7(e1372) double-mutant worms. Dauer larvae were observed at low frequency in the daf-7(e1372) single mutant but at higher frequency in the ubh-1(tm526);daf-7(e1372) double mutant (Fig. 2C). Such an increase in dauer formation in the double mutant was also observed at 20°C (Fig. 2D). These results suggest a possibility that ubh-1 positively regulates the DAF-7/TGF-β signal downstream of DAF-7, thereby negatively regulating dauer formation.

The daf-1 gene encodes a TGF-β type I receptor homolog involved in the regulation of dauer formation by accepting DAF-7/TGF-β secreted by the ASI sensory neuron in response to environmental signals (19, 20). The daf-14 gene encodes a Smad-related protein that is predicted to function as a transducer of the DAF-7/TGF-β-mediated signal that negatively regulates dauer formation and promotes reproductive growth (19, 21). Therefore, the loss-of-function mutants daf-1(m40) and daf-14(m77) showed the Daf-c phenotype. Double mutation of these mutants with ubh-1(tm526) enhanced the Daf-c phenotype, as in the case of the daf-7(e1372) (Fig. 2D).

The daf-8 gene encodes a Smad protein that represses dauer development and is suggested to functionally overlap daf-14 in the TGF-β signaling pathway regulating dauer formation (19, 21). The loss-of-function mutant daf-8(e1393) showed the Daf-c phenotype. In this mutant background, however, double mutation with ubh-1(tm526) showed no enhancement of the Daf-c phenotype at any temperature we analyzed (Fig. 2E).

Transgenic expression of the ubh-1::gfp fusion had no effect on the WT and suppressed the Daf-c phenotype in all of the daf-7(e1372), daf-1(m40), and daf-14(m77) mutants. In the genetic background of daf-8(e1393), however, the Daf-c phenotype was not suppressed (Fig. 2F). Therefore, the enhancement of dauer formation by ubh-1(tm526) might be dependent on DAF-8, suggesting that UBH-1 is involved in the negative regulation of dauer formation by the DAF-7/TGF-β signal via DAF-8.

UCH-L1 activates TGF-β signaling

To investigate whether UCH regulates TGF-β signaling in mammalian cells as well as in C. elegans, human UCH-L1 and UCH-L3, which are the presumable counterparts of C. elegans UBH-1, were individually overexpressed in hepatocellular carcinoma HepG2 cells, and the effects on TGF-β signaling were analyzed using the SBE4-luc Smad3/4-dependent transcriptional reporter gene assay system (22). The SBE4-luc reporter activity induced by TGF-β stimulation was significantly potentiated by UCH-L1, but not by UCH-L3. When the catalytic Cys residue of UCH-L1 was replaced with Ala, overexpression of the inactive UCH-L1C90A did not potentiate TGF-β-induced reporter expression. These results suggest that UCH-L1, rather than UCH-L3, functions as an ortholog of UBH-1 in the mammalian TGF-β signaling pathway and that UCH-L1 enhances TGF-β signaling in an enzyme activity–dependent manner (Fig. 3A).

Then, to investigate whether the effects of UCH-L1 on TGF-β signaling are through Smads, the levels of Smad phosphorylation induced upon TGF-β stimulation were analyzed. HepG2 cells transiently transfected with UCH-L1, UCH-L3, or UCH-L1C90A expression plasmids were treated with TGF-β for 1 h. The levels of phosphorylated Smad2 were raised by TGF-β treatment in the HepG2 cells, which were further up-regulated in the presence of UCH-L1, but not of UCH-L3 or UCH-L1C90A (Fig. 3C). These results suggest that enzymatically active UCH-L1 enhances TGF-β–mediated responses by enhancing Smad activation.
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UCH-L1 interacts with R-Smad

The finding that UCH-L1 potentiates R-Smad phosphorylation motivated us to examine whether R-Smad physically binds to UCH-L1. COS7 cells were transiently transfected with 6Myc–Smad2, –Smad3, or –Smad4 together with UCH-L1–3×FLAG and with or without a constitutively active ALK5 (ALK5ca). Immunoprecipitation and Western blotting analysis revealed that UCH-L1 interacted with all of the analyzed Smads, but Smad2 and Smad4 had slightly augmented binding by coexpression with ALK5ca (Fig. 4A). To further validate endogenous interaction between Smad2/3 and UCH-L1, we used the in situ PLA method (23), which enables detection of the interaction between proteins that reside within less than 40-nm distance. For the negative control of PLA, we generated a cell line with homozygous knockout of UCH-L1 by targeting exon 4 with CRISP-Cas9 gene-editing system (Fig. 4B). A549 cells, which are human lung carcinoma highly expressing UCH-L1 (Fig. S3), were transfected with pX458 plasmid including gRNA.

Figure 2. Genetic analyses of C. elegans ubh-1. A, deletion regions in the ubh-1(tm526), ubh-2(tm2267), and ubh-3(2550) and mutant alleles. The thicker bars represent exons. B, relative RNA levels were determined by RT-qPCR. Each mean level is shown relative to the mean level of ubh-1 mRNA in N2. Error bars show S.E. (n = 8–12). C, fraction of worms at each developmental stage 120 h after hatching at 15 °C. Percentage ratios were calculated on the basis of the total data obtained from three independent experiments. The numbers of worms used for the calculation were 234, 412, 151, and 333 for N2, ubh-1(tm526), daf-7(e1372), and ubh-1(tm526)/daf-7(e1372), respectively. D, effects of the ubh-1(tm526) mutation on dauer formation in the genetic background of loss-of-function mutation of daf-7, daf-1, and daf-14. The mean fraction of the dauers was measured 72 h after hatching at 20.0 °C for daf-7(e1372) and daf-14(m77) and at 22.0 °C for daf-1(m40). The data are shown as means ± S.E. E, effects of the ubh-1(tm526) mutation on dauer formation in the genetic background of daf-8(e1393) at various temperatures. The data are shown as means ± S.E. F, rescue of the Daf-c phenotype by the ubh-1::gfp transgene in the genetic background of daf-7(e1372), daf-1(m40), daf-14(m77), and daf-8(e1393). The mutant ubh-1(tm526) did not show the Daf-c phenotype at 25.0 °C. The data are shown as means ± S.E. Statistical analyses were performed using the t test. *, P < 0.05; **, P < 0.001.
sequence, and highly GFP expressing cells were sorted by FACS and seeded as single cells to obtain single cell–derived clones. Western blotting analysis and target site sequencing were performed to verify knockout of UCH-L1. Among three clones obtained, clones 11 and 21 were confirmed to have a homzygous knockout, and clone 13 was a biallelic nine-nucleotide deleted clonal cell line. All three clones lost the UCH-L1 protein expression (Fig. 4C), and we used A549ΔUCH-L1 clone 11 for the following experiments. The binding between Smad2/3 and Smad4 was examined as a positive control in the in situ PLA methods. Red dots indicating the binding were observed at 1 h of TGF-β stimulation and decrease with time, and there was not a significant difference between the cell lines (Fig. S4).

As shown in Fig. 4D, UCH-L1–Smad2/3 interaction was detected as red dots without TGF-β treatment, and TGF-β stimulation enhanced the association between UCH-L1 and Smad2/3 in A549 cells after 1 h of TGF-β stimulation (Fig. 4D). Immunofluorescent images were analyzed for the number of dots/cells, and the results were summarized (Fig. 4E). The interaction significantly increased with 1-h stimulation \( (p < 0.1 \times 10^{-14}) \), decreased with time, and returned in 6 h to the same level as before the stimulation (Fig. 4, D and E). These data indicated that UCH-L1 constantly interacts with Smad2/3 and that TGF-β activation induces a transient increase in their binding.

Depletion of UCH-L1 down-regulates TGF-β signaling

To examine the effects of UCH-L1 deficiency on TGF-β signaling, Smad phosphorylation induced upon TGF-β stimulation was investigated in A549 and A549ΔUCH-L1 cells. After starvation for 16 h, these cells were stimulated with TGF-β for the indicated times. Phosphorylation of Smad2 and Smad3 was detected 1 h after the TGF-β treatment and then decreased with time (Fig. 5A). Interestingly, the Smad2 phosphorylation was down-regulated in the A549ΔUCH-L1 cells as compared with the A549 cells, whereas the Smad3 phosphorylation was not affected. Furthermore, the protein expression of Smad2, but not of Smad3, normalized by β-actin was also reduced in A549ΔUCH-L1 cells (Fig. 5, B and C). These results suggest that UCH-L1 was involved in promoting the stabilization of Smad2.

UCH-L1 is a DUB for R-Smads

To clarify whether UCH-L1 possesses DUB activity, we assessed its deubiquitinating activity using a biotin–Ub–VME probe, which typically binds covalently and irreversibly to active DUBs (24). A549ΔUCH-L1 cells were transfected with UCH-L1–3×FLAG or UCH-L1C90A–3×FLAG. The cell lysates were immunoprecipitated with anti-DYKDDDDK tag antibody beads and then incubated with or without a biotin–Ub–vinyl methyl ester (VME) probe. As shown in Fig. 5D, UCH-L1, but not the UCH-L1C90A mutant, interacted with the probe, indicating its DUB activity. We next checked whether UCH-L1 serves as a DUB for Smad2 and Smad3. 293T cells were cotransfected with HA–ubiquitin, UCH-L1–3×FLAG, or UCH-L1C90A–3×FLAG, and with 6Myc–Smad2 or –Smad3 and were stimulated with TGF-β in the presence or absence of a 20S proteasome inhibitor, MG132. The cell lysates were
immunoprecipitated with anti-Myc antibody to concentrate the Smad proteins. Transiently expressed Smad2 and Smad3 were modified by ubiquitin attachment, which was slightly increased by stimulation with TGF-β. In the presence of UCH-L1, polyubiquitination of Smad2 and Smad3 was reduced, but the effect was stronger with Smad2 than with Smad3. Furthermore, the UCH-L1C90A mutant did not suppress polyubiquitination. The proteasome inhibitor MG132 increased the polyubiquitination of Smad proteins in 293T cells (Fig. 5, E and F). These data suggest that UCH-L1 deubiquitinates polyubiquitinated R-Smads to protect them from degradation.

UCH-L1 confers diversity of TGF-β signaling in the cancer microenvironment

UCH-L1 is thought to have an oncogenic effect on several tumors (25), and recent evidence has implicated UCH-L1 in the promotion of metastasis via stabilization of HIF-1α (26). In addition, hypoxic conditions can enhance TGF-β signaling (27). Therefore, we examined how the depletion of UCH-L1 affects the TGF-β signaling in a hypoxic environment. Upon culturing A549 and A549UCH-L1 cells under 5% O2 hypoxia, elevated UCH-L1 expression was observed in A549 cells (Fig. 6, A and B). This phenomenon is not specific to A549. Human
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293T embryonic kidney–derived cell line and mouse Lewis lung carcinoma cells also showed up-regulation of UCH-L1 expression under hypoxic condition (Fig. S5). Then A549 and A549<sup>UCH-L1</sup> cells were starved for 16 h and stimulated with TGF-β for the indicated times. Phosphorylation of Smad2 and Smad3 were maximal 1 h of stimulation with TGF-β. Interestingly, A549<sup>UCH-L1</sup> cells showed not only reduced activated Smad2 phosphoprotein but also reduced total Smad2 protein expression levels (Fig. 6, C and D), although Smad3 was not affected (Fig. 6C and Fig. S6). These findings indicate the possibility that UCH-L1 may contribute more to the stability of Smad2 in a hypoxic environment.

We next assessed the transcriptional levels of a TGF-β target gene in response to TGF-β stimulation in A549 and A549<sup>UCH-L1</sup> cells under normoxic and hypoxic conditions. The mRNA level of PAI-1 was increased by the TGF-β stimulation both under normoxic and under hypoxic conditions; however, in A549<sup>UCH-L1</sup> cells, the level of PAI-1 transcription was dramatically reduced in the hypoxic condition (Fig. 6F). These data suggested that UCH-L1 may be necessary for the TGF-β signal to adapt to the hypoxic environment.

To further analyze whether hypoxia affects TGF-β–mediated responses, we examined TGF-β–induced cell motility by a wound-healing assay. A549 cells moved to fill the gap, and TGF-β stimulation increased the velocity of the cell motility under hypoxic conditions. In contrast, A549<sup>UCH-L1</sup> cells did not respond to TGF-β under hypoxic conditions and hardly moved. Again, these data suggested that the presence of UCH-L1 in a hypoxic environment affects tumor cell responsiveness to TGF-β signaling (Fig. 6F).

We then asked whether the increased expression of UCH-L1 with retroviral infection can restore the responsiveness to TGF-β in A549<sup>UCH-L1</sup> cells. When we re-expressed UCH-L1 in the A549<sup>UCH-L1</sup> cells, termed A549<sup>UCH-L1</sup>/UCH-L1(WT) cells, both the phosphorylation of Smad2 and the TGF-β–induced transcriptional activity were significantly restored (Fig. 6, G and H). However, the control A549<sup>UCH-L1</sup>/Vec and the UCH-L1<sup>C90A</sup> re-expressing A549<sup>UCH-L1</sup>/UCH-L1(C90A) cells had no effect on TGF-β–induced transcriptional activity. Together, these results suggest that catalytic activity of UCH-L1 is involved in the functional regulation of TGF-β signaling.

**Knockout of UCH-L1 inhibits tumorigenicity in vivo**

To investigate the effects of UCH-L1 on tumorigenicity in vivo, lung adenocarcinoma A549 and A549<sup>UCH-L1</sup> cells were subcutaneously injected into BALB/c nu/nu mice. Fig. 7A shows a comparison of the increase in the volume of tumors derived from these two cell lines over a period of 45 days. After the transplantation, the mice-transplanted A549 cells appeared to promote more tumor growth than did the A549<sup>UCH-L1</sup>–transplanted group. The tumorigenicity of the A549<sup>UCH-L1</sup> cells was significantly lower than that of the control cells. 45 days after implantation, fresh-frozen sections were prepared from these tumors and immunohistochemically examined for the expression of UCH-L1, HIF-1α, phospho-Smad2 (PS2), and Smad2. Because all the antibodies were rabbit–derived, the serial sections were stained with each antibody together with anti–PECAM-1 antibody, which shows continuity in the structure of blood vessels. The sections from A549 tumor tissues were subjected to immunohistochemical analysis; UCH-L1– and phospho-Smad2–positive areas were found to be colocalized at high HIF-1α expression sites (Fig. 7C). However, the expression of HIF-1α was not detected in the A549<sup>UCH-L1</sup>–transplanted group, and the number of phospho-Smad2 positive cells were also reduced (Fig. 7D). Furthermore, Western blotting analysis of the tumor tissue showed a decrease in Smad2, but not in Smad3, protein (Fig. S7), similarly to our previous findings in *in vitro*. These results suggest that UCH-L1, HIF-1α, and TGF-β signaling cooperate in tumorigenesis.

To assess whether the DUB activity of UCH-L1 is essential for tumorogenesis, A549<sup>UCH-L1</sup>/UCH-L1(WT) or A549<sup>UCH-L1</sup>/UCH-L1(C90A) cells were subcutaneously transplanted into BALB/c nu/nu mice. Expression of WT UCH-L1 in A549<sup>UCH-L1</sup> cells restored tumorigenetic potential; however, a UCH-L1 mutant defective in DUB activity was not able to do so (Fig. 7, D and E). Thus, the DUB activity of UCH-L1 is needed for efficient tumorigenesis of A549 in vivo.

**Discussion**

In the present study, we showed that the structurally related *C. elegans* UBH-1 and human homolog UCH-L1 both positively regulate DAF-7/TGF-β signaling. Although nematodes are invertebrates, many of the cellular and molecular mechanisms including signal transduction in them are markedly similar to those in humans. In the *C. elegans* genome, three genes encode a UCH-L1/UCH-L3 homolog. These genes, namely *ubh-1*, *ubh-2*, and *ubh-3*, form an operon. Among the gene products, the protein encoded by the first gene, UBH-1, is the one most homologous to human UCH-L1 and UCH-L3. In addition to the phylogenetic relevance (Fig. S1), UBH-1 showed enzymatic properties analogous to human UCH-L1/L3, such as C-terminal homology and phospho-Smad2–positive areas were found to be colocalized at high HIF-1α expression sites.

**Figure 5.** UCH-L1 deubiquitinates R-Smads and enhances TGF-β signaling. A, effect of UCH-L1 on TGF-β–induced Smad2 phosphorylation. A549 and A549<sup>UCH-L1</sup> cells were stimulated with 5 ng/ml TGF-β for 1, 3, and 6 h. These cells were lysed and subjected to Western blotting analysis using anti-pSmad2 (PS2), pSmad3 (PS3), Smad2, and UCH-L1 antibodies. B, quantification for Smad2 protein expression. The intensity of Smad2 was normalized using the intensity of the band corresponding to β-actin. Each relative intensity was calculated by comparing it with the value for the cells that were not treated with 5 ng/ml TGF-β. C, quantification for Smad3 protein expression. The intensity of Smad3 was normalized using the intensity of the band corresponding to β-actin. Each relative intensity was calculated by comparing it with the value for the cells that were not treated with 5 ng/ml TGF-β. D, identification of deubiquitinating activity of UCH-L1. A549<sup>UCH-L1</sup> cells were transfected with FLAG-tagged UCH-L1 or UCH-L1<sup>C90A</sup>. The cell lysates were treated with or without a biotin–VME probe and subjected to Western blotting analysis using a biotin antibody. UCH-L1–FLAG or UCH-L1<sup>C90A</sup>–FLAG was measured by Western blotting analysis with anti-UCH-L1 antibody. E and F, 293T cells were transfected with the indicated expressing plasmids. MG132 was added 6 h before, and 5 ng/ml TGF-β was added 90 min before they were harvested. The cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc antibody. The expression of UCH-L1–3×FLAG and Myc–Smads was measured by Western blotting analysis with the indicated antibodies. IB, immunoblotting; IP, immunoprecipitation.
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**Figure A**

A549 and A549ΔUCH-L1

|          | Normo | Hypo |
|----------|-------|------|
| UCH-L1   |       |      |
| β-Actin  |       |      |

|          | -24 kDa | -42 kDa |

**Figure B**

Relative UCH-L1 expression

|          |             |
|----------|-------------|
| Normo    | 100         |
| Hypo     | 300         |

**Figure C**

TGF-β: 0 1 3 6

|          | A549 | A549ΔUCH-L1 |
|----------|------|-------------|
| pSmad2   |      |             |
| pSmad3   |      |             |
| Smad2/3  |      |             |
| Smad3    |      |             |
| UCH-L1   |      |             |
| β-Actin  |      |             |

**Figure D**

Relative level of Smad2/β-Actin

**Figure E**

Relative PAI-1 expression/GAPDH

| TGF-β  | Normoxia | Hypoxia |
|--------|----------|---------|
| 0      |          |         |
| 3      |          |         |
| 6      |          |         |

**Figure F**

Migration (µm)

| TGF-β  | A549 | A549ΔUCH-L1 |
|--------|------|-------------|
| 0      |      |             |
| 3      |      |             |
| 6      |      |             |

**Figure G**

TGF-β: 0 1 3 6

|          | A549 | A549ΔUCH-L1 |
|----------|------|-------------|
| pSmad2   |      |             |
| pSmad3   |      |             |
| Smad2/3  |      |             |
| Smad3    |      |             |
| UCH-L1   |      |             |
| β-Actin  |      |             |

**Figure H**

Relative PAI-1 expression/GAPDH

| TGF-β  | A549 | A549ΔUCH-L1 |
|--------|------|-------------|
| 0      |      |             |
| 6      |      |             |
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(Table S1) but also to UCH-L1 in its high expression in neural cells (Fig. 1E). In this study, we found that UBH1-1 positively regulates the DAF-7/TGF-β signal in the dauer formation of C. elegans and that UCH-L1 rather than UCH-L3 up-regulates TGF-β signaling in human cells.

Post-translational modification with ubiquitin plays crucial roles in regulation of various signaling pathways and biological processes. Several DUBs, which remove monoubiquitin or poly-ubiquitin chains from target proteins, are known to regulate TGF-β signaling to affect cellular homeostasis (28, 29). For example, OTUB1 (OTU domain-containing ubiquitin aldehyde-binding protein 1), one of the DUBs, reduces polyubiquitination of Smad2/Smad3 by inhibiting an E2 ligase independently of its DUB activity (30). On the other hand, UCH-L5/UCH-L37, a member of the UCH family, is involved in cancer and fibrosis by enhancing TGF-β signaling (31, 32). UCH-L5 interacts with I-Smad and mediates ALK5 stabilization (33).

In addition, UCHL5 can inhibit degradation of Smad2 and Smad3 by its DUB activity. Thus, misregulation of TGF-β signaling by hyperactive or hypoactive DUB could be closely related to diseases including cancer. Similarly to UCH-L5/UCH-L37, which interacts with the proteasome by its C-terminal extension, UCH-L1 without such a C-terminal extension also enhanced TGF-β signaling depending on its enzyme activity (Fig. 3B). Thus, both UCH-L1 and UCH-L5/UCH-L37 enhance TGF-β signaling in a DUB activity-dependent manner, whereas OTUB1 regulates TGF-β signaling in a DUB activity-independent manner (32). In any case, strict control of the amount of Smad proteins is considered to be associated with tumorigenicity.

Many DUBs, i.e. UCH-L37 (33), USP4 (34), USP11 (35), USP15 (36), and AMSH (37), have been identified as potent TGF-β/Smad modulators. Many of them target receptors; however, from the result obtained in C. elegans, UBH1/UCH-L1 rather target Smad proteins (Fig. 2, E and F). UCH-L1 bound to Smad2 and Smad3 even without TGF-β stimulation, and the binding was enhanced by TGF-β stimulation. Phosphorylation of Smads was maximal at 1 h after stimulation and decreased at later time points. Following the decrease in phosphorylation, the number of PLA dots, indicating the interaction between UCH-L1 and Smad2/3, also decreased over time. This suggests that UCH-L1 controls Smad protein stability. Indeed, endogenous Smad2/3 expression was reduced upon depletion of UCH-L1 in A549 cells. Therefore, UCH-L1 may be a pivotal regulator to keep TGF-β signaling in check.

UCH-L1 deficiency caused no prominent phenotype under normoxic conditions but dramatically suppressed the TGF-β-induced transcriptional response under hypoxic conditions (Fig. 6, E and F). Remarkably, we found that hypoxia, which is a characteristic of the tumor microenvironment (38, 39), enhances UCH-L1 expression (Fig. 6A and Fig. S5). In addition, we previously reported that long-term exposure to 5% O2 hypoxia enhances TGF-β signaling in Lewis lung cancer cells (27), which also increased UCH-L1 protein expression under 5% O2 hypoxia. The effect of TGF-β is often cell type- or context-dependent, which might be affected by UCH-L1 as part of it. Also, UCH-L1 may be an enzyme that exerts its effects in an environmentally context-dependent manner. In fact, Goto et al. (26) showed that UCH-L1 enhanced HIF-1 activity under 0.1% O2 hypoxic conditions but did not detect endogenous UCH-L1 protein expression in HeLa or MCF7 cells.

UCH-L1 is involved in cancer progression and is a prognostic marker for endometrial cancer and urothelial carcinoma (ENSG00000154277). An interesting article was published during review of this manuscript reporting that UCH-L1 promotes TGF-β-induced breast cancer metastasis in triple-negative breast cancer patients (40). A correlation between increased UCH-L1 protein levels and cancer has been shown in mice, such as xenograft models with EMT6 or B16F10 cells overexpressing UCH-L1 (26, 41), indicating that UCH-L1–transgenic mice tend to develop tumors. Conversely, UCH-L1 knockdown has been shown to suppress tumorogenesis (42). Kabuta et al. (42) demonstrated that UCH-L1 enhances cyclin-dependent kinase activity independently of its DUB activity. Although it is not defined whether UCH-L1 is involved in lung cancer, high expression of UCH-L1 has been reported as a poor prognostic marker in non–small cell lung carcinoma patients (43); thus, further validation is needed for UCH-L1 in lung cancer. For that, a good candidate is A549 cells, which are cells of lung adenocarcinoma, which is classified as non–small cell lung cancer and is the most frequently found lung cancer. We examined UCH-L1 expression in various non–cancer and cancer-derived cell lines and found that A549 and nontumorigenic MCF10A1 cells are highly expressed UCH-L1, but this was not observed in other colon cancer or breast cancer cells that we examined. Although knockdown of UCH-L1 suppressed tumor formation, tumor growth was detected in their xenograft models. On the other hand, we found that tumor growth in the xenograft model with A549 cells is almost completely suppressed when

**Figure 6. UCH-L1 is enhanced under hypoxic conditions and promotes TGF-β signaling.** A, induction of UCH-L1 expression under hypoxic conditions. A549 and A549UCH-L1 cells were cultured under normoxic (Normo, 21% O2) or hypoxic (Hypo, 5% O2) conditions for 1 week, and the expression of UCH-L1 was subjected to Western blotting analysis. B, quantification for UCH-L1 protein expression in A549 cells under normoxic or hypoxic conditions. The intensity of UCH-L1 was normalized using the intensity of the band corresponding to β-actin. C, Western blotting analysis of phospho-Smad2 in A549 and A549UCH-L1 cells stimulated by 5 ng/ml TGF-β for the indicated times. The expressions of phospho-Smad2, phospho-Smad3, Smad2/3, and β-actin were detected by Western blotting analysis with the indicated antibodies. D, quantification of Smad2 protein expression. The intensity of Smad2 was normalized using the intensity of the band corresponding to β-actin. Each relative intensity was calculated by comparing it with the value for cells that were not treated with 5 ng/ml TGF-β. E, qPCR analysis of TGF-β target gene expression. A549 and A549UCH-L1 cells under normoxic or hypoxic conditions were treated with 5 ng/ml TGF-β for the indicated times. Expression levels of SERPIN1 encoding PAI-1 genes are depicted as fold induction relative to the expression levels without TGF-β stimulation. F, wound-healing assay in the presence or absence of TGF-β under normoxic or hypoxic conditions. A549 and A549UCH-L1 cells were stimulated with or without 5 ng/ml TGF-β for 24 h. Results representative of three independent experiments are shown.
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A

![Graph showing tumor volume over days for A549 and A549^UCH-L1](image)

B

![Images of A549 and A549^UCH-L1 tumors](image)

C

![Immunofluorescence images showing expression of UCH-L1, HIF-1α, PS2, and Smad2 in A549 and A549^UCH-L1](image)

D

![Graph showing tumor volume over days for A549^UCH-L1/WT and A549^UCH-L1/C90A](image)

E

![Images of A549^UCH-L1/WT and A549^UCH-L1/C90A tumors](image)
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UCH-L1 in the A549 cells is knocked out (Fig. 7B). This difference may be due to the difference in the cell line used or to whether the gene is knocked out or knocked down. The in vivo results were more pronounced than the in vitro ones, possibly because of regulation of other signaling pathways under physiological conditions in multiple cell types. In fact, we confirmed an increase in pAkt, which was also reported in other cancer cells (44, 45), from A549-derived tumor tissue (Fig. S7, A–C). Furthermore, the cancer tissues in vivo were under a hypoxic environment, which may have enhanced the expression of UCH-L1 (Fig. 7C). These subtle differences in UCH-L1 protein levels may affect the cellular behavior. In fact, UCH-L1 targeting signals are diverse and thus require further validation in terms of cancer treatment.

In conclusion, UCH-L1 interacts with Smad2 and Smad3 and stabilizes in particular Smad2 to promote TGF-β signaling, for which DUB activity of UCH-L1 is essential. Furthermore, UCH-L1 is indispensable for lung-cancer cell survival under hypoxic conditions. These findings suggest that targeting UCH-L1 and its DUB activity could be a new potential therapeutic strategy for lung cancer therapy. Future studies are needed on their target proteins in hypoxic conditions in cancer cells.

Materials and methods

Strains and general methods for C. elegans

Standard methods were used for maintaining and manipulating C. elegans (46). The C. elegans Bristol N2 (WT) and rfe-3 (pk1426) strains was obtained from the Caenorhabditis Genetics Center (University of Minnesota). The following strains were obtained from the National Bioresource Project for the Experimental Animal “Nematode C. elegans” (Tokyo Women’s Medical University School of Medicine): ubh-1(tm526), ubh-2 (tm2267), and ubh-3(tm2550). Transgenic lines were generated by injecting the DNA at a concentration of 10 or 20 ng/μl into the syncytial gonad, as previously described (47).

Preparation of recombinant UBH proteins

The UBH-1, UBH-2, and UBH-3 proteins were expressed as fusion proteins with a His6 tag. The open reading frames of the ubh-1, ubh-2, and ubh-3 transcripts were amplified by RT-PCR using primers as follows: for ubh-1, 5′-GGCCGCCATATG-GGCCCTCATAGGAC-3′ and 5′-GGCCGGATCTGATTTG-AACAAGTCCATGC-3′; for ubh-2, 5′-GGGGCCATGGG-TAGCCGAAACGAGG-3′ and 5′-CCCGTGCAGAATCC-CAACAGTGGC-3′; and for ubh-3, 5′-GGCGGATATGAAGCTC-3′ and 5′-GGCGGATATGTTGCTC-3′. These cells were subcloned into pET22b (Novagen). The NcoI/SalI and NdeI/BamHI fragments of the pET22b expression vector were ligated into the His6 tag coding sequence of UBH1/UCH-L1, UCHL1(C90A), UCHL1(WT), and UCHL1(C90A) cells were transplanted subcutaneously into Balb/c nu/nu mice. Tumor size was measured from above the skin every 3 days. Tumor volumes were calculated using the formula: V = Length × Width × Height / 2. The data presented are means ± S.D. (n = 6). E. coli RosettaTM(DE3) cells were transformed with the resulting plasmids. The transformed cells were cultured in M9ZB medium (1% NZ amine, 0.5% NaCl, 0.5% yeast extract, 0.01% NH4Cl, 0.3% KH2PO4, 1.52% Na2HPO4, 0.4% glucose, and 1 mM MgSO4) containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol with vigorous shaking at 15, 23, and 18°C, respectively, in the cases of UCH-1, UCH-2, and UCH-3. When turbidity at 600 nm reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for 16 h. The cells were harvested by centrifugation and suspended in a lysis buffer (50 mM Tris/ HCl buffer, pH 8.0, containing 1 mM EDTA, and 0.1 M NaCl) containing 10 mg/ml lysozyme. The suspension was kept on ice for 30 min, sonicated for 20 s seven times, and centrifuged at 18,000 × g for 20 min. The supernatant was applied to a column of Ni2+-chelating Sepharose (Amersham Biosciences). The column was equilibrated with the lysis buffer and washed with the buffer. The proteins were eluted by a stepwise elution with an elution buffer (100 mM sodium phosphate, pH 6.0, 300 mM NaCl) containing 0, 50, 100, 200, 250, 350, and 500 mI4-imidazole. Fractions containing the recombinant protein were concentrated with Amicon Ultra-15 10,000 molecular weight cutoff (Millipore). The concentrated solutions were diluted with 100 volumes of 50 mM Tris/HCl, pH 7.6, containing 0.5 mM EDTA and 1 mM DTT and then concentrated again.

Enzyme assay

C. elegans ubiquitin, NEDD8, and SUMO fused with an HSV tag and a His6 tag were expressed in E. coli. Their open reading frames were amplified by RT-PCR using the following primers: for ubiquitin (ubq-2), 5′-GGCCGCCATATGGCAAATTCCTGGT-CAAGAC-3′ and 5′-GGCGGATATTGCATGATTTG-CACGA-3′; for NEDD8 (ned-8), 5′-GGCGGATATGGCATTCAACATTTAAAACC-3′ and 5′-CCGGGAATTCCGCA-AATCTCCGGGAGAGGC-3′; and for SUMO (smo-1), 5′-GGCGGATATGGCCGATAGTCAGCTC-3′ and 5′-GCGGATATGGCCCATAGTCAGCTC-3′. These cells were subcloned into pET22b (Novagen). The nucleotide sequences were confirmed by DNA sequencing. The amplified product of ubh-1 was digested with Ndel and BamHI and then was inserted in the pET22b expression vector. The Ncol/Sall and Ndel/BamHI fragments of ubh-2 and ubh-3 PCR products were inserted in the pET21d and pET22b vectors, respectively. The nucleotide sequences were confirmed by DNA sequencing. E. coli RosettaTM(DE3) cells were transformed with the resulting plasmids. The transformed cells were cultured in M9ZB medium (1% NZ amine, 0.5% NaCl, 0.5% yeast extract, 0.01% NH4Cl, 0.3% KH2PO4, 1.52% Na2HPO4, 0.4% glucose, and 1 mM MgSO4) containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol with vigorous shaking at 15, 23, and 18°C, respectively, in the cases of UCH-1, UCH-2, and UCH-3. When turbidity at 600 nm reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for 16 h. The cells were harvested by centrifugation and suspended in a lysis buffer (50 mM Tris/HCl buffer, pH 8.0, containing 1 mM EDTA, and 0.1 M NaCl) containing 10 mg/ml lysozyme. The suspension was kept on ice for 30 min, sonicated for 20 s seven times, and centrifuged at 18,000 × g for 20 min. The supernatant was applied to a column of Ni2+-chelating Sepharose (Amersham Biosciences). The column was equilibrated with the lysis buffer and washed with the buffer. The proteins were eluted by a stepwise elution with an elution buffer (100 mM sodium phosphate, pH 6.0, 300 mM NaCl) containing 0, 50, 100, 200, 250, 350, and 500 mM imidazole. Fractions containing the recombinant protein were concentrated with Amicon Ultra-15 10,000 molecular weight cutoff (Millipore). The concentrated solutions were diluted with 100 volumes of 50 mM Tris/HCl, pH 7.6, containing 0.5 mM EDTA and 1 mM DTT and then concentrated again.
plasmids. The transformed cells were cultured in M9ZB medium containing 50 μg/ml ampicillin and 30 μg/ml chloramphenicol with vigorous shaking at 37 °C. When turbidity at 600 nm was reached, 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for 3 h. The cells were harvested by centrifugation and suspended in the lysis buffer. The suspensions were frozen and thawed three times, sonicated for 20 s seven times, and centrifuged at 18,000 × g for 20 min. The fusion proteins were purified through a column of Ni²⁺-chelating Sepharose (Amersham Biosciences). The fractions containing the recombinant protein were dialyzed against 100 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 1 mM DTT.

Expression plasmids for UCH-L1 and UCH-L3

Human UCH-L1 and UCH-L3 cDNAs were cloned by PCR with forward primers with a NotI site and reverse primers with a XbaI site. The amplified products were digested with the corresponding restriction enzymes and subcloned into the NotI–XbaI site of the p3×FLAG-CMV™-14 mammalian expression vector (E7908, Sigma–Aldrich). The UCH-L1 (C90A) mutant was obtained using a PrimeStar HS DNA polymerase kit (Takara). The primers used for alanine mutation of the catalytic Cys90 residue of UCH-L1 were as follows: UCH-L1–C90A forward, 5′-GGGAGTTCCGCTGGCACAATCG-GACATTATCC-3′; and UCH-L1–C90A reverse, 5′-ATTGGTGACACTCCATGCCCGTACTG-3′. All DNAs were verified by sequencing after PCR amplification. UCH-L1 and its mutants were inserted into pCD83 (48). (CAGA)12–luc, SBE 3′-luc, 6MyC–Smad2, 6MyC–Smad3, 6MyC–Smad4, ALK5ca/V5, ALK5ca/HA, and ALK6ca/V5 have been described previously (49).

Cell culture and transfections

All cell lines except for A549 cells, which were obtained from Dr. Y. Urano (Tokyo University), were obtained from the Netherlands Cancer Institute. HepG2, A549, 293T, and COS7 cells were cultured in Dulbecco’s modified Eagle’s medium (Nacalai Tesque) containing 10% fetal calf serum (Invitrogen) and 1 mM minimal essential medium nonessential amino acids (Nacalai Tesque). For the reporter assay, HepG2 cells were seeded at 1.5 × 10⁵ cells/well in 12-well plates 1 day before transfection. The cells were transfected using polyethyleneimine (PolySciences). Where indicated, 5 ng/ml TGF-β1 (Wako) was added to the wells 24 h after the transfection. Subsequently, the cells were cultured in Dulbecco’s modified Eagle’s medium containing 0.3% fetal calf serum for 16 h. In all the experiments, β-gal (pCH110, GE Healthcare) activity was measured to normalize for transfection efficiency. Each transfection was carried out in triplicate and repeated at least twice.

Establishment of UCH-L1 knockout cells

A pair of gRNA oligonucleotides for each targeting site was annealed and ligated to a BbsI-treated pSpCas9(BB)-2A-GFP (pX458) vector (50), and the sequences of the gRNAs were verified by sequencing analysis. To generate the KO cell line, A549[UCH-L1], the CRISPR design tool was used to identify the gRNA sequence, and the following sequence was used as the target gRNA: 5′-GACAGTCACGAAAGCCACAC-3′. A549 cells were transfected with a pX458 vector, and 1 day after the transfection, cells that highly expressed GFP with Cas9 were isolated by FACS (SH800, Sony) and cultured. The edited locus was amplified using primers (5′-GGCGTACTCTACGA-ACCAG-3′ and 5′-AAATGCAGGCTCCTCCCTT-3′) and confirmed by Sanger sequencing.

Establishment of UCH-L1 rescued cells

Human UCH-L1 (WT or C90A) cells were subcloned into a pCX4pur mammalian expression vector (51). Each of the following plasmids was transfected into 4.0 × 10⁵ cells of Plat-A cells on a 6-well plate with FuGENE 6 (Promega); pCX4pur/empty(Vec), pCX4pur/UCH-L1(WT), and pCX4pur/UCH-L1 (C90A). 2 days after the transfection, the medium containing retrovirus was collected and filtered through a 0.22-μm filter and added to the A549[UCH-L1] cells for viral transduction. After 24 h of transduction, the cells were trypsinized and seeded onto a 10-cm dishes and selected in 1 mg/ml puromycin for 3–4 days. Stable cell lines expressing UCH-L1–3×FLAG protein were identified by Western blotting analysis with anti-FLAG and anti–UCH-L1 antibodies.

Immunoprecipitation and Western blotting analysis

To detect protein interactions, the plasmids were transfected into COS7 cells (5 × 10⁵ cells/6-cm dish) using polyethyleneimine. 40 h after the transfection, the cells were lysed in 500 μl of TNE buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 100 units/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF, and 20 mM β-glycerophosphate). The cell lysates were preclarified with protein G–Sepharose beads (GE Healthcare) for 30 min at 4 °C and then incubated with anti-FLAG antibody (Sigma) for 2 h or overnight at 4 °C. Subsequently, protein G–Sepharose beads were added to the reaction mixture and incubated overnight at 4 °C. After washing the immunoprecipitates with high-salt buffer (20 mM Tris, pH 7.4, 500 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 2.5 μg/ml aprotinin, 2 mM sodium vanadate, 40 mM NaF,
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and 20 mM β-glycerophosphate) three times and with TNE buffer three times, the immunoprecipitated proteins and aliquots of the total cell lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to an Ultra Cruz nitrocellulose pure transfer membrane (Santa Cruz). The immunoprecipitated proteins and aliquots of the total cell lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to an Ultra Cruz nitrocellulose pure transfer membrane (Santa Cruz). The membranes were probed with primary antibodies. The primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies and a chemiluminescent substrate (Thermo).

**In vitro deubiquitination assay**

To detect the DUB activity, A549\textsuperscript{DUCH-L1}/empty (Vec), A549\textsuperscript{DUCH-L1}/UCH-L1 (WT), or A549\textsuperscript{DUCH-L1}/UCH-L1 (C90A) cells were seeded (5 × 10\textsuperscript{5} cells/10-cm dish) and lysed the next day in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 500 mM NaCl, 5 mM EDTA, 1 mM DTT, 1× protease inhibitor mixture; Nakalai Tesque). The cell lysates were incubated with DYKDGDGDG tag antibody beads (Wako) for 2 h at 4 °C. The samples were divided into two tubes, and one of the two (470 μg of protein) was added a biotin–A-IgG conjugated to a VME probe (final concentration, 1 μM), and incubated for 1 h at 37 °C. The immunoprecipitated proteins and aliquots of the total cell lysates were boiled for 5 min in sample buffer, separated by SDS-PAGE, and transferred to an Ultra Cruz nitrocellulose pure transfer membrane (Santa Cruz). The membranes were probed with primary antibodies. The primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies and a chemiluminescent substrate (Thermo).

**Antibodies**

Antibodies were obtained from the following sources: mouse monoclonal anti–FLAG-M2 antibody (F3165) from Sigma; mouse monoclonal anti-Myc9E10 (sc-40), mouse monoclonal anti–β-actin (sc-69879) from Santa Cruz; mouse monoclonal anti-HA 12CA5 (catalog no. 11583816001) and rat monoclonal anti-HA 3F10 (catalog no. 11867423001) antibodies from Roche; mouse monoclonal anti-V5 antibody (catalog no. 017-235939) from Wako; mouse monoclonal anti-Smad2/3 (catalog no. 610843) and rat monoclonal anti-PECAM-1 (MEC13.3) antibodies from BD Transduction Laboratories; rabbit monoclonal UCH-L1 (catalog no. 11896) and rabbit polyclonal HIF-1α (36169S) from Cell Signaling Technology; sheep anti-mouse IgG (catalog no. NA-931-1ML) from GE Healthcare. The rabbit polyclonal phosphorylated Smad2 antibody, termed PS2, and the rabbit polyclonal Smad2 antibody were homemade (52).

**Proximity-ligation assay**

The cells on coverglasses coated with 0.1% gelatin were cultured with Dulbecco’s modified Eagle’s medium. Then the coverglasses were washed once with PBS, fixed for 10 min with 4% paraformaldehyde (Wako), washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and again washed three times with PBS. The procedures were performed according to the manufacturer’s instructions (Olink Bioscience). To visualize the fluorescence, a BZ-9000 fluorescence microscope (Keyence) was used.

**RNA purification and quantitative PCR (qPCR)**

Total RNA was extracted using a NucleoSpin® RNA Plus kit (Takara). Reverse transcription was performed with a PrimeScript II first strand cDNA synthesis kit (Takara). qPCR was performed with a KAPA SYBR Fast qPCR kit (Kapa). All reactions were carried out on a LightCycler® 96 (Roche). Each sample was analyzed in triplicate at least twice for each PCR measurement. Melting curves were checked to ensure specificity. Relative quantification of mRNA expression was calculated using the standard curve method with the β-actin level. Before qPCR, the DNA fragment amplified using each primer set was detected to be a single band with the correct size by agarose gel electrophoresis. The following primer sets were used to amplify Smad7 and β-actin cDNAs: 5’-GGGTCTGTGTCAACC-GTATC-3’ and 5’-GTAATGTGAATCTCGACTGCC-3’ for human PAI-1 (53) and 5’-GAAGGTTGAGTTCGAGTCC-3’ and 5’-GAAGATGTTGATGGATTTC-3’ for human glyceraldehyde-3-phosphate dehydrogenase.

**In vivo xenografts**

Male BALB/c nude mice (weight, 20–24 g) were purchased from Orientalbio Co., Ltd., and housed for 1 week. A549, A549\textsuperscript{DUCH-L1}, A549\textsuperscript{DUCH-L1}/UCH-L1 (WT), or A549\textsuperscript{DUCH-L1}/UCH-L1(C90A) cells (5 × 10\textsuperscript{5} cells) in 100 μl of PBS–Matrigel (Corning) (v/v, 1:1) were injected subcutaneously into the mice. Tumor volumes (V) were calculated using the following formula: \( V = \text{Length} \times \text{Width} \times \text{Width} \times 0.5 \). The grown cancer tissues were surgically removed and embedded into a frozen section compound (Leica). Fresh-frozen sections (4 μm) were cut with a CM1850 cryostat (Leica), mounted on Cryofilms (Leica), and fixed in 100% ethanol. The films were washed three times with PBS, permeabilized with 0.1% Triton X-100 (Sigma) for 5 min, and blocked with blocking reagent (PerkinElmer) for 1 h at 37 °C. Rabbit anti–HIF-1α (1:200), anti–UCH-L1 (1:200), and anti-PS2 (1:200), anti-Smad2 (1:200), and rat anti-PECAM1 (1:200) antibodies in blocking reagent were added and incubated overnight at 4 °C. The films were washed three times with PBS and then incubated with Alexa 488–conjugated goat anti-rabbit IgG (Molecular Probes) or Alexa 594–conjugated goat anti-rat IgG (Molecular Probes) antibodies at 1:200 for 1 h at room temperature. After the nuclei were stained with 2 μg/ml 4’,6’-diamino-2-phenylindole for 10 min, the samples were washed three times with PBS, and the fluorescence signals were visualized by microscopy (Keyence). All animal manipulations were approved by the president of Tokyo University of Pharmacy and Life Sciences after review by the institutional animal care and use committee (permission number L18-03 and L19-17) and carried out according to the Tokyo University of Pharmacy and Life Sciences Animal Experimentation Regulations.
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Statistical analysis

The data were expressed as means ± S.D. unless otherwise mentioned. Significance was assessed using the Student’s t test. Probability values below 0.05, 0.01, and 0.001 were considered significant.

Data availability

All data generated during this study are included in this article and in the supporting information.

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Abbreviations—The abbreviations used are: DUB, deubiquitinating enzymes; ALK5, activin receptor-like kinase-5; ALK5ca, constitutively active ALK5; CRISP-Cas9, clustered regularly interspaced short palindromic repeats/CRISPR associated proteins 9; gRNA, guide RNA; HIF-1, hypoxia-inducible factor-1; NEDD, neural precursor cell expressed developmentally down-regulated protein; PAI-1, plasminogen activator inhibitor-1; PLA, proximity-ligation assay; PS2, phospho-Smad2; qPCR, quantitative PCR; R-Smad, receptor-regulated Smad; SBE, Smad-binding element; SUMO, small ubiquitin-related(like) modifier; TGF-β, transforming growth factor-β; Ub, ubiquitin; UCH-L1, ubiquitin C-terminal hydrolase-L1; USP, ubiquitin-specific protease; Vec, vector; VME, vinyl methyl ester.

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