Published in final edited form as:

Cancer Res. 2019 April 15; 79(8): 1913–1924. doi:10.1158/0008-5472.CAN-18-3037.

The deubiquitylase OTUB1 mediates ferroptosis via stabilization of SLC7A11

Tong Liu^a, Le Jiang^a, Omid Tavana^a, and Wei Gu^{a,1}

^aInstitute for Cancer Genetics, Department of Pathology and Cell Biology, and Herbert Irving Comprehensive Cancer Center, College of Physicians & Surgeons, Columbia University, 1130 Nicholas Ave, New York, NY 10032, USA

Abstract

Although cell cycle arrest, senescence, and apoptosis are established mechanisms of tumor suppression, accumulating evidence reveals that ferroptosis, an iron-dependent, non-apoptotic form of cell death, represents a new regulatory pathway in suppressing tumor development. Ferroptosis is triggered by lipid peroxidation and is tightly regulated by SLC7A11, a key component of the cystine-glutamate antiporter. Although many studies demonstrate the importance of transcriptional regulation of SLC7A11 in ferroptotic responses, it remains largely unknown how the stability of SLC7A11 is controlled in human cancers. In this study, we utilized biochemical purification to identify the ubiquitin hydrolase OTUB1 as a key factor in modulating SLC7A11 stability. OTUB1 directly interacted with and stabilized SLC7A11; conversely, OTUB1 knockdown diminished SLC7A11 levels in cancer cells. OTUB1 was overexpressed in human cancers, and inactivation of OTUB1 destabilized SLC7A11 and led to growth suppression of tumor xenografts in mice, which was associated with reduced activation of ferroptosis. Notably, overexpression of the cancer stem cell marker CD44 enhanced the stability of SLC7A11 by promoting the interaction between SLC7A11 and OTUB1; depletion of CD44 partially abrogated this interaction. CD44 expression suppressed ferroptosis in cancer cells in an OTUB1-dependent manner. Together, these results show that OTUB1 plays an essential role in controlling the stability of SLC7A11 and the CD44-mediated effects on ferroptosis in human cancers.

Keywords

p53; ferroptosis; ROS; OTUB1; SLC7A11; oxidative stress; tumor suppressor

Introduction:

Ferroptosis is a regulated form of non-apoptotic cell death driven by the accumulation of lipid-based reactive oxygen species (ROS) tightly linked with oxidative stress responses and cystine metabolism (1–4). Solute carrier family 7, membrane 11 (SLC7A11), a 12-pass

1 Corresponding author: Wei Gu, Mailing Address: Institute for Cancer Genetics, Department of Pathology and Cell Biology, and Herbert Irving Comprehensive Cancer Center, College of Physicians & Surgeons, Columbia University, 1130 Nicholas Ave, New York, NY 10032, USA, wg8@cumc.columbia.edu, Tel. 212-851-5282, Fax 212-851-5284.

There are no potential conflicts of interest disclosed.

Further information and requests for reagents may be directed to the corresponding author, Dr. Wei Gu (wg8@columbia.edu).

transmembrane protein, is a key component of the amino acid transporter system x_c^- together with its binding partner CD98 (*SLC3A2*) (4, 5). The primary function of system x_c^- is to uptake cystine in exchange for glutamate. Cystine is essential for glutathione synthesis, a major cofactor of the endogenous antioxidant program (1). A key feature of the ferroptosis process is the accumulation of lipid peroxidates, normally dissipated by the antioxidant enzyme glutathione peroxidase 4 (GPX4) (2, 6, 7). GPX4 is a phospholipid peroxidase that catalyzes lipid peroxides, in the presence of glutathione as an essential cofactor. Recent studies demonstrate that inhibition or loss of GPX4 directly leads to ferroptosis activation because of accumulation of lipid peroxides (3). The inability of the cells to catalyze the reduction of lipid peroxides can also be a consequence of dysfunction in cystine metabolism. For example, downregulation of *SLC7A11* ultimately leads to loss of intracellular cystine levels and subsequent depletion of glutathione biosynthesis, indirectly causing suppression of GPX4 activity and subsequently ferroptosis activation (1, 2, 6).

Accumulating evidence indicate that ferroptosis acts as an independent mechanism of tumor suppression (3). Cell-cycle arrest, senescence and apoptosis have been well accepted as major barriers to cancer development; however, recent studies indicate that loss of cell cycle arrest, apoptosis, and senescence are not sufficient to abrogate the tumor suppression activity of p53 (8, 9). Although the precise mechanism by which p53 suppresses tumor growth through metabolic regulation needs to be further elucidated, we and others recently showed that p53-mediated down-regulation of *SLC7A11* may contribute significantly to its tumor suppression function (8, 10–13). *SLC7A11* is highly expressed in many types of human cancers associated with poor patient survival (5, 14–16). The levels of *SLC7A11* can be induced by oxidative stress and metabolic stress such as glucose starvation and amino acid deprivation (4,17). Upon stress, the *SLC7A11* promoter is significantly activated mainly by NRF2 (nuclear factor erythroid 2-related factor) and Activating transcription factor 4 (ATF4) (18–20). Indeed, several studies indicate that activation of *SLC7A11* by either NRF2 or ATF4 is well correlated with their abilities in modulating ferroptosis and tumor growth (19–21). Notably, our recent study also showed that the tumor suppressor ARF directly interacts with, and suppresses NRF2-mediated transactivation function (22). ARF-mediated suppression of *SLC7A11* expression induced by NRF2 is critically involved in ARF-dependent tumor growth repression in a p53-independent manner.

Nevertheless, transcriptional regulation of *SLC7A11* expression in human cancers is well established but the mechanisms by which *SLC7A11* activity is regulated remain largely unclear. Here, we identify OTUB1 as a key regulator of *SLC7A11* function. OTUB1, an ovarian tumor (OTU) family member deubiquitinase, was previously implicated as a regulator of the p53 pathway by modulating the activities of Mdm2 and Mdmx (23, 24). We found that OTUB1 directly interacts with *SLC7A11* and regulates *SLC7A11* stability in a p53-independent manner. Moreover, depletion of endogenous OTUB1 diminishes *SLC7A11* activity in human cancer cells. OTUB1 is overexpressed in a variety of human cancers. Importantly, OTUB1 inactivation sensitizes cancer cells to ferroptosis through downregulating *SLC7A11* function. Loss of OTUB1 suppresses tumor growth in xenograft mouse models but these effects are largely abrogated by *SLC7A11* overexpression, validating a critical role for the OTUB1-*SLC7A11* interaction in tumorigenesis. Finally, the *SLC7A11*-OTUB1 interaction is also regulated by CD44, a key marker of cancer stem cells.

We further showed that OTUB1 is essential for CD44-mediated inhibitory effects on ferroptosis in human cancer cells. Thus, these results demonstrate that OTUB1 plays a major role in modulating SLC7A11 activity and ferroptotic responses in human cancer cells and reveals that OTUB1 is a potential target in cancer therapy.

Materials and Methods:

Cell culture and stable lines

HEK293, SK-N-BE(2)C, U2OS, HCT116, SKRC-42 and H1299 cancer lines were obtained from American Type Culture Collection (ATCC) in 2014 and 2015, and have been proven to be negative for mycoplasma contamination. T24, UM-UC-3 and SW780 bladder cancer cell lines were obtained from Dr. Abate-Shen's lab of Columbia University Medical Center in 2017. No cell lines used in this work were listed in the ICLAC database. All cells were cultured in DMEM with 10% FBS (all from Gibco) at 37 °C incubator with 5% CO₂. OTUB1 CRISPR-cas9-knockout SK-N-BE(2)C, U2OS, H1299, T24 and UM-UC-3 cells were generated by transfecting OTUB1 double nickase plasmid (sc-407665-NIC; Santa Cruz). To generate the SLC7A11 stable cell line, wild-type Flag-HA-SLC7A11 was transfected into H1299 cells/OTUB1 CRISPR cells or T24 cells/OTUB1 CRISPR cells followed by selection and maintenance with 1mg/mL G418 (Sigma) in DMEM medium containing 10% FBS. Single clones were selected and screened by western blot.

Plasmids

Flag-OTUB1 and its mutants were generously gifted from Dr. MS Dai (Oregon Health&Science University). Flag-HA-SLC7A11 was previously described (8). Full-length SLC7A11 was subcloned into pcDNA3.1/v5-His-Topo vector (Invitrogen). OTUB1 was amplified by PCR from Flag-OTUB1 expression vector and subcloned into the SFB vector. CD44 was obtained from Addgene (#19127), and full length CD44 was subcloned into the pcDNA3.1/v5-His-Topo vector (Invitrogen).

Purification of SLC7A11-complexes from human cells

The epitope-tagging strategy to isolate SLC7A11-containing protein complexes from human H1299 cells was performed as previously (22), please see details in supplementary file.

Western Blotting and Antibodies.

Protein extracts were analyzed by Western Blotting according to standard protocols using primary antibodies specific for OTUB1 (ab175200; Abcam), SLC7A11 (#12691; Cell Signaling), CD44 (BBA10; R&D systems), vinculin (V9264; Sigma-Aldrich), Beta-actin (a5441; Sigma-Aldrich), Flag (f7425; Sigma-Aldrich) and HA (11867423001; Sigma-Aldrich). HRP-conjugated anti-mouse (na931v; GE Healthcare) and anti-rabbit (na934v; GE Healthcare), anti-rat (Southern Biotech;3050-05) were used.

Ablation of endogenous OTUB1 and CD44 family by RNAi

Knock-down of OTUB1 protein was performed by transfection of H1299, SK-N-BE(2)C T24, UM-UC-3 and SW780 cells with siRNA duplex oligo set (ON-TARGET plus

SMARTpool: OTUB1, L-021061-00-0005, Dharmacon) and CD44 (ON-TARGET plus SMARTpool: CD44, L-009999-00-0005) with Lipofectamine 3000 (Invitrogen) for 72h according to the manufacturer's protocol.

RNA extraction and qRT-PCR and sequencing of tumor samples

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was generated using SuperScript™ IV VILO™ Master Mix (Invitrogen). For the qRT-PCR analysis of human transcripts the following primers were used:

PTGS2 forward	5'-CTTCACGCATCAGTTTTTCAAG-3'
PTGS2 reverse	5'-TCACCGTAAATATGATTTAAGTCCAC-3'
GAPDH forward	5'-ATCAATGGAAATCCCATCACCA-3'
GAPDH reverse	5'-GACTCCACGACGTACTCAGCG-3'

Cell death assay

For cell death assays, cells were treated with TBH, erastin or cystine starvation as indicated. Cells were trypsinized, collected and stained with trypan blue, and then counted with a hemocytometer using the cell number counter (Life technologies countess II). Living cells and dead cells were all counted according to the cell size, and cells stained blue were considered as dead cells. The cell death assays were also further confirmed with the similar results by FACS. To validate whether these are ferroptotic cell death, we treated the cells with Ferrostatin-1 or other cell death inhibitors. If the cell death can be completely rescued by Ferrostatin-1 but not by other cell death inhibitors, the cell death is ferroptotic.

Drugs and inhibitors

For ROS generation, tert-butyl hydroxide solution (TBH, Sigma) was used at different doses depending on the experiment; see respective figure legends. Erastin (329600, EMD, Milipore) was used at different doses depending on the experiment. Ferrostatin-1 (ferroptosis inhibitor; Xcess Biosciences) was used at different doses depending on the experiment; see respective figure legends. For cystine starvation, cells were pre-washed with PBS 3 times and supplied with cystine-deficient DMEM with 10% FBS (ME110123L1, GIBCO) for indicated time.

GST pull-down assays

GST and GST-tagged fusion proteins were purified using GST resin (Novagen) according to the manufacturer's instruction. To purify the Flag-HA-SLC7A11 proteins, the 293T cells transfected with Flag-HA-SLC7A11 were lysed in BC100 buffer and then subjected with anti-Flag (M2) antibody-coupled beads for immunoprecipitation. The bound proteins were eluted with BC100 buffer 2% NP-40 with FLAG peptide. GST or GST-OTUB1 were incubated with the purified Flag-HA-SLC7A11 overnight at 4 °C in BC100 buffer. After washing three times, the bound proteins were eluted for 2 h at 4 °C in elution buffer (20 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) and subjected to Western blot analysis.

Cystine uptake assays

Cells were plated in a six-well plate and cultured in regular DMEM medium overnight. Cells were washed twice in prewarmed Na⁺-free uptake buffer containing 137 mM choline chloride, 3 mM KCL, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM D-glucose, 0.7 mM K₂HPO₄ and 10 nM HEPES (pH7.4). Cells were then incubated in 1 ml uptake buffer at 37 °C for 10 min. The buffer was replaced with 600 µl uptake buffer containing [¹⁴C] cystine (0.2 µCi ml⁻¹) (PerkinElmer) and incubated for 10 min. Cells were rinsed twice with cold cystine uptake buffer followed by addition of NaOH (0.1 M) to lyse the cells. Radioactivity (DPM) was measured using Tri-Carb liquid scintillation analyzer (PerkinElmer, Model 4810TR).

Mouse xenograft

T24 control cells, OTUB1 CRISPR cells and OTUB1 CRISPR cells stably transfected with SLC7A11 were trypsinized and counted. 5.0×10^6 cells were mixed with Matrigel (BD Biosciences) at 1:1 ratio (v/v) and injected subcutaneously into seven-week old nude mice (NU/NU; Charles River). Mice were fed with regular chow. After nine weeks, the mice were killed and the tumors were weighed and recorded. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

Quantification and Statistical Analysis

Results are presents as the mean $\pm$ s.d. Differences were determined by using a two-tailed, unpaired Student's t-test with a confidence interval (CI) of 95%. $P \leq 0.05$ was denoted as statistically significant.

Results

OTUB1 is a *bona fide* binding partner of SLC7A11 both *in vitro* and *in vivo*.

To dissect the mechanisms by which SLC7A11 is regulated in human cancer, we sought to isolate SLC7A11-associated protein complexes from human tumor cells. To this end, cell extracts from a p53-null H1299 lung carcinoma cell line that stably expresses a human SLC7A11 protein with N-terminal Flag and HA epitopes (Flag-HA-SLC7A11) (Figure 1A) were subjected to two-step affinity chromatography as previously described (22, 25). The affinity-purified SLC7A11-associated proteins were analyzed by liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). As expected, we identified SLC3A2, a known binding partner of SLC7A11 in the cystine-glutamate antiporter (5, 26), by both Western Blot analysis (Figure S1) and mass spectrometric analysis (Figure S1). In addition, mass spectrometric analysis of a protein band migrating at ~34 kD (Figure 1B) revealed 15 peptide sequences matching OTUB1, a deubiquitinating enzyme of the ovarian tumor (OTU) family (23, 27, 28) (Figure 1B). To validate the *in vivo* interaction between SLC7A11 and OTUB1, we first transfected native H1299 cells with an OTUB1 expression vector in the presence or absence of a vector encoding Flag-tagged SLC7A11. As shown in Figure 1C, OTUB1 was readily detected in the immunoprecipitated complexes of Flag-SLC7A11. Conversely, SLC7A11 was co-immunoprecipitated with Flag-tagged OTUB1 in a similar fashion (Figure 1D). To evaluate this interaction under more physiological conditions, we performed co-immunoprecipitation assays with endogenous proteins from human

neuroblastoma SK-N-BE(2)C cells. As shown in Figure 1E, the endogenous OTUB1 protein was co-precipitated by an SLC7A11-specific antibody, while endogenous SLC7A11 was co-precipitated by an OTUB1-specific antibody (Figure 1F). To ascertain whether OTUB1 and SLC7A11 interact directly, we performed *in vitro* GST pull-down assays by incubating a GST-fusion protein containing full-length OTUB1 with purified Flag-SLC7A11. As shown in Figure 1G, SLC7A11 strongly bound immobilized GST-OTUB1 but not GST alone. These data demonstrate that OTUB1 is a *bona fide* binding partner of SLC7A11 both *in vitro* and *in vivo*.

OTUB1 acts as a major regulator for SLC7A11 stability in human cancer cells.

To understand the functional consequences of this interaction, we first examined whether expression of the OTUB1 deubiquitinase affects SLC7A11 protein levels in human cells. Indeed, the steady-state levels of SLC7A11 were markedly increased (Figure 2A), and the half-life of SLC7A11 was significantly extended (Figure 2B and Figure S2A), upon OTUB1 co-expression. The steady-state levels of ubiquitinated SLC7A11 were also reduced by OTUB1 expression (Figure 2C, lane 3 vs. lane 4), suggesting that OTUB1 stabilizes SLC7A11 by directly reducing its ubiquitination levels. Conversely, RNAi-mediated depletion of endogenous OTUB1 with a pool or any of four distinct oligonucleotides resulted in stark decreases in SLC7A11 protein levels (Lanes 2–6, Figure 2D). Similar results were obtained with other human cancer cell lines, including neuroblastoma SK-N-BE(2)C (Figure S2B) and osteosarcoma U2OS (lane 2 vs. lane 1, Figure 2E) cells. As shown in Figure 2E, the steady-state levels of SLC7A11 in OTUB1-depleted cells were significantly restored upon treatment with the MG132 proteasome inhibitor (lane 4 vs. lane 2), indicating that OTUB1 depletion promotes SLC7A11 degradation in a proteasome-dependent manner. OTUB1 is a deubiquitinating enzyme that promotes protein stabilization through unconventional mechanisms (23, 27, 28). Several studies have shown that OTUB1 modulates the ubiquitination levels of target proteins independent of its deubiquitinase activity (29–30). For example, the C to A point mutant at the enzymatic domain OTUB1(C91A) fully retains the ability to stabilize the target proteins. Interestingly, OTUB1 can also suppress the ubiquitination of the protein targets by directly interacting and inhibiting E2-conjugating enzymes recruited by E3 ligases. The D to A point mutant OTUB1(D88A) can disrupt the interaction between OTUB1 and E2-conjugating enzymes. Thus, these two functional mutants OTUB1(C91A) and OTUB1(D88A) can separate the two different activities of OTUB1 in protein stabilization. To this end, we tested the effects of two different OTUB1 missense mutants on SLC7A11 stability (Figure 2F). As shown in Figure 2G, the stability of SLC7A11 was effectively rescued by OTUB1(C91A) but not by OTUB1(D88A), suggesting that OTUB1 promotes SLC7A11 stabilization independent of its deubiquitinase activity. Moreover, as shown in Figure S2C, co-immunoprecipitation assays revealed that both functional mutants OTUB1(C91A) and OTUB1(D88A) can interact with SLC7A11 although the binding affinity between OTUB1(D88A) and SLC7A11 was apparently lower than the one between OTUB1(C91A) and SLC7A11. Taken together, although the precise mechanism by which OTUB1 induces SLC7A11 stabilization requires further elucidation, it is very likely that the binding between OTUB1 and SLC7A11 as well as OTUB1's ability of inhibiting E2-conjugating enzymes recruited by the unknown E3 ligase contribute to SLC7A11 stabilization induced by OTUB1.

OTUB1 inactivation promotes ferroptosis in human cancer cells primarily by down-regulating SLC7A11 levels.

Since previous studies from us and others demonstrated that SLC7A11 is critically involved in ferroptosis (8, 20–22,31,32), we examined the role of OTUB1 in modulating ferroptotic responses. To this end, we used the CRISPR/Cas9 method to generate OTUB1-knockout subclones of H1299 cells. Consistent with the RNAi-mediated depletion data, SLC7A11 levels were drastically reduced in all OTUB1-knockout subclones (Figure S3A). Next, we tested whether OTUB1 depletion renders cells susceptible to ROS-induced ferroptosis. In these experiments, ROS levels were induced by treatment with *tert*-Butyl hydroperoxide (TBH), as described previously (8,22). As shown in Figure 3A, high levels of cell death were observed upon ROS induction in all OTUB1-knockout subclones but not in parental H1299 control cells (Figure S3B). As expected, cell death was completely inhibited by the ferroptosis inhibitor Ferrostatin-1 (32) (Figure 3B). Similar results were also obtained when cells were treated with erastin, a small molecule that can induce ferroptosis by inhibiting the cystine/glutamate antiporter (32) (Figure 3C). Since SLC7A11 is a key component of the antiporter, we examined whether cystine starvation-induced ferroptosis is also regulated upon OTUB1 depletion (33). Again, significantly greater levels of ferroptosis were observed upon cystine starvation in OTUB1-knockout cells relative to their parental H1299 control cells (Figure 3D). Since ferroptosis is iron-dependent, we also tested whether the cell death observed in OTUB1-null cells is affected by the iron chelator. As shown in Figure S3C, S3D and S3E, indeed, the ferroptotic cell death in OTUB1 KO cells under different treatment conditions (TBH, cystine starvation and erastin) were all rescued by the iron chelator DFO.

To corroborate these findings, we also used CRISPR technology to generate OTUB1-knockout subclones of human neuroblastoma SK-N-BE(2)C cells, each of which displayed a significant reduction in steady-state SLC7A11 levels (Figure S4A). Again, high levels of cell death were observed in OTUB1-knockout cells, but not the parental control cells, upon treatment with either erastin (Figure 3E, Figure 3F and Figure S4B) or TBH (Figure 3G and Figure S4C). In both cases, the ferroptotic nature of cell death was confirmed by the suppression with Ferrostatin-1 (Figure 3F and Figure 3G). To further validate that OTUB1 depletion promotes ferroptosis through its effect on SLC7A11 stability, we examined whether ROS-induced ferroptosis in OTUB1-knockout cells can be suppressed by ectopic expression of SLC7A11. To this end, we transfected OTUB1-knockout cells with an expression vector encoding SLC7A11, and then tested for ferroptosis upon TBH treatment (Figure 3H and Figure S4D). Indeed, OTUB1-knockout SK-N-BE(2)C cells were rescued from ROS-initiated ferroptosis by ectopic SLC7A11 expression (Figure 3H). Further analysis also validated that the levels of cystine uptake activity were also downregulated in OTUB1-null cells (Figure 3I). Together, these data suggest that OTUB1 inactivation promotes ferroptosis in human cancer cells primarily by down-regulating SLC7A11 levels.

OTUB1 is overexpressed in human cancers and the OTUB1-SLC7A11 interaction is critical for tumor growth.

Examination of the ONCOMINE data base revealed that OTUB1 expression is markedly elevated in human bladder cancers (Figure 4A) (34) and to a lesser degree in head and neck squamous cell carcinomas and ovarian cancers (Figure S5A and Figure S5B) (35–36). To

ascertain whether OTUB1 also regulates ferroptotic responses in human bladder cancer cells, we first tested whether loss of OTUB1 expression affects SLC7A11 levels in this setting. As shown in Figure 4B, SLC7A11 protein levels were significantly reduced upon RNAi-mediated knockdown of endogenous OTUB1 in the human bladder cancer cell lines T24 (lanes 1 and 2), UM-UC-3 (lanes 3 and 4) and SW780 (lanes 5 and 6). To further validate the role of OTUB1 in modulating ferroptosis, we established OTUB1-null T24 and UM-UC-3 cells by CRISPR-mediated knockout of OTUB1 expression. As expected in T24 cells, loss of OTUB1 expression diminished the levels of endogenous SLC7A11 (Figure S6A); ROS-mediated ferroptosis was significantly enhanced in all clones of OTUB1-null cells (Figure S6B); the same results were observed in OTUB1-null UM-UC-3 cells (Figure S7A, Figure S7B and Figure S7C). Moreover, to examine the role of downregulation of SLC7A11 in ferroptosis by OTUB1 depletion, we ectopically expressed SLC7A11 in OTUB1-null cells (Figure 4C). Indeed, high levels of ferroptosis were readily induced in OTUB1-null T24 cells (lanes 1, 2, Figure 4C, Figure 4D), while ectopic overexpression of SLC7A11 rescued OTUB1-knockout T24 cells from ferroptosis (lane 3, 4, Figure 4C; Figure 4D).

Since SLC7A11 is overexpressed in many types of human cancers (8, 15, 16, 26), downregulation of SLC7A11 stability by OTUB1 depletion may act to curb tumor growth *in vivo* by promoting ferroptosis. In support of this hypothesis, we examined whether OTUB1 inactivation in human cancer cells induces tumor growth suppression in mouse xenograft models. As shown in Figure 4E, the growth of T24 xenografts in mice was dramatically repressed by CRISPR-mediated knockout of OTUB1 expression (panel 2 vs. panel 1, and Figure 4F). Moreover, this repression of tumor xenograft growth was largely abrogated by SLC7A11 overexpression (panel 3 vs. panel 2, Figure 4E and Figure 4F), indicating that loss of OTUB1 inhibits tumor growth mainly through stabilization of SLC7A11. In addition, the induction of *Ptgs2*, a marker of ferroptosis (37), observed in OTUB1-knockout tumors was ablated by SLC7A11 overexpression (Figure 4G and Figure 4H), suggesting that ferroptosis indeed plays an important role in tumor growth suppression.

The OTUB1-SLC7A11 interaction is tightly regulated by CD44 in human cancer cells.

To further elucidate the significance of SLC7A11 stability control in tumorigenesis, we searched for potential regulation of the OTUB1-SLC7A11 interaction by other factors in human cancers. A recent study showed that SLC7A11 interacts with a CD44 variant (CD44v) and promotes cellular defense against reactive oxygen species (ROS) in human gastrointestinal cancer cells with a high level of CD44 variant expression (37). CD44 is a single-pass type I transmembrane protein that functions as a cellular adhesion molecule and a receptor for hyaluronic acid. Although normally expressed in hematopoietic cells, numerous studies indicate that the standard CD44 isoform and its splicing variants CD44v are both overexpressed in many types of cancers (38,39). CD44 also serves as a key cell surface marker of cancer stem cells and has been shown to be critically involved in tumor development (40, 41). Notably, Western Blot analysis of all human cancer cell lines used in this study detected high levels of the standard CD44 isoform (also called CD44s) while the levels of CD44v were barely detectable (Figure S8A). Since only a CD44v isoform was reported to interact with SLC7A11 in human gastrointestinal cancer cells (42), it was

unclear whether SLC7A11 is also regulated by the standard CD44 isoform. To this end, we first examined whether SLC7A11 interacts with the standard CD44 isoform by co-immunoprecipitation assays. Indeed, Western Blot analysis revealed that the standard CD44 isoform was readily detected in the immunoprecipitated complexes of Flag-SLC7A11 (Figure S8B). More importantly, siRNA-mediated knockdown of endogenous CD44 decreases the steady-state levels of endogenous SLC7A11 in H1299 cells (Figure 5A) and sensitizes these cells to ferroptosis (Figure 5B). Together, these data demonstrate that the standard CD44 isoform can regulate the protein stability of SLC7A11 and the sensitivity of tumor cells to ferroptosis.

Next, we examined whether OTUB1 can influence CD44 regulation of SLC7A11 stability and ferroptosis. As shown in Figures 5C and 5D, CD44 expression significantly increased the levels of SLC7A11 (lanes 1–3, Figure 5D) in T24 cells but CD44-mediated stabilization of SLC7A11 was largely abrogated by OTUB1 knockout-T24 cells (lanes 4–6, Figure 5D). And CD44-mediated stabilization of SLC7A11 was abrogated by OTUB1 knockout in H1299 cells as well (Figure 5E). To elucidate the molecular mechanism that underlies this effect, we examined whether CD44 modulates the SLC7A11-OTUB1 interaction by co-immunoprecipitation analysis. As shown in Figure 5F, the levels of SLC7A11 were upregulated by CD44 expression (Input: lanes 1 and 2 vs. lanes 3 and 4, Figure 5F). To avoid the issue that increased SLC7A11 protein levels are the only factor for the increase of the OTUB1-SLC7A11 interaction, we normalized the amounts of SLC7A11 after immunoprecipitation (IP: lanes 1 and 2 vs. lanes 3 and 4, Figure 5F). Then, we examined the proteins levels of OTUB1 co-immunoprecipitated with the same amount of SLC7A11 proteins. Indeed, the levels of OTUB1 co-immunoprecipitated with the same amount of SLC7A11 proteins were increased upon CD44 expression. Moreover, as shown in Figure 5G, the levels of SLC7A11 were downregulated by CD44 knockdown (Input: lane 1 vs. lane 2 Figure 5G). By the same method, to avoid the issue that decreased SLC7A11 protein levels are the only factor for the decrease of the OTUB1-SLC7A11 interaction in CD44 knockdown cells (Input: lane 1 vs. lane 2, Figure 5G), we normalized the amounts of SLC7A11 after immunoprecipitation (IP: lane 3 vs. lane 4, Figure 5G). Then, we examined the proteins levels of OTUB1 co-immunoprecipitated with the same amount of SLC7A11 proteins. Indeed, the levels of OTUB1 co-immunoprecipitated with the same amount of SLC7A11 proteins were reduced upon CD44 knockdown. Taken together, these data demonstrate that CD44 can enhance the OTUB1-SLC7A11 interaction even with the same amount of SLC7A11 protein.

Our data showed that CD44 can interact with SLC7A11 while SLC7A11 also interacts with OTUB1. As expected, inactivation of OTUB1 leads to destabilization of SLC7A11 but the levels of endogenous CD44 were not affected in OTUB1-null cells (Figure S8C), suggesting that OTUB1 has no effect on CD44 stability. Interestingly, by co-immunoprecipitation assays, we found that CD44 also interacts with OTUB1 although the binding affinity between OTUB1 and CD44 is not very high (Figure 6A). Moreover, we found that the N-terminal domain of SLC7A11 is required for interacting with OTUB1 (Figure 6B and Figure 6C). In contrast, the C-terminal domain of SLC7A11 is critical for binding CD44 (Figure 6D). Thus, OTUB1 and CD44 interacts with the different domains of SLC7A11. Since CD44 can stabilize SLC7A11 by enhancing the binding between SLC7A11 and OTUB1

(Figure 5F), it is very likely that CD44, OTUB1 and SLC7A11 form a stable three-protein complex that leads to more effective stabilization of SLC7A11.

Finally, to further elucidate the significance of these interactions, we tested whether CD44 can promote tumor cell growth by inhibiting ferroptosis and whether OTUB1 is critical for CD44-mediated efforts. To this end, we examined the role of OTUB1 in modulating CD44-mediated ferroptotic response in native T24 and OTUB1-null T24 cells. As shown in Figure 6E, levels of erastin-induced ferroptosis in the parental T24 control cells were dramatically reduced from 52% to 23 %, suggesting that expression of CD44 inhibits ferroptosis by stabilizing SLC7A11. In contrast, CD44 had no dramatic effect on ferroptosis in OTUB1-null T24 cells (Figure 6F). These data demonstrate that the SLC7A11-OTUB1 interaction is modulated by CD44 in human cancer cells and that OTUB1 is required for CD44-mediated suppression of ferroptosis in human cancer cells. Together, these data reveal a new pathway critically involved in ferroptotic responses in human cancer cells.

Discussion

The emerging role of ferroptosis linked cell metabolism and tumor suppression has been a topic of great interest (1–3). Many studies indicate that SLC7A11 acts as a key factor in modulating ferroptotic responses in human cancers. Through biochemical purification, we have identified OTUB1 as a bona fide regulator of SLC7A11. Interestingly, we also found that OTUB1 plays a key role in CD44-mediated effects on SLC7A11 stability and ferroptotic responses in human cancer cells. In particular, our results show that (i) OTUB1 is a *bona fide* binding partner of SLC7A11 both *in vitro* and *in vivo*; (ii) OTUB1 acts as a major regulator for SLC7A11 activity in human cancer cells; (iii) OTUB1 inactivation promotes ferroptosis in human cancer cells primarily by down-regulating SLC7A11 levels; (iv) OTUB1 is overexpressed in human cancers and the OTUB1-SLC7A11 interaction is critical for tumor growth; (v) The OTUB1-SLC7A11 interaction is tightly regulated by CD44 in human cancer cells. Thus, these results have significant implications regarding how SLC7A11 function is regulated in human cancers (Figure 7).

Accumulating evidence indicates that SLC7A11 acts as a potential biomarker for human cancers critically involved in tumorigenesis. By promoting cystine uptake for the synthesis of reduced glutathione (GSH), high SLC7A11 expression can protect cancer cells from oxidative stress and ferroptosis. Thus, the precise mechanism by which SLC7A11 is regulated in human cancers requires further elucidation. Our study implicates OTUB1 as a key regulator of SLC7A11 protein stability that is overexpressed in several types of human cancers. Importantly, inhibition of OTUB1 leads to destabilization of SLC7A11, enhanced sensitivity to ferroptosis, and suppression of *in vivo* tumor growth. Interestingly, by promoting the interaction between SLC7A11 and OTUB1, the CD44 cellular adhesion molecule can also enhance SLC7A11 stability and inhibit ferroptosis. Thus, our study identifies a novel regulatory pathway that modulates the sensitivity of tumor cells to ferroptotic death by governing the protein stability of SLC7A11. Notably, a recent study showed that the function of SLC7A11 is also regulated by mTORC2-mediated phosphorylation. It will be interesting to know whether the OTUB1-SLC7A11 interaction is regulated by this modification (43).

Since high levels of cell proliferation are generally accompanied by increased ROS production, cancer cells employ various strategies to protect themselves from oxidative stress (39). CD44 is a multi-functional protein that appears to promote tumorigenesis through a variety of mechanisms (38–41, 44). In this study, we demonstrate that, by promoting the interaction between SLC7A11 and OTUB1, CD44 serves as a positive regulator of SLC7A11 activity by facilitating the recruitment of OTUB1 and thereby reducing the sensitivity of cancer cells to oxidative stress and ferroptotic death (Figure 5 and Figure 6E and 6F). Like CD44, OTUB1 is also overexpressed in human cancers and acts to suppress ferroptosis by promoting SLC7A11 stability. Although OTUB1 belongs to the OTU family of deubiquitinases, recent studies indicate that OTUB1 stabilizes its protein substrates by blocking the activity of E2 conjugating enzymes required for their polyubiquitination rather than, as anticipated, through its enzymatic activity as a deubiquitinase (29–31). For example, during DNA damage responses OTUB1 can suppress RNF168-dependent polyubiquitination of histones by binding and inhibiting the E2 ubiquitin conjugating enzyme Ubc13/UBE2N (27). OTUB1 is also reported to stabilize several oncoproteins, including cIAP, FOXM1 and Mdmx (24, 45–47). Nonetheless, OTUB1-mediated regulation of SLC7A11 is likely to play a major role in tumorigenesis since the *in vivo* tumor growth suppression induced by OTUB1 depletion is readily reversed by SLC7A11 overexpression. Thus, modulating the OTUB1/SLC7A11 pathway, either by destabilizing SLC7A11 levels or inhibiting OTUB1 function, should prove to be a viable strategy for cancer therapy. Finally, OTUB1 has also been implicated in the DNA damage response. It will be interesting to know whether SLC7A11 stability as well as cysteine metabolism is also regulated by DNA damage.

Solute carrier (SLC) transporters are a huge diverse group that classified into 52 families and over 400 members in total (48). Ubiquitination is one of the most popular post-translational modifications which promotes protein degraded via the proteasome, and this process can be reversed by de-ubiquitinases (DUBs). For example, OAT1 (SLC22A6) was ubiquitinated by NEDD4 (49); Glutamate transporter 1 (GLT1) also called SLC1A2 was ubiquitinated by NEDD4–2 (50). It will be interesting to test whether these E3 ligases are also involved in ubiquitination and degradation of SLC7A11. In summary, our study demonstrates that OTUB1 plays an essential role in controlling the stability of SLC7A11 and is also critically involved in CD44-mediated effects on ferroptosis in human cancer cells. OTUB1 is overexpressed in human cancers; OTUB1 inactivation induces ferroptosis by destabilizing SLC7A11, resulting in tumor growth suppression in xenograft mouse models. Thus, by elucidating a new layer of ferroptosis regulation through SLC7A11 stability control in human cancers, our study suggests OTUB1 as a potential target in cancer therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Mushui Dai for OTUB1 constructs. We also thank Dr. Richard Bear for critical discussion and Dr. Michael Shen and Dr. Corey Abate-Shen for providing human bladder cancer cell lines for this study. This work was supported by the National Cancer Institute of the National Institutes of Health under Award 5R01CA190477,

5RO1CA085533, 5RO1CA216884, and 5RO1CA224272 to W.G. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

1. Xie Y, Hou W, Song X, Yu Y, Huang J, Sun X, et al. Ferroptosis: process and function. *Cell death and differentiation* 2016;23:369–79 [PubMed: 26794443]
2. Gao M, Jiang X. To eat or not to eat-the metabolic flavor of ferroptosis. *Current opinion in cell biology* 2017;51:58–64 [PubMed: 29175614]
3. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell* 2017;171:273–85 [PubMed: 28985560]
4. Zhang Y, Shi J, Liu X, Feng L, Gong Z, Koppula P, et al. BAP1 links metabolic regulation of ferroptosis to tumour suppression. *Nature cell biology* 2018;20:1181–92 [PubMed: 30202049]
5. Conrad M, Sato H. The oxidative stress-inducible cystine/glutamate antiporter, system x(c) (–): cystine supplier and beyond. *Amino acids* 2012;42:231–46 [PubMed: 21409388]
6. Yang WS, Stockwell BR. Ferroptosis: Death by Lipid Peroxidation. *Trends in cell biology* 2016;26:165–76 [PubMed: 26653790]
7. Angeli JPF, Shah R, Pratt DA, Conrad M. Ferroptosis Inhibition: Mechanisms and Opportunities. *Trends in pharmacological sciences* 2017;38:489–98 [PubMed: 28363764]
8. Jiang L, Kon N, Li T, Wang SJ, Su T, Hibshoosh H, et al. Ferroptosis as a p53-mediated activity during tumour suppression. *Nature* 2015;520:57–62 [PubMed: 25799988]
9. Li T, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, et al. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. *Cell* 2012;149:1269–83 [PubMed: 22682249]
10. Jennis M, Kung CP, Basu S, Budina-Kolomets A, Leu JI, Khaku S, et al. An African-specific polymorphism in the TP53 gene impairs p53 tumor suppressor function in a mouse model. *Genes & development* 2016;30:918–30 [PubMed: 27034505]
11. Saint-Germain E, Mignacca L, Vernier M, Bobbala D, Ilangumaran S, Ferbeyre G. SOCS1 regulates senescence and ferroptosis by modulating the expression of p53 target genes. *Aging* 2017;9:2137–62 [PubMed: 29081404]
12. Wang SJ, Li D, Ou Y, Jiang L, Chen Y, Zhao Y, et al. Acetylation Is Crucial for p53-Mediated Ferroptosis and Tumor Suppression. *Cell reports* 2016;17:366–73 [PubMed: 27705786]
13. Gao Q, Zhu X, Chen J, Mao C, Zhang L, Xu Z. Upregulation of P53 promoted G1 arrest and apoptosis in human umbilical cord vein endothelial cells from preeclampsia. *Journal of hypertension* 2016;34:1380–8 [PubMed: 27115339]
14. Lo M, Wang YZ, Gout PW. The x(c)-cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases. *Journal of cellular physiology* 2008;215:593–602 [PubMed: 18181196]
15. Huang Y, Dai Z, Barbacioru C, Sadee W. Cystine-glutamate transporter SLC7A11 in cancer chemosensitivity and chemoresistance. *Cancer research* 2005;65:7446–54 [PubMed: 16103098]
16. Liu XX, Li XJ, Zhang B, Liang YJ, Zhou CX, Cao DX, et al. MicroRNA-26b is underexpressed in human breast cancer and induces cell apoptosis by targeting SLC7A11. *FEBS letters* 2011;585:1363–7 [PubMed: 21510944]
17. Koppula P, Zhang Y, Shi J, Li W, Gan B. The glutamate/cystine antiporter SLC7A11/xCT enhances cancer cell dependency on glucose by exporting glutamate. *The Journal of biological chemistry* 2017;292:14240–9 [PubMed: 28630042]
18. Suzuki T, Motohashi H, Yamamoto M. Toward clinical application of the Keap1-Nrf2 pathway. *Trends in pharmacological sciences* 2013;34:340–6 [PubMed: 23664668]
19. Ye P, Mimura J, Okada T, Sato H, Liu T, Maruyama A, et al. Nrf2- and ATF4-dependent upregulation of xCT modulates the sensitivity of T24 bladder carcinoma cells to proteasome inhibition. *Molecular and cellular biology* 2014;34:3421–34 [PubMed: 25002527]
20. Chen D, Fan Z, Rauh M, Buchfelder M, Eyupoglu IY, Savaskan N. ATF4 promotes angiogenesis and neuronal cell death and confers ferroptosis in a xCT-dependent manner. *Oncogene* 2017;36:5593–608 [PubMed: 28553953]

21. Fan Z, Wirth AK, Chen D, Wruck CJ, Rauh M, Buchfelder M, et al. Nrf2-Keap1 pathway promotes cell proliferation and diminishes ferroptosis. *Oncogenesis* 2017;6:e371 [PubMed: 28805788]
22. Chen D, Tavana O, Chu B, Erber L, Chen Y, Baer R, et al. NRF2 Is a Major Target of ARF in p53-Independent Tumor Suppression. *Molecular cell* 2017;68:224–32e4 [PubMed: 28985506]
23. Sun XX, Dai MS. Deubiquitinating enzyme regulation of the p53 pathway: A lesson from Otub1. *World journal of biological chemistry* 2014;5:75–84 [PubMed: 24920999]
24. Chen Y, Wang YG, Li Y, Sun XX, Dai MS. Otub1 stabilizes MDMX and promotes its proapoptotic function at the mitochondria. *Oncotarget* 2017;8:11053–62 [PubMed: 28035068]
25. Dai C, Tang Y, Jung SY, Qin J, Aaronson SA, Gu W. Differential effects on p53-mediated cell cycle arrest vs. apoptosis by p90. *Proceedings of the National Academy of Sciences of the United States of America* 2011;108:18937–42 [PubMed: 22084066]
26. Guo W, Zhao Y, Zhang Z, Tan N, Zhao F, Ge C, et al. Disruption of xCT inhibits cell growth via the ROS/autophagy pathway in hepatocellular carcinoma. *Cancer letters* 2011;312:55–61 [PubMed: 21906871]
27. Nakada S, Tai I, Panier S, Al-Hakim A, Iemura S, Juang YC, et al. Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. *Nature* 2010;466:941–6 [PubMed: 20725033]
28. Wiener R, Zhang X, Wang T, Wolberger C. The mechanism of OTUB1-mediated inhibition of ubiquitination. *Nature* 2012;483:618–22 [PubMed: 22367539]
29. Juang YC, Landry MC, Sanches M, Vittal V, Leung CC, Ceccarelli DF, et al. OTUB1 co-opts Lys48-linked ubiquitin recognition to suppress E2 enzyme function. *Molecular cell* 2012;45:384–97 [PubMed: 22325355]
30. Sun XX, Challagundla KB, Dai MS. Positive regulation of p53 stability and activity by the deubiquitinating enzyme Otubain 1. *The EMBO journal* 2012;31:576–92 [PubMed: 22124327]
31. Wiener R, DiBello AT, Lombardi PM, Guzzo CM, Zhang X, Matunis MJ, et al. E2 ubiquitin-conjugating enzymes regulate the deubiquitinating activity of OTUB1. *Nature structural & molecular biology* 2013;20:1033–9
32. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 2012;149:1060–72 [PubMed: 22632970]
33. Dixon SJ, Patel DN, Welsch M, Skouta R, Lee ED, Hayano M, et al. Pharmacological inhibition of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis. *eLife* 2014;3:e02523 [PubMed: 24844246]
34. Blaveri E, Simko JP, Korkola JE, Brewer JL, Baehner F, Mehta K, et al. Bladder cancer outcome and subtype classification by gene expression. *Clinical cancer research: an official journal of the American Association for Cancer Research* 2005;11:4044–55
35. Estilo CL, Oc P, Talbot S, Socci ND, Carlson DL, Ghossein R, et al. Oral tongue cancer gene expression profiling: Identification of novel potential prognosticators by oligonucleotide microarray analysis. *BMC cancer* 2009;9:11 [PubMed: 19138406]
36. Bonome T, Levine DA, Shih J, Randonovich M, Pise-Masison CA, Bogomolny F, et al. A gene signature predicting for survival in suboptimally debulked patients with ovarian cancer. *Cancer research* 2008;68:5478–86 [PubMed: 18593951]
37. Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, et al. Regulation of ferroptotic cancer cell death by GPX4. *Cell* 2014;156:317–31 [PubMed: 24439385]
38. Senbanjo LT, Chellaiah MA. CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells. *Frontiers in cell and developmental biology* 2017;5:18 [PubMed: 28326306]
39. Orian-Rousseau V CD44 Acts as a Signaling Platform Controlling Tumor Progression and Metastasis. *Frontiers in immunology* 2015;6:154 [PubMed: 25904917]
40. Nagano O, Okazaki S, Saya H. Redox regulation in stem-like cancer cells by CD44 variant isoforms. *Oncogene* 2013;32:5191–8 [PubMed: 23334333]
41. Thapa R, Wilson GD. The Importance of CD44 as a Stem Cell Biomarker and Therapeutic Target in Cancer. *Stem cells international* 2016;2016:2087204 [PubMed: 27200096]

42. Ishimoto T, Nagano O, Yae T, Tamada M, Motohara T, Oshima H, et al. CD44 variant regulates redox status in cancer cells by stabilizing the xCT subunit of system xc(−) and thereby promotes tumor growth. *Cancer cell* 2011;19:387–400 [PubMed: 21397861]
43. Gu Y, Albuquerque CP, Braas D, Zhang W, Villa GR, Bi J, et al. mTORC2 Regulates Amino Acid Metabolism in Cancer by Phosphorylation of the Cystine-Glutamate Antiporter xCT. *Molecular cell* 2017;67:128–38e7 [PubMed: 28648777]
44. Godar S, Ince TA, Bell GW, Feldser D, Donaher JL, Bergh J, et al. Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 2008;134:62–73 [PubMed: 18614011]
45. Goncharov T, Niessen K, de Almagro MC, Izrael-Tomasevic A, Fedorova AV, Varfolomeev E, et al. OTUB1 modulates c-IAP1 stability to regulate signalling pathways. *The EMBO journal* 2013;32:1103–14 [PubMed: 23524849]
46. Karunarathna U, Kongsema M, Zona S, Gong C, Cabrera E, Gomes AR, et al. OTUB1 inhibits the ubiquitination and degradation of FOXM1 in breast cancer and epirubicin resistance. *Oncogene* 2016;35:1433–44 [PubMed: 26148240]
47. Zhou Y, Wu J, Fu X, Du W, Zhou L, Meng X, et al. OTUB1 promotes metastasis and serves as a marker of poor prognosis in colorectal cancer. *Molecular cancer* 2014;13:258 [PubMed: 25431208]
48. Lin L, Yee SW, Kim RB, Giacomini KM. SLC transporters as therapeutic targets: emerging opportunities. *Nature reviews Drug discovery* 2015;14:543–60 [PubMed: 26111766]
49. Xu D, Wang H, You G. An Essential Role of Nedd4–2 in the Ubiquitination, Expression, and Function of Organic Anion Transporter-3. *Molecular pharmaceuticals* 2016;13:621–30 [PubMed: 26651153]
50. Zhang Y, He X, Meng X, Wu X, Tong H, Zhang X, et al. Regulation of glutamate transporter trafficking by Nedd4–2 in a Parkinson's disease model. *Cell death & disease* 2017;8:e2574 [PubMed: 28151476]

Significance

This study identifies OTUB1 as a key regulator of ferroptosis and implicates it as a potential target in cancer therapy.

Figure 1. OTUB1 is a *bona fide* binding partner of SLC7A11 both *in vitro* and *in vivo*.
A. Schematic representation of the SLC7A11 protein used for protein complex purification.
B. Coomassie blue staining of affinity-purified protein complexes from Flag-HA-SLC7A11 H1299 stable cell line (lane 3) and the parental H1299 cell line (lane 2) in the upper panel. Specific SLC7A11-interacting protein bands were analyzed by LC-MS/MS and identified OTUB1 peptide sequences in the lower panel.
C. Western Blot analysis for OTUB1 after immunoprecipitation (IP) of Flag-SLC7A11, with anti-Flag (M2) antibody-coupled beads, from H1299 cells transfected with Flag-SLC7A11 and OTUB1 individually or together. 1% of the sample was loaded as input.
D. Western Blot analysis for SLC7A11 after immunoprecipitation of Flag-OTUB1, with anti-Flag (M2) antibody-coupled beads, from H1299 cells transfected with Flag-SLC7A11 and OTUB1 individually or together. 2.5% of the sample was loaded as input.

E. Western Blot analysis for endogenous OTUB1 after immunoprecipitation of endogenous SLC7A11 from parental SK-N-BE(2)C cells.

F. Western Blot analysis for endogenous SLC7A11 after immunoprecipitation of endogenous OTUB1 from parental SK-N-BE(2)C cells.

G. Western Blot analysis for pulldown of purified Flag-SLC7A11 incubated with purified GST or GST-OTUB1 fusion protein. 1% of the cell lysate was loaded as input. Ponceau staining of GST and GST-OTUB1 is shown in the bottom panel.

Figure 2. OTUB1 acts as a major regulator for SLC7A11 stability in human cancer cells.
A. Western Blot analysis for SLC7A11(Long Exposure, LE and Short Exposure, SE) and Flag-OTUB1, from H1299 cells transfected with an SLC7A11-expressing plasmid and either an empty vector or increasing amounts of a Flag-OTUB1-expressing vector.
B. Densitometry quantification of SLC7A11 protein levels calculated using ImageJ software (National Institutes of Health [NIH], Bethesda, MD) and plotted for half-life determination corresponding to Supplemental Fig. 2A. Error bars represent the mean $\pm$ SEM of 3 independent experiments.
C. Western Blot analysis for HA-ubiquitin(Ub) after incubation of anti-Flag-coupled (M2) beads with lysates from HEK293 cells transfected with empty vector (–) or those expressing Flag-SLC7A11 either alone or together with OTUB1.

D. Western Blot analysis for SLC7A11 and OTUB1 from H1299 cells transfected with control siRNA (ctrl) or a pool of OTUB1-specific siRNAs or the individual OTUB1 oligos composing the pool for 72 hours.

E. Western Blot analysis for SLC7A11 and OTUB1 from U2OS cells transfected with control siRNA (ctrl) or OTUB1 siRNA and treated with DMSO/MG132 10 μ g/ml for 10 hours.

F. A schematic structure of wild-type OTUB1 (top panel), E2-conjugating enzyme binding defect mutant, OTUB1 D88A (middle panel) and catalytic activity defect mutant, OTUB1 C91A (bottom panel).

G. Western Blot analysis for SLC7A11 and Flag-OTUB1 WT, D88A and C91A, from H1299 cells transfected with an SLC7A11-expressing plasmid and either an empty vector or indicated Flag-OTUB1-expressing vectors (WT, D88A, C91A).

Figure 3. OTUB1 inactivation promotes ferroptosis in human cancer cells primarily by down-regulating SLC7A11 levels.

A. Representative phase-contrast images of H1299 parental cells and OTUB1 CRISPR clones treated with TBH (40 μM) and Ferr-1 (2 μM) as indicated for 6 hours.

B.C.D. H1299 control cells and OTUB1-null cells were treated with TBH (40 μM) for 6 hours, erastin (25 μM) for 16 hours and cystine starvation for 48 hours or together with Ferr-1 (2 μM) as indicated respectively. Quantification of cell death from three replicates is shown; error bars represent the mean ± s.d.

E. Representative phase-contrast images of SK-N-BE(2)C parental cells and OTUB1-null cells treated with erastin (35 μM) and Ferr-1 (2 μM) as indicated for 20 hours.

F.G. SK-N-BE(2)C parental cells and OTUB1-null cells were treated with erastin (35 μM) for 20 hours and TBH (40 μM) for 7 hours. Quantification of cell death from three replicates is shown; error bars represent the mean ± s.d.

H. SK-N-BE(2)C parental cells and OTUB1-null cells were transfected with empty vector or a SLC7A11 expressing plasmid and treated with TBH (250 μ M) and Ferr-1 (2 μ M) as indicated for 7 hours. Quantification of cell death from three replicates is shown; error bars represent the mean $\pm$ s.d.

I. Cystine uptake levels (D.P.M., disintegrations per minutes) were measured in U2OS control cells or U2OS OTUB1-null treated with erastin (30 μ M) for 6 hours as indicated.

Figure 4. OTUB1 is overexpressed in human cancers and the OTUB1-SLC7A11 interaction is critical for tumor growth.

A. Box plots derived from gene expression data in ONCOMINE (<https://www.oncomine.org/resource/login.html>), comparing the expression of OTUB1 mRNA in 3 normal human samples (left plots) and 39 cancer tissues (right plots) from bladder cancer samples.

B. Western blot analysis for OTUB1 and SLC7A11 from T24, UM-UC-3 and SW780 cells transfected with control siRNA (ctrl) or a pool of OTUB1-specific siRNAs for 72 hours.

C. Western Blot analysis for OTUB1 and SLC7A11 in T24 control cells and OTUB1-null cells that were transfected with either an empty vector or SLC7A11.

D. T24 control cells and OTUB1-null cells were transfected with SLC7A11 as in Fig 4C and then treated with TBH (10 μ M) and Ferr-1 (2 μ M) as indicated for 16 hours. Quantification of cell death from three technical replicates is shown; error bars represent the mean $\pm$ s.d.

E. Image of xenograft tumors that were inoculated into nude mice with T24 control cells, OTUB1-null cells and OTUB1-null cells stably transfected with SLC7A11 expression vector for 9 weeks.

F. Weight (mg) of tumors as shown in Fig 4E was determined and compared, error bars represent the mean $\pm$ s.d. from 5 tumors per group.

G. PTGS2 mRNA level from tumors was determined by qPCR; three replicates are shown; error bars represent the mean $\pm$ s.d.

H. Western Blot analysis for OTUB1 and SLC7A11 from xenograft tumors obtained from the groups of T24 control cells, OTUB1-null cells and OTUB1-null cells stably transfected with SLC7A11 expression vector.

Figure 5. The OTUB1-SLC7A11 interaction is tightly regulated by CD44 in human cancer cells.

A. Western Blot analysis for CD44 and SLC7A11 from H1299 cells transfected with control siRNA or a pool of CD44-specific siRNAs.

B. H1299 cells transfected with control siRNA or a pool of CD44 siRNAs and then treated with TBH (40 μ M) and Ferr-1 (2 μ M) as indicated for 6 hours. Quantification of cell death from three technical replicates is shown; error bars represent the mean $\pm$ s.d.

C. Western Blot analysis for SLC7A11, OTUB1 and CD44 from H1299 cells transfected with HA-SLC7A11 and either an empty vector or increasing amounts of a Flag-CD44 expressing vector.

D. Western Blot analysis for SLC7A11, OTUB1 and CD44 from T24 control cells and OTUB1-null cells transfected with HA-SLC7A11 and either an empty vector or increasing amounts of a CD44-expressing vector as indicated.

E. Western Blot analysis for HA-SLC7A11, OTUB1 and CD44 from H1299 control cells and OTUB1 CRISPR cells transfected with HA-SLC7A11 and either an empty vector or a CD44-expressing vector.

F. Western Blot analysis for OTUB1 after immunoprecipitation of SLC7A11, with anti-HA antibody-coupled beads, from HA-SLC7A11 stable H1299 cells transfected with CD44 and Flag-OTUB1 as indicated. The amount of SLC7A11 was normalized after immunoprecipitation. 1% of the sample was loaded as input.

G. Western Blot analysis for OTUB1 after immunoprecipitation of Flag-SLC7A11, with anti-Flag (M2) antibody-coupled beads, from H1299 cells transfected with control siRNA or a pool of CD44-specific siRNAs, Flag-SLC7A11 and OTUB1 individually or together as indicated. The amount of SLC7A11 was normalized after immunoprecipitation. 1% of the sample was loaded as input.

Figure 6. CD44 and OTUB1 interact with different domains of SLC7A11.

A. Western Blot analysis for CD44 after immunoprecipitation of SFB-OTUB1, with Streptavidin-coupled beads, from HEK293 cells transfected with CD44 and SFB-OTUB1 individually or together as indicated. 1% of the sample was loaded as input.

B. A schematic structure of wild-type Flag-HA tagged SLC7A11 Full length (FL, top panel), 1–43 amino acids N terminal deletion (NT), 471–501 amino acids C terminal deletion (CT) (bottom panel). 1% of the sample was loaded as input.

C. Western Blot analysis for HA-SLC7A11/ NT/ CT after immunoprecipitation (IP) of SFB-OTUB1 with Streptavidin-coupled beads, from HEK293 cells transfected with HA-SLC7A11 FL or NT/ CT and SFB-OTUB1 individually or together. 1% of the sample was loaded as input.

- D. Western Blot analysis for CD44 after immunoprecipitation (IP) of HA-SLC7A11, with anti-HA antibody-coupled beads, from HEK293 cells transfected with HA-SLC7A11 FL or NT/ CT and CD44 individually or together. 1% of the sample was loaded as input.
- E. T24 cells transfected with or without CD44 and then treated with erastin (15 μ M) as indicated for 70 hours. Quantification of cell death from three replicates is shown; error bars represent the mean $\pm$ s.d.
- F. T24 OTUB1 CRISPR cells transfected with or without CD44 and then treated with erastin (15 μ M) as indicated for 70 hours. Quantification of cell death from three replicates is shown; error bars represent the mean $\pm$ s.d.

Figure 7. Model of Deubiquitination of SLC7A11 by OTUB1 inhibits ferroptosis and promotes tumorigenesis.

Schematic model where OTUB1 stabilizes SLC7A11 through deubiquitination of SLC7A11, which is enhanced by CD44. OTUB1 inhibits ferroptosis and promotes tumorigenesis.