USP15 antagonizes CRL4^{CRBN}-mediated ubiquitylation of glutamine synthetase and neosubstrates

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Targeted protein degradation by the ubiquitin–proteasome system represents a new strategy to destroy pathogenic proteins in human diseases, including cancer and neurodegenerative diseases. The immunomodulatory drugs (IMiDs) thalidomide, lenalidomide, and pomalidomide have revolutionized the treatment of patients with multiple myeloma (MM) and other hematologic malignancies, but almost all patients eventually develop resistance to IMiDs. CRBN, a substrate receptor of CUL4-RBX1-DDB1-CRBN (CRL4^{CRBN}) E3 ubiquitin ligase, is a direct target for thalidomide teratogenicity and antitumor activity of IMiDs (now known as Cereblon E3 ligase modulators: CELMoDs). Despite recent advances in developing potent CELMoDs and CRBN-based proteolysis-targeting chimeras (PROTACs), many questions apart from clinical efficacy remain unanswered. CRBN is required for the action of IMiDs, but its protein expression levels do not correlate with intrinsic resistance to IMiDs in MM cells, suggesting other factors involved in regulating resistance to IMiDs. Our recent work revealed that the CRL4^{CRBN}-p97 pathway is required for degradation of natural substrate glutamine synthetase (GS) and neosubstrates. Here, I show that USP15 is a key regulator of the CRL4^{CRBN}-p97 pathway to control stability of GS and neosubstrates IKZF1, IKZF3, CK1-α, RNF166, GSPT1, and BRD4, all of which are crucial drug targets in different types of cancer. USP15 antagonizes ubiquitylation of CRL4^{CRBN} target proteins, thereby preventing their degradation. Notably, USP15 is highly expressed in IMiD-resistant cells, and depletion of USP15 sensitizes these cells to lenalidomide. Inhibition of USP15 represents a valuable therapeutic opportunity to potentiate CELMoD and CRBN-based PROTAC therapies for the treatment of cancer.

ubiquitin | CRBN | IMiDs | PROTACs | USP15

Ubiquitylation of protein substrates is catalyzed by the ubiquitin-specific E1, E2, and E3 ligases and can be reversed by deubiquitinating enzymes (DUBs) (1, 2). The key components of the ubiquitin–proteasome system (UPS), including over 600 E3 ligases, ∼100 DUBs, and the proteasome have recently emerged as therapeutic targets for treatment of cancer and other diseases (3–5). However, there are fundamental gaps in our knowledge and understanding of how E3s and DUBs control degradation of individual protein substrates. CRBN, a substrate receptor of the CUL4-RBX1-DDB1-CRBN (CRL4^{CRBN}) E3 ubiquitin ligase, is a direct target for thalidomide teratogenicity (6). Subsequently, CRBN was shown as the target that accounts for the antitumor activity of immunomodulatory drugs (IMiDs) in multiple myeloma (MM) (7, 8). These drugs directly bind CRBN and promote the recruitment of neosubstrates, including the transcription factors Ikaros (IKZF1) and Aiolos (IKZF3) (9–11) and casein kinase 1-α (CK1-α) (12), leading to ubiquitylation and subsequent degradation of neosubstrates. This accounts for antitumor activity of IMiDs (9, 10, 12). The crystal structure of CRBN bound to DDB1 and IMiDs revealed that the glutarimide rings of IMiDs bind a hydrophobic pocket in the thalidomide-binding domain of CRBN, whereas the phthalimide portion is largely exposed to solvent, critical for the recruitment of neosubstrates to the complex (13, 14). Multiple neosubstrates, such as ZNF692, SALL4, and RNF166, have been reported to be targeted for IMiD-induced ubiquitylation and subsequent degradation by the proteasome (15). More recently, novel thalidomide analogs, now known as the Cereblon E3 ligase modulators (CELMoDs), including CC-122, CC-220, and CC-885 (16), have been developed to target pathogenic proteins for degradation via a molecular glue mechanism (17). For example, CC-885 induces degradation of the translation termination factor GSPT1, a new CRL4^{CRBN} neosubstrate (18). In addition, CRBN is also a target for the development of proteolysis-targeting chimera (PROTAC) technology, which relies on linking a drug that binds to a protein of interest to IMiDs (19–21). Many CRBN-based PROTACs, including the potent BRD4 protein degrader dBET1 (22), have been developed for the degradation of target proteins in cancer and other human diseases (21, 23).

DUBs are divided into five families based on their specific structural domains: ubiquitin C-terminal hydrolases, ubiquitin-specific proteases (USPs), ovarian tumor proteases, Josephins, and JAB1/MPN/MOV34 metalloenzymes (24). Genetic alterations and overexpression of USP15 have been reported in many human cancers, in particular glioblastoma (GBM), breast and ovarian cancer (25), leukemia and lymphomas (26), and MM (27). USP15 amplification confers poor prognosis in patients with GBM (25). High expression of USP15 was significantly correlated with poor survival rate within the pancancer patient cohort (26), representing a key feature of oncogene activity. Since *Usp15*$^{-/-}$ mice were viable (26, 28–31), targeting USP15 in cancer could achieve major advantages for an optimal therapeutic window. USP15 has been shown to deubiquitylate and stabilize protein substrates in different signaling pathways such as TGF-β (25, 32, 33), MDM2 (28), and NF-κB (34). However,

Significance

CRBN, a substrate receptor of CUL4-RBX1-DDB1-CRBN (CRL4^{CRBN}) E3 ubiquitin ligase, is a direct target for thalidomide teratogenicity and antitumor activity of immunomodulatory drugs (IMiDs) in multiple myeloma (MM) and other hematologic malignancies. IMiDs bind CRBN to recruit neosubstrates for ubiquitylation and subsequent degradation. However, it is not known whether a deubiquitinating enzyme might be involved in this process. The present study revealed that USP15 antagonizes CRL4^{CRBN}-mediated ubiquitylation of natural substrate glutamine synthetase and neosubstrates, thereby preventing their degradation. Targeting USP15 sensitizes IMiD-resistant MM cells to IMiDs by promoting degradation of neosubstrates. These results suggest USP15 protein expression as a predictive biomarker of response or resistance to IMiDs and highlight the potential of USP15 as a key cancer drug target.

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the molecular mechanism underlying the oncogenic activity of USP15 remains poorly understood.

In mammals, glutamine synthetase (GS) is the only enzyme that is capable of the de novo synthesis of glutamine and detoxifies glutamate and ammonia. Mutations and deregulation of GS have been linked to human diseases, including congenital glutamine deficiency, Alzheimer's disease, and cancers (35–40). Pioneering studies by Stadtman et al. demonstrated that bacterial GS is regulated by reversible adenylation (41). In contrast to the well-defined regulation of bacterial GS, the molecular mechanism underlying the regulation of GS activity in mammals is poorly understood. Over 60 y ago, the mammalian GS was found to be inactivated by extracellular glutamine (42, 43). Subsequent studies done prior to the discovery of UPS suggested that glutamine promotes GS degradation through an unknown mechanism (44, 45). Our recent studies, focused on identification of endogenous substrates for $CRL4^{CRBN}$, reported that GS and AMPK-γ are endogenous substrates of $CRL4^{CRBN}$ (46, 47). Strikingly, we uncovered that the $CRL4^{CRBN}$-p97 pathway is required for degradation of GS and neosubstrates (48). However, molecular control of this pathway remains unknown.

Results

USP15 Is a GS-Interacting Partner. To identify a player that participates in regulating GS degradation in the $CRL4^{CRBN}$-p97 pathway, I first sought to identify GS-interacting proteins. Two independent mass spectrometry (MS) analyses of immunoprecipitation (IP) of endogenous USP15 prepared from LN-229 cells or two leukemia cell lines (MV-4-11 and Kasumi-1) revealed that GS was one of the top USP15-interacting proteins (26, 49). To validate these proteomic data, I performed IP experiments and found that $USP15^{Flag}$ interacts with GS^{Myc} (Fig. 1*A*). Strikingly, endogenous GS interacts with endogenous USP15 in different cell lines (Fig. 1 *B* and *C*). Most recently, quantitative in vivo interactome profiling of mouse tissues has further confirmed that endogenous USP15 interacts with endogenous GS in the heart, kidneys, lungs, and thymus in mice (50). To investigate whether the ubiquitylation status of GS could be involved in the interaction between USP15 and GS, IP experiments were carried out using protein extracts from 293FT cells pretreated with the NEDD8-activating enzyme inhibitor MLN4924 (51), the DUB inhibitor PR-619, or the p97 inhibitor CB-5083 (52), followed by glutamine treatment. As shown in Fig. 1*D*, glutamine enhanced the interaction between endogenous USP15 and endogenous unmodified forms of GS, which was greatly reduced in cells pretreated with MLN4924, suggesting that this interaction depends on ubiquitylation. In addition, CB5083, and to a lesser extent PR-619, decreased the interaction between USP15 and GS in glutamine-stimulated cells (Fig. 1*D*), similar to the binding mode of p97 to GS observed in our recent study (48). It is likely that CB5083- or PR-619–treated cells accumulated other p97 substrates to compete with ubiquitylated GS subunits for the binding to p97 and USP15. Consistent with the USP15-GS binding mode (Fig. 1*D*), endogenous USP15 interacted directly or indirectly with endogenous p97 (Fig. 1*D*). These results suggest that USP15 binds to ubiquitylated GS subunits, which are associated with p97 complex.

In-depth analysis of the ubiquitinome using stable isotope labeling of amino acids in cell culture (SILAC)-based quantitative MS from Jurkat cells treated with the proteasome inhibitor MG-132 (53) or PR-619 for 4 h revealed that while increased ubiquitylation of GS was observed in cells treated with MG132, PR-619 treatment decreased GS ubiquitylation (54), suggesting that GS is rapidly ubiquitylated and degraded in PR-619–treated cells, leading to a decrease in abundance of both native and ubiquitylated forms of GS. Since USP15 is inhibited by the broad-spectrum DUB inhibitors NSC632839 (55) and PR-619 (56, 57), I evaluated their effects on GS ubiquitylation and abundance. Consistent with this proteomic study (54), inhibition of DUB activity by NSC632839 or PR-619 promoted glutamine-induced ubiquitylation of GS (Fig. 1*E*). In addition, NSC632839 and PR-619 treatment resulted in decreased protein levels of endogenous GS (*SI Appendix*, Fig. S1 *A* and *B*). I next tested whether the action of DUB inhibitors on acceleration of glutamine-induced GS degradation is indeed dependent on the E3 ubiquitin ligase $CRL4^{CRBN}$. I cotreated cells with NSC632839 or PR-619 and MLN4924 and found that MLN4924 blocked the action of DUB inhibitors on glutamine-induced GS degradation (*SI Appendix*, Fig. S1 *C* and *D*). Together, these results suggest that USP15 may regulate GS ubiquitylation and degradation in an E3 ligase activity–dependent manner.

Depletion of USP15 Induces GS Degradation. Next, I studied the molecular mechanism by which USP15 might control GS stability

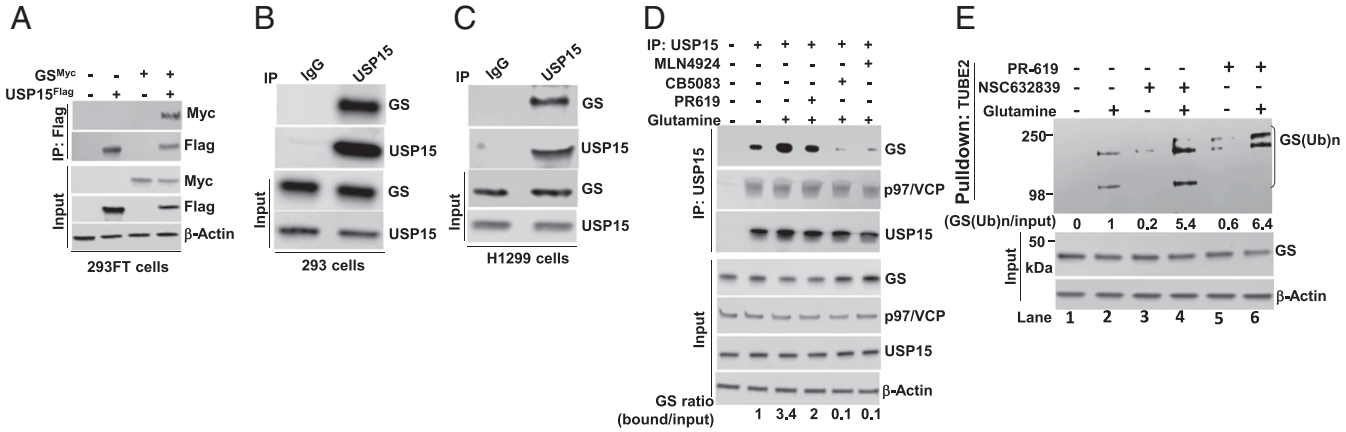

Fig. 1. Validation of USP15 as a GS-interacting protein. (*A*) 293FT cells were transfected with GS^{Myc} and $USP15^{Flag}$. After 48 h, cell extracts were immunoprecipitated with anti-Flag antibody. (*B* and *C*) Cell extracts from 293 cells (*B*) or H1299 cells (*C*) were immunoprecipitated with rabbit IgG control or USP15 antibody and then analyzed by IB with the indicated antibodies. (*D*) 293FT cells were starved of glutamine for 24 h, pretreated with or without MLN4924 (2 μM), CB5083 (10 μM), or PR-619 (20 μM) for 30 min, and then treated with 4 mM glutamine for 2 h. Protein extracts were immunoprecipitated with IgG control (lane 1) or USP15 antibody (lanes 2 through 6) and analyzed by IB with the indicated antibodies. The relative ratio of endogenous GS interacted with endogenous USP15 to input GS, normalized to that of untreated cells in lane 2, is shown. (*E*) MCF7 cells, starved of glutamine for 24 h, were treated with MG132 (10 μM) and/or 40 μM NSC632839 and 20 μM PR-619 for 0.5 h, followed by 4 mM glutamine treatment for 2 h. Total ubiquitylated proteins were purified using TUBE2-agarose and then analyzed by IB with the indicated antibodies. The relative ratio of ubiquitylated GS, GS(Ub)n to input GS, normalized to that of glutamine-treated cells in lane 2, is shown.

via the $CRL4^{CRBN}$-p97 pathway using RNA interference (RNAi) and CRISPR-Cas9 gene editing approaches. I first disrupted the USP15-GS interaction by depletion of USP15 using doxycycline (Dox)-inducible short hairpin RNA (shRNA) in the TRIPZ system. Consistent with the results obtained with the DUB inhibitors, a similar result was obtained upon shRNA knockdown of USP15. Depletion of USP15 by shRNA induced GS degradation (*SI Appendix*, Fig. S2 *A–D*). Most importantly, the steady-state level of GS was significantly down-regulated in *USP15*-knockout (KO) 293FT cells generated by CRISPR-Cas9 (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S2*E*). The cycloheximide (CHX) chase experiments revealed that GS protein was markedly degraded in *USP15*-KO cells (Fig. 2*C*). The USP15-dependent degradation of GS is a posttranslational regulation because the messenger RNA (mRNA) level of *GS* remained unchanged in *USP15*-KO 293FT cells (*SI Appendix*, Fig. S2*F*). As shown in Fig. 2 *D–F*, the down-regulation of endogenous GS protein levels observed in *USP15*-KO 293FT cells was rescued by blocking GS ubiquitylation using MLN4924 or the GS inhibitor L-methionine sulfoximine (MSO) (48), suggesting that USP15 controls GS degradation in downstream of $CRL4^{CRBN}$.

USP15 Directly Deubiquitylates Polyubiquitylated GS In Vivo and In Vitro. Since degradation of GS is dependent on the UPS, I next sought to examine the effect of USP15 depletion on GS ubiquitylation. Indeed, a significant increase in ubiquitylated GS forms was detected in $USP15^{-/-}$ 293FT cells (Fig. 3*A* and *SI Appendix*, Fig. S3*A*). To further define the molecular events in which USP15 participated in regulation of GS ubiquitylation, I performed TUBE2 pull-down experiments using $USP15^{-/-}$ 293FT cells grown in complete medium with 0.5 mM glutamine (the same glutamine concentration in human plasma) and found that the accumulation of ubiquitylated GS was observed in cells treated with CB-5083 (Fig. 3*B* and *SI Appendix*, Fig. S3*B*, lane 2) but not in cells cotreated with MLN4924 and MSO, which were recently reported to inhibit GS ubiquitylation (48), suggesting that USP15 controls GS ubiquitylation by acting downstream of $CRL4^{CRBN}$ and upstream of p97, possibly dependent on its DUB activity. Consistent with this notion, overexpression of wild-type (WT) $USP15^{Flag}$ reduced polyubiquitylated GS, while a catalytically inactive C298A-$USP15^{Flag}$ mutant failed to deubiquitylate polyubiquitylated GS in $USP15^{-/-}$ 293FT (Fig. 3*C* and *SI Appendix*, Fig. S3*C*). Most importantly, recombinant USP15 enzyme (rUSP15) purified from insect cells completely deconjugated polyubiquitylated GS in vitro (Fig. 3*D* and *SI Appendix*, Fig. S3*D*). These findings strongly indicate that USP15 is a component of the $CRL4^{CRBN}$-p97 pathway and that it deubiquitylates polyubiquitylated GS in vivo and in vitro and stabilizes GS protein levels in cells.

GBM is the most frequent adult primary malignant brain tumor (58), and it is the second leading cause of cancer mortality in adults under 35 y of age (59). GBM and many other tumors expressing high GS levels can synthesize glutamine de novo, grow, and proliferate in the absence of exogenous glutamine (38–40). Interestingly, USP15 amplification confers poor prognosis in patients with GBM (25). The data observed in Fig. 2 *A–C* provide compelling evidence that USP15 is a key component of the $CRL4^{CRBN}$-p97 pathway to regulate GS stability. I found that depletion of USP15 by shRNAs in LN229 cells resulted in a marked reduction in the steady-state level of GS (*SI Appendix*, Fig. S3*E*). Depletion of USP15 by shRNAs or inhibition of GS by the GS inhibitor MSO in control LN229 cells exhibited a severe defect in cell viability (Fig. 3*E*). In line with previous research (25, 39), these results confirm the important role of the USP15–GS axis in maintaining GBM cell viability. Strikingly, depletion of both USP15 by shRNAs and GS by MSO produced synergistic inhibition of cell viability in LN229 cells (Fig. 3*E*). Taken together, these results indicate the importance of USP15 in the regulation of glutamine metabolism via the GS enzyme that is essential for tumor growth.

USP15 Regulates $CRL4^{CRBN}$-Dependent Ubiquitylation and Degradation of Neosubstrates. Several groups reported that the molecular glue degrader CELMoDs and the PROTAC degrader dBET1 degrade $CRL4^{CRBN}$ neosubstrates or target proteins via molecular glue and PROTAC mechanisms, respectively (9–12, 15, 18, 22). Notably, we uncovered the $CRL4^{CRBN}$-p97 pathway, which is required for degradation of both endogenous substrate GS and neosubstrates of $CRL4^{CRBN}$ (46, 48). I next sought to investigate a potential role for USP15 in regulating ubiquitylation of

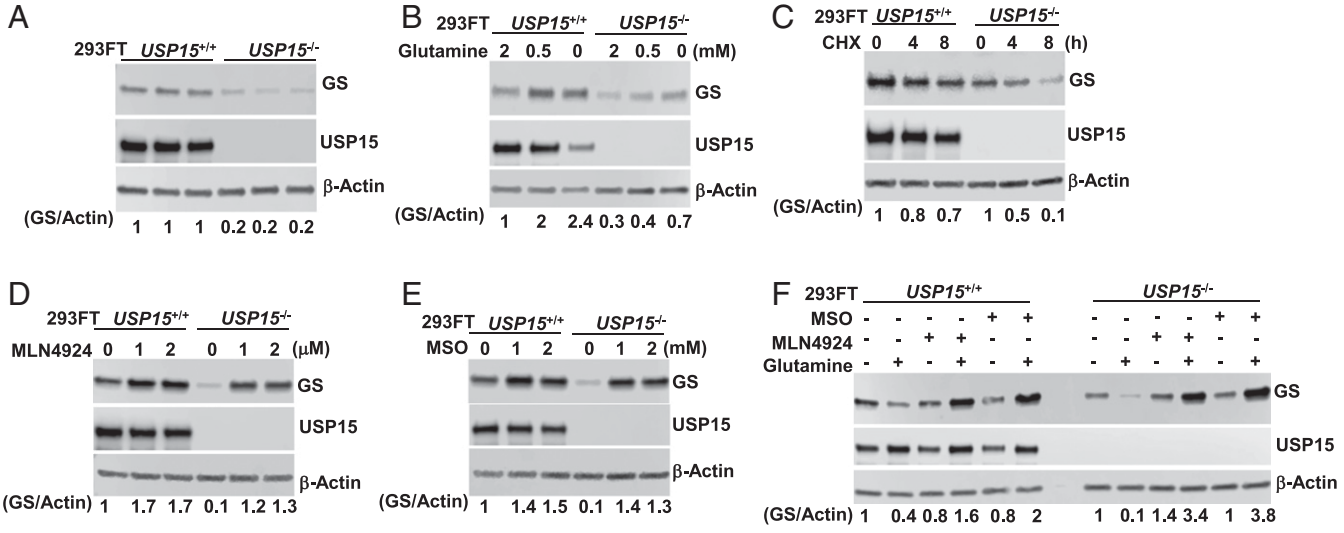

Fig. 2. Disruption of USP15 loci by CRISPR-Cas9 induces GS degradation. (*A* and *B*) $USP15^{+/+}$ and $USP15^{-/-}$ 293FT cells (triplicate) were cultured in 2 mM glutamine (*A*) or grown in different glutamine concentrations (*B*). (*C*) Same as *A*, except that cells were treated with CHX. Cell extracts were analyzed by IB. The relative ratio of GS:Actin is shown. (*D–F*) Rescue of endogenous GS protein levels in *USP15*-KO cells. $USP15^{+/+}$ and $USP15^{-/-}$ 293FT cells were grown in complete medium and then treated with 2 μM MLN4924 (*D*) or 2 mM MSO (*E*) for 10 h. (*F*) Same as *D* and *E*, except that cells were starved of glutamine for 24 h and then pretreated with 2 μM MLN4924 or 2 mM MSO for 30 min, followed by 4 mM glutamine treatment for 8 h. Cell extracts were analyzed by IB with the indicated antibodies. The relative ratio of GS:Actin is shown.

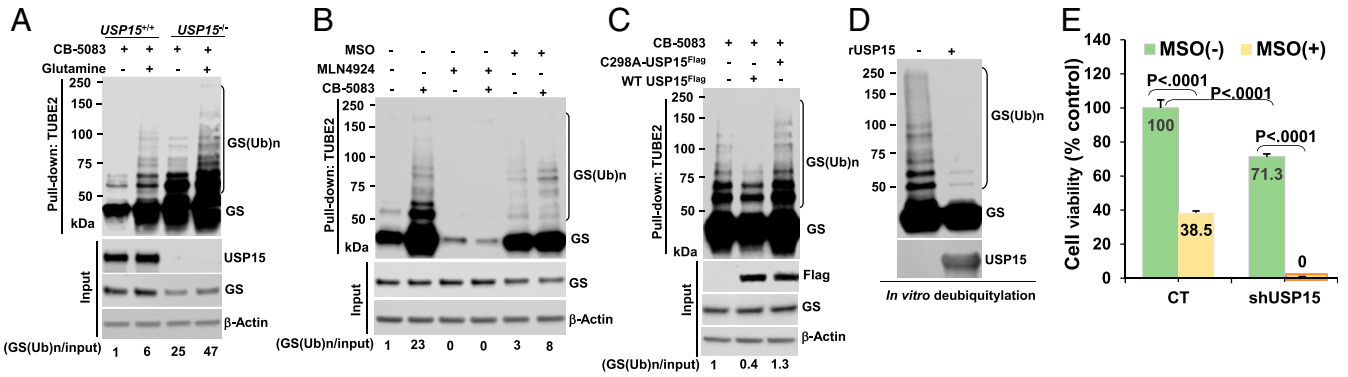

Fig. 3. USP15 deubiquitylates ubiquitylated GS in vivo and in vitro. (*A*) Glutamine-starved *USP15*$^{+/+}$ and *USP15*$^{-/-}$ 293FT cells were treated with 10 μM CB-5083 for 30 min, followed by 4 mM glutamine treatment for 3 h. (*B*) Cells, grown in medium with 0.5 mM glutamine, were pretreated with 2 μM MLN4924 or 2 mM MSO for 0.5 h, followed by treatment with CB-5083 for 6 h. Total ubiquitylated proteins were purified using TUBE2-agarose. The relative ratio of ubiquitylated GS, GS(Ub)n to input GS is shown. (*C*) *USP15*$^{-/-}$ 293FT cells were transfected with control plasmid, WT USP15^{Flag}, or C298A-USP15^{Flag} mutant plasmid. After 48 h, cells were treated with CB-5083 for 6 h. Total ubiquitylated proteins were affinity-purified using TUBE2-agarose. The relative ratio of ubiquitylated GS, GS(Ub)n to input GS is shown. (*D*) In vitro deubiquitylation assay. *USP15*$^{-/-}$ 293FT cells, starved of glutamine for 24 h, were pretreated with CB-5083 for 0.5 h, followed by 4 mM glutamine treatment for 4 h. Total ubiquitylated proteins were affinity-purified using TUBE2-agarose beads, which were subsequently treated with or without rUSP15 protein at 37 °C for 1 h and then analyzed by Western blot. (Ub)n, polyubiquitin. (*E*) LN229 cells were transduced with the GIPZ lentiviral vectors that expressed either control (CT) shRNA or *USP15* shRNA clones 4 and 6 (shUSP15). An equal number of cells (1×10^6 cells in 10-cm plates) were then shifted to medium without glutamine in the presence (+) or absence (–) of the GS inhibitor MSO (2 mM) for 5 d, after which cell viability was quantified by staining with 0.4% Trypan blue. Cell viability was normalized to MSO-untreated control (–), which was set to 100%. Error bars represent the SEM of triplicates ($P < 0.0001$ by *t* test).

CRL4^{CRBN} neosubstrates. As shown in Fig. 4 *A*–*E* and *SI Appendix*, Fig. S4 *A*–*E*, lenalidomide (Len), CC-885, and dBET1 promoted accumulation of polyubiquitylated forms of all tested neosubstrates including IKZF3, CK1-α, RNF166, GSPT1, and BRD4 in *USP15*-KO 293FT cells pretreated with CB5083 or the proteasome inhibitors MG132 or bortezomib (53), suggesting that USP15 plays an important role in regulating the ubiquitylation status of these target proteins, possibly through its deubiquitylating enzyme activity. I further tested if depletion of USP15 could have an effect on IMiD-induced degradation of neosubstrates. I performed CHX chase experiments to monitor Len-induced degradation of CK1-α (31) and found that Len treatment led to increased degradation of CK1-α in *USP15*-KO 293FT cells (Fig. 4*F*). To further define the sequential events in which USP15 controls CRL4^{CRBN}-dependent ubiquitylation and degradation of neosubstrates, I performed TUBE2 pull-down using cell extracts of *USP15*$^{-/-}$ 293FT cells treated with different inhibitors and observed that polyubiquitylated GSPT1 was accumulated in the cells cotreated with CC-885 and MG132 or CB-5083, which was completely abolished in the presence of MLN4924 (Fig. 4*G* and *SI Appendix*, Fig. S4*F*). In addition, CC-885–induced degradation of GSPT1 was blocked in cells cotreated with CC-885 and MG132 or CB-5083 (Fig. 4 *G*, *Lower*). These results indicated that USP15 functions downstream of CRL4^{CRBN} and upstream of p97 and the proteasome to control the stability of all neosubstrates.

USP15 Deubiquitylates Polyubiquitylated Neosubstrates In Vitro. Since USP15 is a deubiquitylating enzyme, I next tested whether it could remove polyubiquitin chains from neosubstrates of CRL4^{CRBN} in vitro. To achieve this goal, I utilized ubiquitin-binding TUBE2 resin to purify polyubiquitylated neosubstrates from cellular extracts of *USP15*-KO cells treated with Len and CC-885 in the presence of p97 inhibitor and then performed the in vitro deubiquitylation assays using rUSP15. USP15 directly deubiquitylates polyubiquitylated CK1-α forms (Fig. 5*A* and *SI Appendix*, Fig. S5*A*) and GSPT1 (Fig. 5*B*, *SI Appendix*, Fig. S5*B*). Strikingly, the activity of USP15 was completely inhibited in the presence of the broad-spectrum DUB inhibitors *N*-Ethylmaleimide (NEM) (60) and PR-619 at a high dose of 100 μM (Fig. 5*B* and *SI Appendix*, Fig. S5*B*). I further demonstrated that PR-619 with the concentration range of 1 to 20 μM substantially suppressed USP15 activity in a dose-dependent manner (Fig. 5*C* and *SI Appendix*, Fig. S5*C*). I next determined whether USP15 might directly antagonize CRL4^{CRBN}-mediated ubiquitylation of IKZF1/3 in vitro. To this end, I performed the "competitive" ubiquitylation/deubiquitylation assays that contain all key components of the UPS, including recombinant E1, E2, CRL4^{CRBN}, neosubstrate IKZF3^{FM} (FM: carboxyl-terminal Flag-Myc) purified from *USP15*$^{-/-}$ 293FT cells and rUSP15 in the presence or absence of Len to mimic the biological properties of in vivo cells. The in vitro competitive ubiquitylation/deubiquitylation assay strongly indicated that Len promoted CRL4^{CRBN}-mediated ubiquitylation of IKZF3 in vitro in an IMiD-dependent manner (Fig. 5*D*), consistent with two previous studies (9, 10). In striking contrast, Len-induced ubiquitylation of IKZF3 was significantly inhibited in the presence of rUSP15 (Fig. 5*D*). Since branched ubiquitin chains have recently been implicated in playing an important role in providing specific signals for efficient proteasomal degradation (61, 62), I next tested whether USP15 could deconjugate different polyubiquitin chains of IKZF3 in vitro. As shown in Fig. 5*E*, USP15 greatly antagonized CRL4^{CRBN}-mediated K48-, K11-, or K63-linked polyubiquitylation of IKZF3. Taken together, these results demonstrated that USP15 is a regulator of the CRL4^{CRBN}-p97 pathway to control CELMoD-induced ubiquitylation and subsequent degradation of neosubstrates.

Depletion of USP15 Promotes IMiD-Induced Degradation of IKZF1/3 in Len-Resistant MM Cell Lines. To gain further insight into the molecular mechanisms underlying IMiD sensitivity and resistance in MM, I first checked the USP15 protein levels in Len-sensitive and Len-resistant MM cell lines by immunoblotting (IB) with antibodies against USP15 and core components of the CRL4^{CRBN}-p97 pathway. While the protein levels of p97 and DDB1 remained unchanged in all tested Len-sensitive and -resistant cell lines, the protein levels of CRBN and CUL4A in Len-resistant cell lines expressed at least equal or higher compared with those in Len-sensitive cell lines (Fig. 6*A* and *SI Appendix*, Table S1). In striking contrast, the protein levels of USP15 in all Len-resistant cell lines were overproduced compared to the levels observed in Len-sensitive cell lines (Fig. 6*A* and *SI Appendix*, Table S1), suggesting that USP15 might play an important role in IMiD resistance. Consistent with the role of USP15 in regulating IMiD-induced ubiquitylation and degradation of neosubstrates in *USP15*$^{-/-}$ 293FT cells

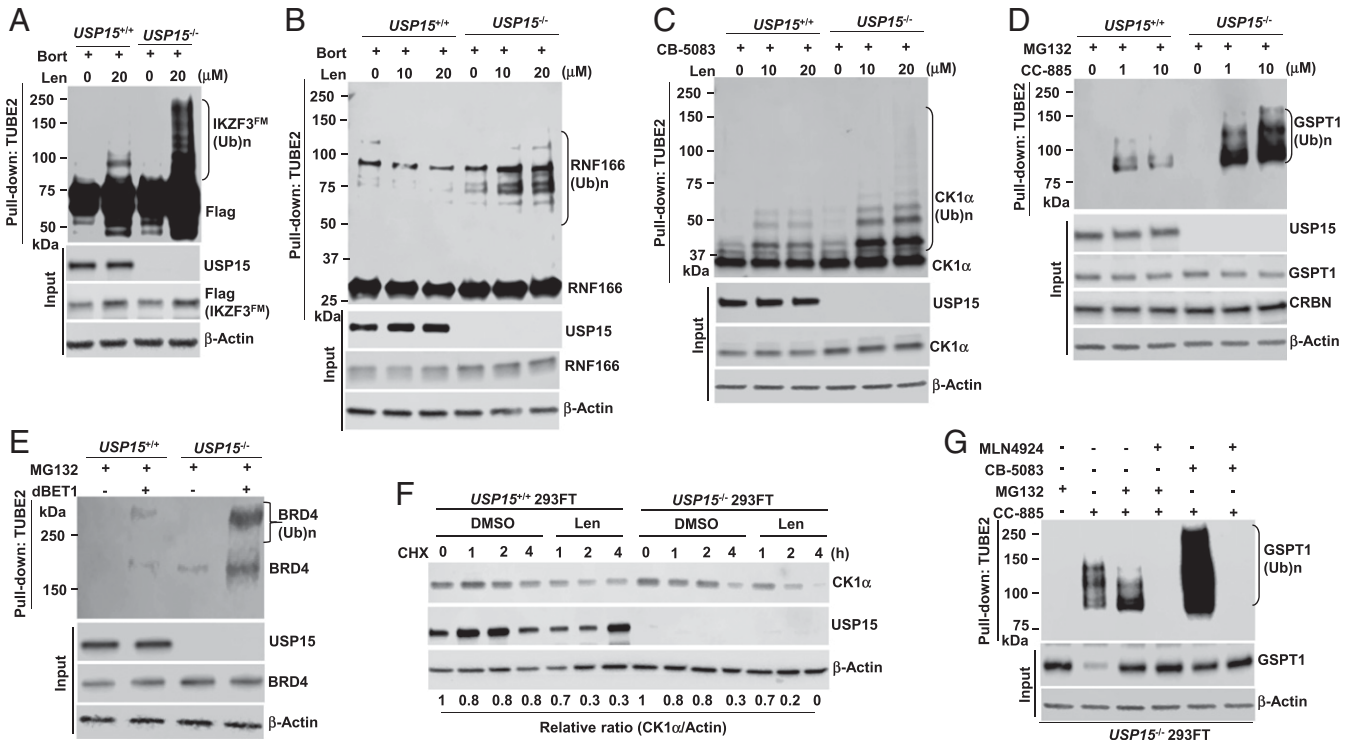

Fig. 4. Depletion of USP15 promoted CELMoD-induced ubiquitylation of CRBN neosubstrates and subsequent degradation. (*A*) USP15$^{+/+}$ and USP15$^{-/-}$ 293FT cells, transfected with IKZF3^{FM} (IKZF3 with a carboxyl-terminal Flag-Myc tag) plasmid for 48 h, were pretreated with bortezomib (Bort, 1 μM) for 0.5 h, followed by Len treatment at 0 and 20 μM for 5 h. (*B*) Cells were pretreated with Bort for 0.5 h, followed by Len treatment at 0, 10, and 20 μM for 5 h. (*C*) Same as *B*, except that cells were pretreated with CB-5083 (10 μM) for 0.5 h, followed by treatment with Len at 0, 10, and 20 μM for 5 h. (*D* and *E*) Same as *B*, except that cells were pretreated with MG132 (20 μM) for 1 h, followed by treatment with CC-885 at 0, 1, and 10 μM for 3 h (*D*) or dBET1, a potent BRD4 degrader (2 μM), for 4 h (*E*). Total ubiquitylated proteins were affinity-purified using TUBE2-agarose. Bound fractions and cell lysates (input) were analyzed by IB with indicated antibodies. (Ub)n: polyubiquitin. (*F*) USP15$^{+/+}$ and USP15$^{-/-}$ 293FT cells were pretreated with 10 μM Len for 0.5 h, followed by addition of CHX. Samples were harvested for IB analysis. The relative ratio of CK1-α:Actin was shown. (*G*) USP15$^{-/-}$ 293FT cells, pretreated with 10 μM CB-5083, 20 μM MG132, and 2 μM MLN4924 for 0.5 h, were treated with 10 μM CC-885 for 3 h. Total ubiquitylated proteins were affinity-purified using TUBE2-agarose.

(Fig. 4 *A*–*F*), knockdown of *USP15* in the Len-resistant RPMI8226 cell line by two different RNAi approaches, including *USP15*-shRNA TRIPZ_2 (Fig. 6*B*) or GIPZ lentiviral shRNAs targeting human *USP15* clones 4 or 6 (shUSP15_4 or 6) (Fig. 6*C*), promoted IMiD-induced degradation of IKZF1/3 proteins. Consistent results were observed in two other Len-resistant cell lines, KMS-11 (Fig. 6*D*) and OPM-1 (Fig. 6*E*). USP15 also contributes to the antitumor activities of IMiDs, since depletion of USP15 also enhanced IKZF1/3 degradation in two different IMiD-sensitive MM cell lines, MM1.S (*SI Appendix*, Fig. S6*B*) and U266 (*SI Appendix*, Fig. S6*C*). These findings suggest that USP15 counteracts IMiD-induced ubiquitylation of CRL4^{CRBN} neosubstrates including IKZF1/3 in MM cells, thus preventing their degradation. Strikingly, depletion of USP15 sensitizes MM.1S cells (*SI Appendix*, Fig. S6*D*) and RPMI8226 to Len (Fig. 6*F*). These results inevitably imply the need for considering a combination of IMiDs and USP15 inhibitor, which may improve clinical outcomes of patients with MM and other hematologic malignancies.

Discussion

Based on the findings reported here and previously (46, 48), a proposed model for the role of USP15 in regulating the stability of natural substrate GS and neosubstrates in the CRL4^{CRBN}-p97 pathway is illustrated in Fig. 7. High glutamine induces GS acetylation at lysines 11 and 14 to create a degron that binds CRBN (46). Subsequently, acetylated GS is ubiquitylated by CRL4^{CRBN}, segregated by p97, and degraded by the proteasome (46). However, in GBM and other cancers, up-regulation of USP15 stabilizes GS, a key metabolic enzyme in glutamine metabolism, which is essential for tumor growth. Most importantly, USP15 antagonizes CELMoD/PROTAC-induced ubiquitylation of target proteins, thereby inhibiting their subsequent degradation. This accounts for intrinsic resistance in IMiD-resistant MM cell lines that greatly overproduce USP15 protein. Inhibition of USP15 represents a valuable therapeutic opportunity to potentiate CELMoD and CRBN-based PROTAC therapies for the treatment of cancer, including MM, deletion 5q [del(5q)] subtype of myelodysplastic syndrome, acute myeloblastic leukemia, and other human diseases.

Although CRBN is essential for antitumor activity of IMiDs (7–10), its mRNA and protein expression levels do not correlate with intrinsic sensitivity or resistance to IMiDs in MM cell lines (63), suggesting that factors other than CRBN might regulate sensitivity and/or intrinsic resistance to IMiDs. Six independent groups, using CRISPR-Cas9 screens to identify genes required for IMiD sensitivity and resistance, have recently reported that regulators of CRL4^{CRBN} and E2 components are necessary for IMiD-dependent CRL4^{CRBN} function (64–69). Other factors, such as overexpression of RUNX proteins and activation of Wnt signaling, ERK, and the IL6/STAT3 pathway, contribute to intrinsic or acquired resistance to IMiDs (70–73). Dual inhibition of DNA methyltransferases and EZH2 can also overcome both intrinsic and acquired resistance of MM cells to IMiDs in a CRBN-independent manner (74). However, there is no direct evidence supporting the major hypothesis that other factors in downstream of CRL4^{CRBN} in the UPS are involved in regulating intrinsic resistance to IMiDs.

Previously, we were particularly interested in understanding the molecular action of IMiDs and performed SILAC-based

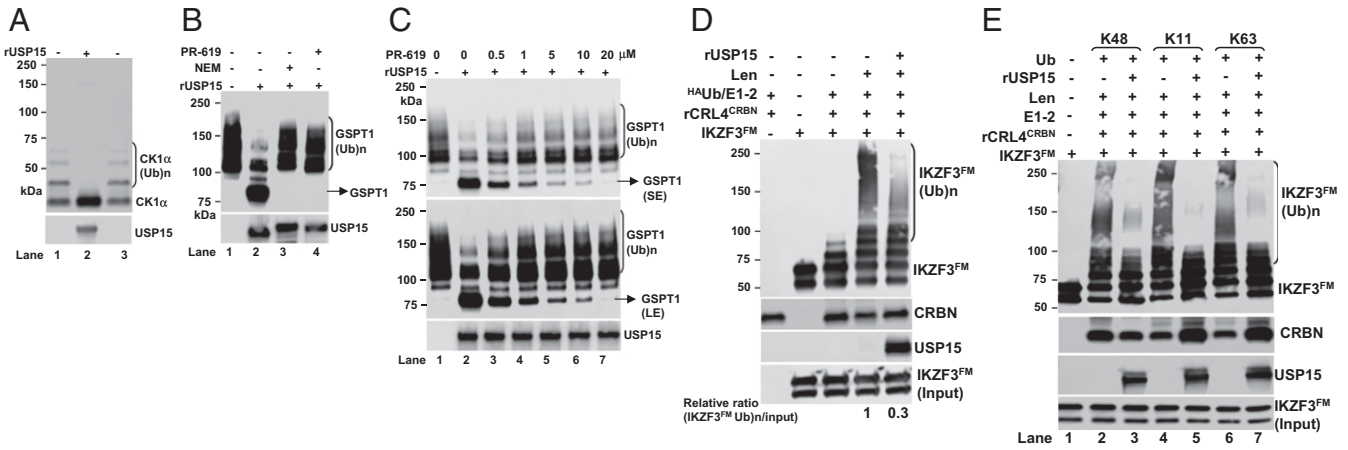

Fig. 5. USP15 deubiquitylates polyubiquitylated neosubstrates in vitro. (*A*) USP15$^{-/-}$ 293FT cells, pretreated with CB-5083 (10 μM) for 0.5 h, were induced with 20 μM Len for 5 h. Total ubiquitylated proteins were affinity-purified using TUBE2-agarose beads. Polyubiquitylated CK1-α proteins, pulled down with TUBE2 resin, were kept on ice as control (lane 1) or treated with or without rUSP15 protein at 37 °C for 0.5 h (lanes 2 and 3, respectively), followed by IB analysis. CK1-α(Ub)n: polyubiquitylated CK1-α. (*B*) USP15$^{-/-}$ 293FT cells, pretreated with CB-5083 for 0.5 h, were treated with CC-885 (10 μM) treatment for 4 h. Total polyubiquitylated proteins, pulled down with TUBE2 resin, were treated with or without rUSP15 protein in the absence or presence of DUB inhibitors NEM (20 mM) and PR-619 (100 μM) at 37 °C for 0.5 h, followed by SDS-PAGE analysis and IB with antibodies against GSPT1, ubiquitin, and USP15. GSPT1(Ub)n: polyubiquitylated GSPT1. Arrow indicates a native form of GSPT1, which was released after deubiquitylation of polyubiquitylated GSPT1 by rUSP15. (*C*) Same as *B*, except that polyubiquitylated proteins, pulled down with TUBE2 resin, were treated with or without rUSP15 protein in the absence or presence of indicated concentrations of PR-619 at 37 °C for 0.5 h, followed by SDS-PAGE and IB analysis. SE and LE: short and long exposures. (*D*) IKZF3^{FM} was purified from USP15$^{-/-}$ 293FT cells. An in vitro competitive ubiquitylation/deubiquitylation of IKZF3^{FM} was carried out for 1 h at 30 °C in the presence or absence of E1, E2, ^{HA}Ub (HA-tagged wild-type ubiquitin), and the recombinant CLR4^{CRBN} complex in a final volume of 30 μL. Where indicated, Len (50 μM) and rUSP15 were added. Reactions were stopped by mixing with an equal amount of 2× SDS sample buffer and analyzed by SDS-PAGE and IB with antibodies against Flag, USP15, and CRBN. (Ub)n indicates polyubiquitylation. The relative ratio of ubiquitylated IKZF3^{FM}, IKZF3^{FM} (Ub)n to input IKZF3^{FM} is shown. (*E*) Same as *D*, except that ubiquitin mutants (Ub) with K48 only (lanes 2 and 3), K11 only (lanes 4 and 5) or K63 only (lanes 6 and 7) were used.

quantitative MS analysis to search for CRBN-interacting partners and found that GS was a potential candidate substrate of CRL4^{CRBN} among CRBN-interacting proteins in thalidomide-treated 293T cells (46). We proved that GS is a natural substrate of CRL4^{CRBN} (46). Strikingly, we used GS, one of the best-characterized natural substrates for CRL4^{CRBN}, to understand regulation of CRL4^{CRBN} downstream and discovered that the CRL4^{CRBN}-p97 pathway essential for degradation of GS and neosubstrates (48). In the present study, I identify USP15 as a GS-interacting partner based on two independent proteomic studies (26, 49). The results presented here provide direct evidence that USP15 deubiquitylates and stabilizes seven tested CRL4^{CRBN} protein targets, including GS, IKZF1, IKZF3, CK1-α, RNF166, GSPT1, and BRD4 via natural substrate, molecular glue, and PROTAC mechanisms. It functions downstream of CRL4^{CRBN} and upstream of p97 and the proteasome. This accounts for intrinsic resistance of IMiDs in MM. Although USP15 plays an essential role in regulating degradation of target

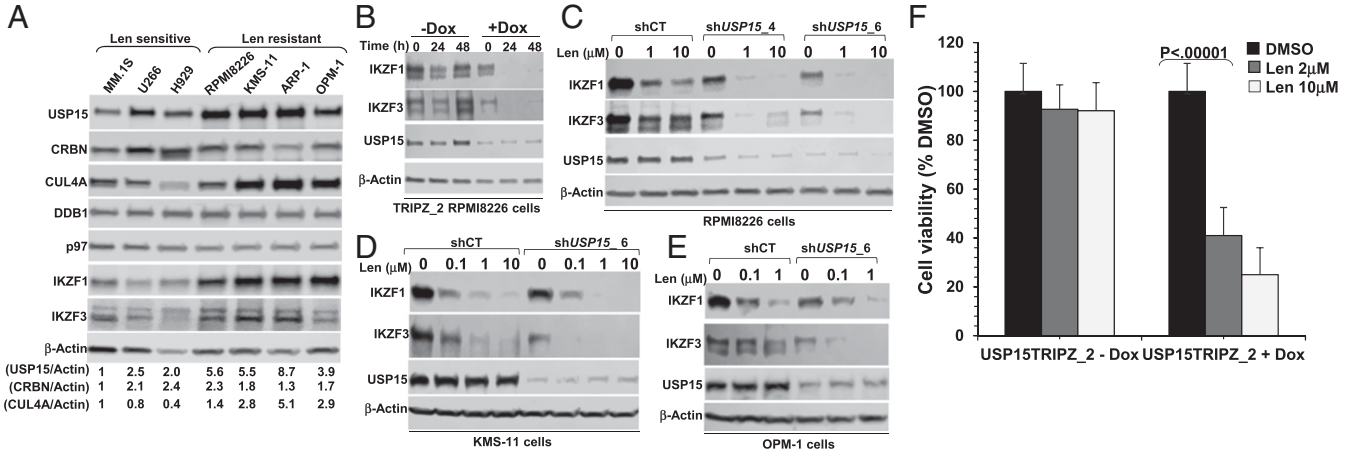

Fig. 6. Depletion of USP15 promotes IMiD-induced degradation of IKZF1/3 in Len-resistant MM cell lines. (*A*) USP15 was highly expressed in Len-resistant MM cell lines. Equal amounts of protein extracts from three Len-sensitive MM cell lines and four Len-resistant MM cell lines were analyzed by IB with the indicated antibodies. The relative ratios of USP15:Actin, CRBN:Actin, and CUL4A:Actin, normalized to MM1S cells, are shown. (*B*) USP15-shRNA TRIPZ_2 RPMI8226 cells were induced with or without Dox (2 μg/mL) for 96 h. Cells were treated with 10 μM Len for 24 and 48 h. (*C*) RPMI8226 cells, stably expressing CT shRNA lentivirus (shCT) or GIPZ lentiviral shRNAs targeting human USP15 clones 4 or 6 (shUSP15_4 or 6), were treated with Len at 0, 1, and 10 μM for 48 h. (*D* and *E*) KMS-11 cells (*D*) or OPM-1 cells (*E*) stably expressing shCT or shUSP15_6 were treated with Len for 24 h. Cell extracts were analyzed by IB with the indicated antibodies. (*F*) Depletion of USP15 sensitizes MM cells to lenalidomide. RPMI8226 cells stably expressing Dox-inducible shRNA TRIPZ targeting USP15 clone 2 (*USP15*TRIPZ_2) were induced with or without Dox (2 μg/mL) for 3 d and then treated with dimethyl sulfoxide (DMSO) or 2 and 10 μM Len for 5 d. Cell viability was quantified by staining with 0.4% Trypan blue. Cell viability was normalized to DMSO-treated CT, which was set to 100%. Error bars represent the SEM of triplicates ($P < 0.00001$ by *t* test).

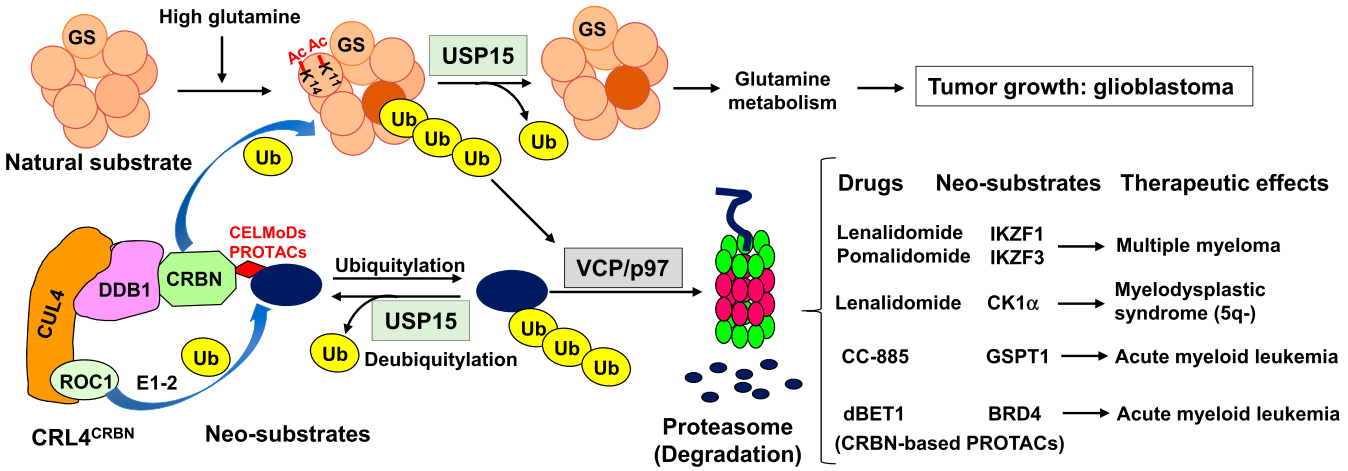

Fig. 7. Proposed model: USP15 is a key regulator of the CRL4^{CRBN}-p97 pathway to control the stability of natural substrate GS and neosubstrates. After treating cells with glutamine or CELMoDs, CRL4^{CRBN} promotes ubiquitylation of GS or neosubstrates, respectively. USP15 antagonizes CRL4^{CRBN}-mediated ubiquitylation of target proteins, thereby preventing their degradation. p97 functions in disassembling ubiquitylated GS oligomers or neosubstrates to facilitate their degradation by the proteasome. See the first paragraph of *Discussion* for more details. Ub, ubiquitin; Ac, acetylated.

proteins in the CRL4^{CRBN}-p97 pathway, further studies will be required to determine whether a combination of IMiDs and USP15 inhibitor may improve clinical outcomes of patients with MM and other types of cancer. These findings also suggest that USP15 protein expression is a potential biomarker of response or resistance to IMiD therapy in patients with MM.

It has been implied that p97 can directly or indirectly interact with E3s and DUBs to edit the ubiquitin chains of ubiquitylated substrate to target it to the proteasome or recycle the substrate (75). For example, many DUBs implicated in endoplasmic reticulum-associated protein degradation associate with p97 either directly or indirectly (76). The COP9 signalosome (CSN), an essential regulator of cullin-RING ubiquitin ligase (CRL) activity, is highly conserved from yeast to humans (77, 78). CSN has been shown to interact with the deubiquitinating enzyme Ubp12p (79) and the *Schizosaccharomyces pombe* ortholog of human USP15 (60). Interestingly, CSN and p97 could form a large adenosine triphosphate-dependent oligomeric complex to control polyubiquitylated substrates via USP15 activity (80). The present study indicates that USP15 interacts with ubiquitylated GS subunits and the p97 complex. Therefore, further work is required to define in greater detail the molecular basis for the binding of USP15 to ubiquitylated target proteins. It will also be of particular interest in future studies to investigate whether USP15 can deubiquitylate other E3-dependent neosubstrates such as neosubstrates of VHL-based PROTACs (81).

In conclusion, USP15 is a key regulator of the CRL4^{CRBN}-p97 pathway to control CRL4^{CRBN}-mediated ubiquitylation and subsequent degradation of natural substrate GS and neosubstrates. The findings presented in this work not only advance our understanding of how USP15 antagonizes CRL4^{CRBN}-dependent degradation of target proteins but also underscore the important contribution of USP15 to the molecular action of IMiDs and highlight the potential of USP15 as a key drug target for cancer therapeutics.

Materials and Methods

Generation of *USP15*-KO 293FT Cells by CRISPR-Cas9 Genome Editing. Cells cultured in a 24-well plate were transiently transfected with 0.5 μg human *USP15* CRISPR-Cas9 Plasmids (catalog No. sc-402416; Santa Cruz Biotechnology) using Fugene HD (Promega). Two days after transfection, a single cell was seeded in a 96-well plate via serial dilutions. After 2 wk, single clones were obtained and expanded to validate the editing of *USP15* by Western blot.

Immunoblot Analysis, IP, and TUBE2 Pull-down. The protocols for immunoblot analysis and IP were performed as described previously (46, 82). The protocols for TUBE2 pull-down were carried out as described recently (48).

In Vitro Deubiquitylation Assay. The in vitro deubiquitylation assay was performed as described in our recent work (48). Briefly, *USP15*$^{-/-}$ 293FT cells were treated with IMiDs or the CELMoDs in the presence of proteasome or p97 inhibitors to accumulate polyubiquitylated target proteins, which were subsequently purified by TUBE2 pull-down (48). The beads containing polyubiquitylated proteins were mixed in 30 μL ubiquitylation buffer, followed by addition of 1 μg rUSP15 protein (catalog No. E-594-050; R&D Systems) and incubated at 37 °C for 0.5 h. rUSP15-treated samples were mixed with 30 μL 2× sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and subjected to Western blot analysis.

The competitive ubiquitylation/deubiquitylation assays were developed based on the in vitro ubiquitylation assays, which were described in two recent studies (46, 47). Briefly, IKZF3^{FM} was purified from transiently transfected *USP15*$^{-/-}$ 293FT cells. The in vitro competitive ubiquitylation/deubiquitylation assays were carried out for 1 h at 30 °C in a final volume of 30 μL containing IKZF3^{FM}, E1, E2 (UbcH5a and UbcH3/Cdc34), recombinant CLR4^{CRBN} complex, ^{HA}Ub (HA-tagged wild-type ubiquitin) or ubiquitin mutants with K48 only, K11 only or K63 only, rUSP15 (1 μg), and 50 μM Len. Reactions were stopped by mixing with equal amount of 2× SDS sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and IB with antibodies against Flag, USP15, and CRBN.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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