p97/VCP promotes degradation of CRBN substrate glutamine synthetase and neosubstrates

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Glutamine synthetase (GS) plays an essential role in metabolism by catalyzing the synthesis of glutamine from glutamate and ammonia. Our recent study showed that CRBN, a direct protein target for the teratogenic and antitumor activities of immunomodulatory drugs such as thalidomide, lenalidomide, and pomalidomide, recognizes an acetyl degron of GS, resulting in ubiquitylation and degradation of GS in response to glutamine. Here, we report that valosin-containing protein (VCP)/p97 promotes the degradation of ubiquitylated GS, resulting in its accumulation in cells with compromised p97 function. Notably, p97 is also required for the degradation of all four known CRBN neo-substrates [Ikaros family zinc finger proteins 1 (IKZF1) and 3 (IKZF3), casein kinase 1α (CK1α), and the translation termination factor GSPT1] whose ubiquitylation is induced by immunomodulatory drugs. Together, these data point to an unexpectedly intimate relationship between the E3 ubiquitin ligase CRL4^{CRBN} and p97 pathways.

VCP/p97 | glutamine synthetase | CRBN | substrates | degradation

Glutamine plays important roles in many cellular processes, including oxidative metabolism and ATP generation, biosynthesis of proteins, lipids, and nucleic acids, and cell growth and proliferation through the regulation of the mTOR signaling pathway, translation, and autophagy (1, 2). In mammals, it is the most abundant amino acid in plasma with a concentration of 0.5–0.9 mM (3), accounting for ∼20% of its free amino acid pool. Glutamine synthetase (GS) is the only enzyme that is capable of de novo synthesis of glutamine and also functions to detoxify glutamate and ammonia, depending on tissue localization. Skeletal muscles and lungs are major sites of glutamine synthesis, whereas cells of the gut and the immune system, such as lymphocytes and macrophages, consume large amounts of glutamine in plasma (4). GS protects neurons against excitotoxicity by converting glutamate into glutamine in brain, detoxifies ammonia in liver, and maintains physiologic pH in kidney (5). In an attempt to investigate the role of GS in development, He et al. generated GS-knockout mice and reported that GS is essential in early embryogenesis, because deletion of the murine *GLUL* gene causes lethality at the blastocyst stage (embryonic day 3.5) (6). Interestingly, mouse ES cells maintain pluripotency and proliferate when grown in the absence of exogenous glutamine (7). However, inhibition of GS with the small molecule methionine sulfoximine (MSO) is sufficient to block the proliferation of ES cells in glutamine-free medium (7). In humans, congenital systemic glutamine deficiency caused by homozygous GS mutations results in multiorgan failure and neonatal death (8).

Recent studies highlight the importance of glutamine metabolism in metabolic reprogramming, because many tumor cells display "glutamine addiction" (9). Activation of oncogenes such as MYC, KRAS, and HIF1α and/or loss of tumor suppressor genes including p53 can directly mediate the reprogramming of glutamine metabolism by selectively activating their downstream signaling or metabolic pathways (1, 4, 10, 11). As a result, some tumor cells require large amounts of exogenous glutamine to generate building blocks and energy for their growth and survival. In contrast, various tumor cell lines with high expression levels of GS enzyme can synthesize glutamine de novo and can grow and proliferate in the absence of exogenous glutamine (12–14).

Befitting its critical role in nitrogen metabolism, GS activity is tightly regulated. Pioneering studies by Stadtman's group (15) and others demonstrated that bacterial GS is subject to complex feedback regulation by glutamine and downstream metabolites by reversible adenylylation and deadenylylation of a specific tyrosine residue, resulting in the inactivation of GS (16–18). In contrast to the well-defined regulation of bacterial GS, the molecular mechanism underlying the regulation of GS activity in mammalian cells is poorly understood. Before the discovery of ubiquitin-dependent proteolysis, it was proposed that glutamine inactivates GS through an uncharacterized degradation mechanism (19–22). Interestingly, the C-terminal region of bacterial GS, which contains the tyrosine that is adenylylated, is missing in mammalian GS. In contrast, eukaryotic GS has a highly conserved N-terminal extension that does not exist in prokaryotic GS (23). We recently reported that endogenous GS protein levels in multiple cell types and different mouse tissues are negatively regulated by glutamine via the E3 ubiquitin ligase CRL4^{CRBN} (24). CRBN, a direct protein target for thalidomide teratogenicity and antitumor activity of immunomodulatory drugs, including lenalidomide and pomalidomide and a novel CRBN modulator CC-885 (25–31), recognizes an acetylated motif (called an "acetyl degron") of GS, leading to ubiquitylation and subsequent degradation of GS in response to glutamine (24). However, the molecular events that take place at each step of the

Significance

We have recently reported that glutamine synthetase (GS) is negatively regulated by glutamine through a feedback loop involving the E3 ubiquitin ligase CRL4^{CRBN}. However, the molecular events that take place at each step of the pathway are not well understood. Here, we show that valosin-containing protein (VCP)/p97, is required for GS degradation. It acts downstream of CRL4^{CRBN}. p97 extracts ubiquitylated GS subunits from the decamer so that they can be degraded by the proteasome. Interestingly, p97 is also required for immunomodulatory drug-induced degradation of all four known CRL4^{CRBN} neosubstrates, including Ikaros family zinc finger proteins 1 (IKZF1) and 3 (IKZF3), casein kinase 1α (CK1α), and the translation termination factor GSPT1, which accounts for antitumor activity of these drugs. Our findings could have important implications for patient responsiveness to cancer therapy with immunomodulatory drugs.

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pathway are not well understood. For example, one of the fundamental questions is how the ubiquitin–proteasome system (UPS) manages to degrade individual subunits of a homodecameric enzyme complex.

Valosin-containing protein (VCP)/p97, a homohexameric AAA ATPase, promotes a number of cellular processes, including ubiquitin-dependent protein degradation, endoplasmic reticulum-associated degradation (ERAD), and autophagy (32). p97 working in concert with different adaptors mediates the extraction of ubiquitylated proteins from organelles, chromatin, and protein complexes and delivers them for proteasome- and autophagy-mediated protein degradation. One of the major functions of p97 is thought to be the disassembly of protein complexes, presumably by converting chemical energy generated from ATP hydrolysis into mechanical force used for conformational changes of target proteins (33). Mutations in p97 cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD) (34, 35) and a small fraction of familial amyotrophic lateral sclerosis (ALS) cases (36). Transgenic and knockin mouse models have been generated to investigate how these mutations contribute to the pathogenesis of IBMPFD and ALS (37–40). Because of its pivotal role in maintaining the cellular protein homeostasis important for tumor cell growth and survival, p97 is of particular interest as an anticancer drug target. Recently we developed the reversible and ATP-competitive p97 inhibitors DBeQ and ML240 (41, 42). Subsequent optimization of ML240 resulted in the identification of CB-5083 (43), which is currently being tested in phase I clinical trials. CB-5083 exhibits potent antitumor activity in both multiple myeloma and solid tumor xenograft models (44). However, the precise mechanisms by which p97 regulates substrates under physiological conditions remain poorly understood, and only a limited number of substrates have been studied in great detail.

Results

Glutamine Induces GS Disassembly in a Ubiquitylation- and p97-Dependent Manner. The active sites of the proteasome are enclosed in a chamber, access to which is governed by portals with a diameter of ~13 Å (45, 46). Because this diameter is narrower than the diameter of most globular proteins, substrates must be disengaged from binding partners and unfolded before being threaded into the proteasome's proteolytic chamber. Because GS is a homodecamer composed of two pentameric rings stacked upon each other to form a structure 90 × 110 Å (23), it is not known how the UPS mediates its glutamine-induced degradation. It is possible that a high concentration of glutamine can destabilize the GS oligomer and facilitate the degradation of individual monomers. To test this idea, we performed a series of gel filtration experiments with cell lysates prepared from glutamine-starved cells and glutamine-treated cells in the presence or absence of different chemical inhibitors. Fractions from each sample were collected and analyzed by immunoblotting. As shown in Fig. 1*A*, endogenous GS eluted primarily as a single peak corresponding to a homodecameric protein complex (~440 kDa) in glutamine-starved cells. Upon glutamine treatment, GS fractionated as a broader peak with "tails" of both higher and lower molecular weight (MW), both of which were suppressed by the addition of the NEDD8-activating enzyme inhibitor MLN4924 (Fig. 1 *B–D*) (47). Moreover, glutamine-induced spreading of the GS peak was also blocked by the GS inhibitor MSO, which inhibited glutamine-induced GS ubiquitylation and degradation (Fig. S1) (48). Interestingly, the p97 inhibitor CB-5083 also blocked glutamine-induced spreading of the GS peak (Fig. 1*D*) but did not block GS ubiquitylation (Fig. 1*E*). We show later that this effect of CB-5083 may be caused by the competitive displacement of GS by other substrates that accumulate upon inhibition of p97. Taken together, our data suggest that glutamine-triggered GS ubiquitylation may promote the recruitment of p97 to initiate the disassembly of the decamer, inducing a shift of a fraction of the GS pool to higher and lower MW, respectively.

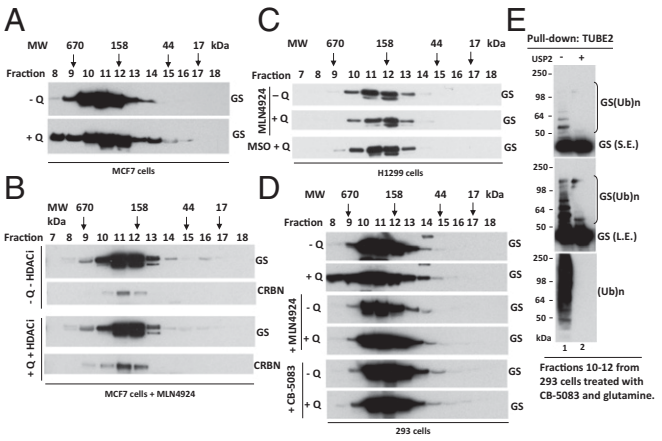

Fig. 1. Glutamine alters the apparent assembly state of GS in an ubiquitylation- and p97-dependent manner. (*A* and *B*) MCF7 cells were starved of glutamine for 24 h and then were pretreated with (*A*) or without (*B*) 2 μM pan cullin–RING ubiquitin ligase inhibitor MLN4924 for 30 min, followed by the addition of 4 mM glutamine plus the histone deacetylase (HDAC) inhibitors suberoylanilide hydroxamic acid (SAHA) (2 μM) and NAM (10 μM) (+Q +HDACi) or not (−Q −HDACi) for 2 h. Cell lysates were fractionated on a Superdex 200 gel filtration column. Individual fractions were concentrated by trichloroacetic acid (TCA) precipitation and analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. (*C*) As in *A* and *B*, except that H1299 cells were starved of glutamine for 24 h and then were pretreated with MLN4924 (2 μM) or the GS inhibitor MSO (2 mM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 2 h. (*D*) As in *A* and *B*, except that HEK293 cells were starved of glutamine for 24 h and then were pretreated with MLN4924 (2 μM) or the p97 inhibitor CB-5083 (10 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 2 h. (*E*) Fractions 10–12, prepared from HEK293 cells treated with CB-5083 and glutamine (used in *D*, bottom panel), were combined and subjected to pulldown with TUBE2 resin, followed by treatment with or without USP2. The bound fractions were analyzed by SDS/PAGE and immunoblotting with antibodies against GS and ubiquitin. L.E., long exposure; S.E., short exposure; (Ub)n, polyubiquitin.

p97 Interacts with Endogenous GS and Promotes Degradation of GS in a Glutamine-Dependent Manner. Based on the above observations, we hypothesized that p97 may be recruited to ubiquitylated GS to regulate its degradation. To investigate this hypothesis, we first performed immunoprecipitation (IP) experiments using an antibody specific to p97 and found that glutamine greatly enhanced the binding of endogenous, apparently unubiquitylated GS to endogenous p97 (Fig. 2*A*). This binding nevertheless was likely dependent on ubiquitylation, because it was greatly reduced by pretreatment of cells with the pan–Cullin-RING ligase (CRL) inhibitor MLN4924 (Fig. 2*A*). The role of ubiquitylation in GS binding to p97 is considered in more detail in the next section. GS also was recovered, albeit in reduced amounts, in p97 immunoprecipitates from cells treated with the p97 inhibitor CB-5083 following the activation of GS degradation (Fig. 2*A*). We suggest that this reduction was caused by competition from other p97 substrates that accumulated upon treatment with CB-5083; this notion is consistent with the observation that much greater amounts of UFD1•NPL4 and high-MW ubiquitin conjugates coimmunoprecipitated with p97 from cells treated with CB-5083. In support of this idea, the recovery of GS in p97 immunoprecipitates was essentially eliminated if a bolus of p97 substrates was accumulated by CB-5083 treatment before the activation of GS degradation with glutamine (Fig. S2*A*; note that the design of this experiment is similar to that of Fig. 1*D*).

The enhanced binding of GS to p97 in cells treated with glutamine suggests that p97 might modulate glutamine-induced degradation of GS. Consistent with this idea, inhibition of p97 by CB-5083 or NMS-873 blocked glutamine-induced GS degradation (Fig. 2 *B* and *C* and Fig. S2*B*). Glutamine-induced GS degradation also was inhibited upon depletion of p97 by shRNA knockdown (Fig. 2*D* and Fig. S2*C*).

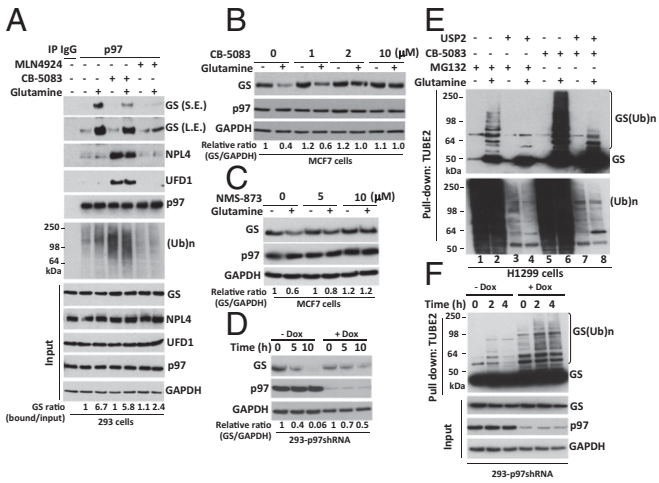

Fig. 2. p97 interacts with endogenous GS and promotes glutamine-induced degradation of GS. (*A*) HEK293 cells were starved of glutamine for 24 h, were pretreated (or not) with MLN4924 (2 μM) for 30 min, and then were treated (or not) with 4 mM glutamine for 2 h. For CB-5083 treatment, after the addition of 4 mM glutamine for 90 min, cells were treated with CB-5083 (10 μM) for 30 min. Protein extracts were immunoprecipitated with mouse IgG control or p97 antibodies, followed by Western blot analysis with the indicated antibodies. The ratio of GS bound to p97 normalized to input GS is shown. L.E., long exposure (the long exposure blot was quantified); S.E., short exposure; (Ub)n, polyubiquitin. (*B* and *C*) MCF7 cells were starved of glutamine for 24 h and were pretreated with the p97 inhibitors CB-5083 (*B*) or NMS-873 (*C*) for 30 min, followed by addition (or not) of 4 mM glutamine for 4 h. Cell extracts were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, p97, and GAPDH. The relative ratios of GS:GAPDH, normalized to lane 1, are shown. (*D*) HEK293 cells stably expressing doxycycline (Dox)-inducible shRNA targeting p97 were either mock-treated or induced with doxycycline (1 μg/mL) for 48 h and then were starved of glutamine for 24 h, followed by the addition of 4 mM glutamine for the indicated times. Cell lysates were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, p97, and GAPDH. The relative ratios of GS:GAPDH, normalized to lane 1, are shown. (*E*) H1299 cells were starved of glutamine for 24 h and then were pretreated with MG132 (10 μM) or CB-5083 (10 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 3 h. Cell lysates were fractionated on a TUBE2 resin, followed by treatment with or without USP2. The bound fractions and lysate samples were analyzed by SDS/PAGE and immunoblotting with antibodies against GS and ubiquitin. Input is shown in Fig. S3*B*. (*F*) HEK293 cells stably expressing doxycycline-inducible shRNA targeting p97 were mock-treated or were induced with doxycycline (1 μg/mL) for 48 h and then were starved of glutamine for 24 h, followed by the addition of 4 mM glutamine for the indicated times. Cell lysates were fractionated on a TUBE2 resin, and both lysate samples and the bound fractions were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, ubiquitin (shown in Fig. S3*C*), p97, and GAPDH.

Because glutamine promotes GS degradation by the UPS, we next sought to determine the effect of p97 inactivation on GS ubiquitylation. We treated glutamine-starved cells with the proteasome inhibitor MG132 or the p97 inhibitor CB-5083 in the presence or absence of 4 mM glutamine and then enriched ubiquitin conjugates on a tandem ubiquitin-binding entity (TUBE2) resin. Immunoblotting of the bound fraction and input (cell lysate) with antibodies against GS or ubiquitin revealed that inhibition of either p97 or the proteasome in glutamine-treated cells resulted in a significant increase in ubiquitin-conjugated GS forms, which were deconjugated with the deubiquitylating enzyme ubiquitin C-terminal hydrolase 2 (USP2) (Fig. 2*E* and Fig. S3*A*). A similar result was obtained upon shRNA knockdown of p97 (Fig. 2*F*). Consistent with the results obtained with p97 ATPase inhibitors, overexpression of the ATPase-deficient $p97^{E578Q}$ mutant (49) caused accumulation of polyubiquitylated GS upon glutamine activation, even though the mutant protein was able to bind GS, albeit with slightly reduced efficiency (Fig. S3 *D* and *E*).

It is worth noting that substantial amounts of apparently unubiquitylated GS coprecipitated with p97 (Fig. 2*A* and Fig. S2*A*). However, large amounts of unmodified GS were also pulled down along with ubiquitylated GS on TUBE2 resin (Fig. 2 *E* and *F* and Fig. S3 *A* and *D*). The association of apparently unmodified GS with TUBE2 resin was specific, in that it was strongly stimulated by glutamine (best seen in Fig. 2*E*). We suggest that p97 is recruited to GS decamers that contain a small number of ubiquitylated GS subunits and a much larger number of unmodified subunits, to extract the ubiquitylated GS subunits from the unmodified subunits.

Accumulation of Ubiquitylated GS upon p97 Inhibition Is Dependent on $CRL4^{CRBN}$ Activity. We recently reported that $CRL4^{CRBN}$ directly mediates the glutamine-induced ubiquitylation of GS (24). Next, we tested whether $CRL4^{CRBN}$ and p97 function on the same pathway to regulate glutamine-induced GS degradation. We reasoned that, if p97 functions downstream of $CRL4^{CRBN}$, the exceptionally strong accumulation of ubiquitylated GS induced by p97 inhibitors should be blunted in cells in which $CRL4^{CRBN}$ is inhibited. To address this issue, MCF7 cells were cotreated with the p97 inhibitors CB-5083 or NMS-873 with or without the pan-CRL inhibitor MLN4924 and then were activated with 4 mM glutamine for 3 h. TUBE2 pulldown experiments showed that the accumulation of polyubiquitylated GS caused by CB-5083 or NMS-873 was completely abolished in MLN4924-treated cells (Fig. 3*A*). In keeping with the point made in the preceding paragraph, loss of ubiquitylated GS caused a parallel reduction in the recovery of apparently unmodified GS on TUBE2 resin. A similar epistatic effect of MLN4924 was observed in cells depleted of p97 by shRNA knockdown (Fig. 3*B*). The effect of MLN4924 was caused specifically by the inhibition of $CRL4^{CRBN}$, because accumulation of ubiquitylated GS in response to NMS873 was blunted

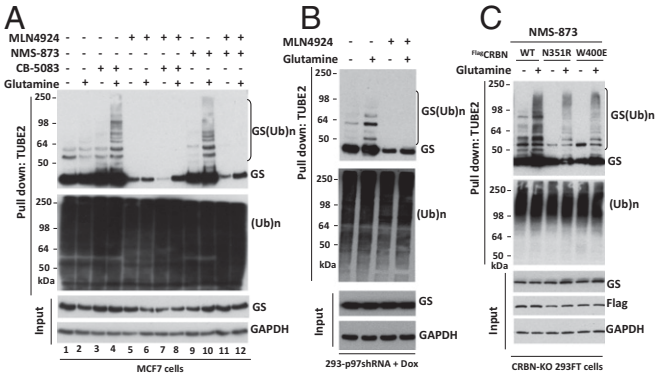

Fig. 3. Inhibition of p97 promotes the accumulation of ubiquitylated GS in a $CRL4^{CRBN}$-dependent manner. (*A*) MCF7 cells were starved of glutamine for 24 h and then were pretreated (or not) with CB-5083 (10 μM), NMS-873 (10 μM), or MLN4924 (2 μM) for 30 min as indicated in the figure, followed by the addition (or not) of 4 mM glutamine for 3 h. Cell lysates were fractionated on a TUBE2 resin, and both lysate samples and the bound fractions were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, ubiquitin, and GAPDH. (Ub)n, polyubiquitin. (*B*) HEK293 cells stably expressing doxycycline (Dox)-inducible shRNA targeting p97 were induced with doxycycline (1 μg/mL) for 48 h and then were starved of glutamine for 24 h. Cells were pretreated (or not) with MLN4924 (2 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 2 h. Cell lysates were fractionated on a TUBE2 resin, and both lysate samples and the bound fractions were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, ubiquitin, and GAPDH. (*C*) CRBN-KO 293FT cells stably expressing WT ^{Flag}CRBN or the indicated mutants were starved of glutamine for 24 h. Cells were pretreated with NMS-873 (10 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 2 h. Cell lysates were fractionated on a TUBE2 resin, and both lysate samples and the bound fractions were analyzed by SDS/PAGE and immunoblotting with antibodies against GS, ubiquitin, Flag, and GAPDH.

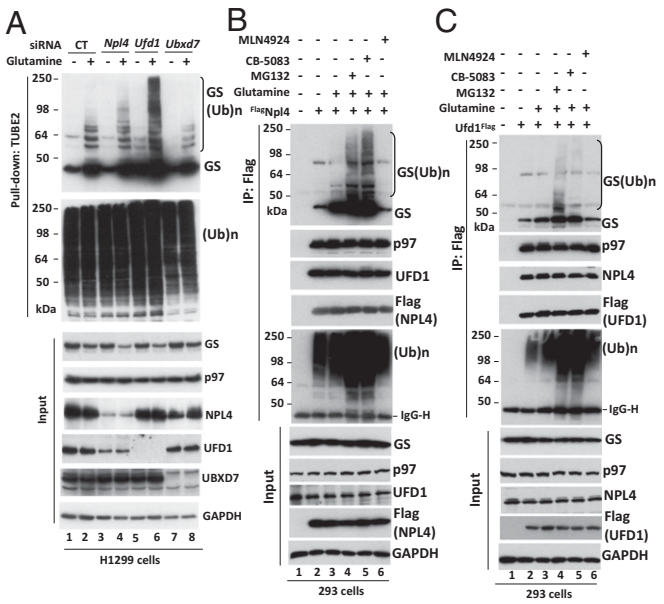

Fig. 4. The p97 adaptor complex UFD1•NPL4 interacts with ubiquitylated GS upon the addition of glutamine. (*A*) H1299 cells were transfected with control (CT) siRNA or with siRNAs that target NPL4, UFD1, or UBXD7. Forty-eight hours after transfection, cells were starved of glutamine for 24 h, followed by the addition (or not) of 4 mM glutamine for 2 h. Total ubiquitinated proteins were affinity-purified from cell lysates using TUBE2-agarose. Bound fractions and cell lysates (input) were analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. (Ub)n, polyubiquitin. (*B* and *C*) HEK293 cells were transiently transfected with empty vector or with plasmids encoding ^{Flag}NPL4 (*B*) or UFD1^{Flag} (*C*). After 24-h transfection, cells were starved of glutamine for 24 h and were pretreated (or not) with MG132 (20 μM), CB-5083 (10 μM), or MLN4924 (2 μM) for 30 min, followed by the addition (or not) of 4 mM glutamine for 2 h. Cell lysates were immunoprecipitated with anti-Flag resin. Precipitated and input fractions were analyzed by SDS/PAGE and immunoblotting with the indicated antibodies.

in CRBN-knockout 293FT cells expressing substrate binding-defective N351R or W400E mutants of CRBN (Fig. 3*C*) (24).

The p97 Adaptor Complex UFD1•NPL4 Interacts with Ubiquitylated GS upon Glutamine Activation. One of the central roles of p97 is to bind and extract ubiquitylated proteins from stable protein complexes, membranes, or chromatin (32). This process typically involves an adaptor protein, which may link ubiquitylated substrates to p97. To test whether a p97 adaptor participates in glutamine-induced GS ubiquitylation and degradation, we used siRNAs to knock down UBX domain-containing protein 7 (UBXD7), a direct binding partner of cullins 2 (CUL2) and 4 (CUL4) (50, 51), and the heterodimer ubiquitin fusion degradation protein (UFD1)•nuclear protein localization protein 4 (NPL4), which is well known to target ubiquitylated substrates for p97-dependent proteasomal degradation (52, 53). Depletion of UFD1, but not UBXD7, caused ubiquitylated GS to accumulate upon glutamine treatment (Fig. 4*A*), suggesting that UFD1•NPL4 participates in glutamine-induced GS degradation. Consistent with observations in the TUBE2 pulldowns, glutamine stimulated the interaction between UFD1•NPL4 and both ubiquitylated and apparently unmodified GS (Fig. 4 *B* and *C*). Inhibition of proteasome by MG132 or p97 by CB-5083 significantly enhanced this interaction, which was abolished by blocking GS ubiquitylation with MLN-4924 (Fig. 4 *B* and *C*). Despite these interactions, depletion of either UFD1 or NPL4 did not block glutamine-induced degradation of GS (Fig. 4*A*). Either UFD1•NPL4 plays a facilitating role that is not essential for GS degradation, or other adaptors can compensate for UFD1•NPL4 when it is absent.

p97 Mediates the Disassembly of GS upon Inhibition of the Proteasome. Our observation that p97 binds ubiquitylated GS and promotes its proteasome-dependent degradation suggests that p97 might mediate the disassembly of ubiquitylated GS subunits from a homodecamer, thereby enabling their degradation by the proteasome. This notion is consistent with the observations reported in Fig. 1 that the appearance of a low-MW pool of GS upon gel filtration of cell lysate was dependent upon glutamine, NEDD8 conjugation, and p97 activity. We next sought to explore more fully whether p97 mediates glutamine-induced disassembly of GS by treating MCF7 cells with glutamine in the presence of proteasome and/or p97 inhibitors. Gel filtration experiments indicated that inhibition of the proteasome accentuated the glutamine-induced spreading of the GS peak into higher- and lower-MW complexes and that this spreading was completely blocked by inhibition of p97 with CB-5083, NMS873, or shRNA knockdown (Fig. 5*A* and Fig. S4). The spreading of the GS peak to lower MW in cells treated with glutamine plus MG132 was likely caused by the disassembly of the decamer into smaller oligomers; we suggest that the spreading to higher MW was caused, at least in part, by the binding of GS to p97, because both proteins coeluted in high-MW fractions, GS bound p97 and UFD1•NPL4 (Figs. 2*A* and 4 *B* and *C*), and the high-MW complex was absent when p97 function was inhibited with small molecules or shRNA knockdown. As a more robust test of this proposal, we treated glutamine-starved cells with or without glutamine and fractionated the cell lysates by gel filtration (Fig. 5*B*). Fractions from across the peak of the glutamine-treated sample were subjected to IP with

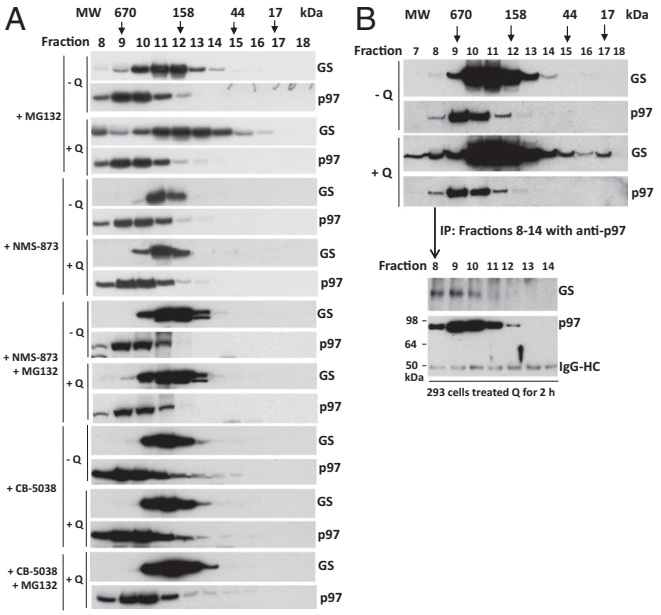

Fig. 5. p97 mediates the disassembly of GS upon inhibition of the proteasome. (*A*) MCF7 cells were grown to 80–90% confluence in 15-cm plates and were starved of glutamine for 24 h. Cells were pretreated (or not) with MG132 (10 μM), NMS-873 (10 μM), or CB-5083 (10 μM) for 30 min and then were treated with 4 mM glutamine (+Q) or not (−Q) for 6 h. Cell lysates were fractionated on a Superdex 200 gel filtration column. Individual fractions were concentrated by TCA precipitation and analyzed by SDS/PAGE and immunoblotting with the indicated antibodies. (*B*) HEK293 cells were starved of glutamine for 24 h and then were treated with 4 mM glutamine (+Q) or not (−Q) for 2 h. Cell lysates were fractionated on a Superdex 200 gel filtration column. (*Upper*) Individual fractions were concentrated by TCA precipitation and analyzed by SDS/PAGE and immunoblotting with antibodies against GS and p97. (*Lower*) Fractions 8–14, prepared from HEK293 cells treated with 4 mM glutamine, were immunoprecipitated with p97 antibody, followed by Western blot analysis with the indicated antibodies. A band at ~50 kDa represents IgG heavy chains (IgG-HC).

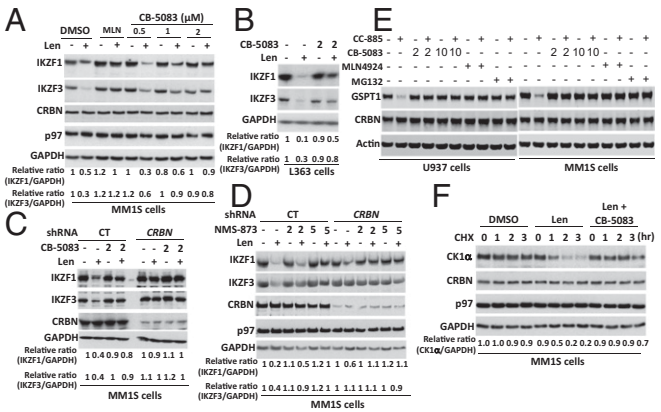

Fig. 6. p97 is required for immunomodulatory drug-induced degradation of CRBN neosubstrates. (*A*) MM1S cells were pretreated with the indicated doses of CB-5083 or 1 μM MLN4924 (MLN) for 30 min, followed by the addition of lenalidomide (Len) (10 μM) for 4 h. Cell lysates were fractionated by SDS/PAGE and immunoblotted for the indicated endogenous proteins. The relative ratios of IKZF1:GAPDH or IKZF3, normalized to lane 1, are shown here and in *B*–*D*. (*B*) L363 cells were pretreated with CB-5083 (2 μM) for 30 min, followed by the addition (or not) of lenalidomide (10 μM) for 4 h. Cell lysates were fractionated by SDS/PAGE and immunoblotted for the indicated endogenous proteins. (*C*) MM1S cells stably expressing control (CT) or CRBN shRNAs were pretreated with CB-5083 (2 μM) for 30 min, followed by the addition (or not) of lenalidomide (10 μM) for 5 h. Cell lysates were analyzed by immunoblotting with the indicated antibodies. (*D*) As in *C*, except that cells were pretreated (or not) with NMS-873 (2 or 5 μM) for 30 min. (*E*) U937 and MM1S cells were pretreated (or not) with CB-5083 (2 or 10 μM), MLN4924 (1 μM), or MG132 (10 μM) for 1 h, followed by treatment with 10 nM CC-885 for an additional 2 h. Whole-cell extracts were subjected to immunoblot analysis. (*F*) Cells were pretreated with CB-5083 (2 μM) and/or lenalidomide (10 μM) for 30 min followed by the addition of cyclohexamide (CHX) (100 μg/mL). At the indicated times after the addition of cyclohexamide, samples were harvested for immunoblot analysis. The relative ratios of CK1α:GAPDH, normalized to lane 1, are shown.

anti-p97 followed by immunoblotting for GS. A clear association of GS with p97 was observed in fractions that correspond to the high-MW tail of the GS peak.

Immunomodulatory Drug-Induced Degradation of CRBN Neosubstrates Requires p97. To investigate a potential role for p97 in regulating the degradation of other $CRL4^{CRBN}$ substrates, we extended our study to immunomodulatory drug-induced degradation of $CRL4^{CRBN}$ neosubstrates (28, 29, 54). CB-5083 blocked the lenalidomide-induced degradation of the lymphoid transcription factors Ikaros family zinc finger proteins 1 (KZF1) and 3 (IKZF3) in MM.1S and L363 multiple myeloma cells (Fig. 6 *A*–*C*). A similar blockade of the lenalidomide effect was observed in MM.1S (Fig. 6*D*) and U266 (Fig. S5*A*) cells treated with NMS873. Control experiments verified that the effect of lenalidomide on IKZF1 and IKZF3 was CRBN-dependent (Fig. 6 *C* and *D*). Inhibition of p97 with CB-5083 or proteasome with MG132 caused the accumulation of ubiquitylated IKZF1 regardless of whether lenalidomide was added (Fig. S5*B*), suggesting that p97 was required for both its constitutive and lenalidomide-induced degradation. To ask if p97 inhibition also affects the degradation of other CRBN neosubstrates induced by the CRBN modulator CC-885, we examined the effect of CB5083 on CC-885–induced degradation of the translation termination factor GSPT1 (30). Similar to MLN4924 and MG132, pretreatment with CB5083 completely blocked the CC-885–induced degradation of GSPT1 in U937 AML cells and MM1S myeloma cells (Fig. 6*E*). Finally, to confirm that CB5083 was indeed blocking neosubstrate proteolysis, we performed a cycloheximide chase experiment to monitor lenalidomide-induced degradation of casein kinase 1α (CK1α) (31). As shown in Fig. 6*F*, lenalidomide induced rapid loss of CK1α, but this effect was completely blocked upon inhibition of p97 with CB5083.

Discussion

In this study, we uncover a role for p97 in regulating GS. As illustrated in Fig. 7, glutamine induces $CRL4^{CRBN}$-dependent ubiquitylation of GS subunits. Subsequently, p97•UFD1•NPL4 binds to ubiquitylated GS and promotes the disassembly of the homodecamer, thereby enabling degradation of the ubiquitylated subunits by the proteasome. Currently, we do not know how acetylation, ubiquitylation, and extraction of individual subunits are coordinated. Pulldown experiments with a ubiquitin-binding resin reveal that large amounts of unmodified GS are retrieved in a manner that depends on factors that promote GS ubiquitylation. This finding suggests that one or a small number of subunits are ubiquitylated per homodecamer. Once these subunits are extracted and degraded by the proteasome, the fate of the remaining unmodified subunits is unclear, but we anticipate that they reassemble to form a smaller number of homodecamers. A number of questions about the mechanism and regulation of GS degradation remain to be addressed, including the means by which glutamine levels are monitored, the mechanism by which p97 extracts ubiquitylated subunits from GS decamers, and the role of adaptor proteins in this process. Interestingly, bioinformatic analysis of cell lines from the Cancer Cell Line Encyclopedia (CCLE) (55) revealed significant covariance between the expression of mRNAs encoding *CRBN* and multiple p97 adaptors (Fig. S6), suggesting diverse functional connections between these pathways.

In the brain, GS is highly expressed in astrocytes and plays a critical role in regulating the glutamine–glutamate cycle between astrocytes and neurons (56). In astrocytes, GS combines glutamate and ammonia to form glutamine, which then is transferred to neurons and converted to glutamate by mitochondrial glutaminase (57). Glutamate is sequestered in synaptic vesicles and then is released as a neurotransmitter into the synaptic cleft, after which it is taken up by astrocytes to complete the cycle. The excessive accumulation of glutamate can induce excitotoxicity or neuronal cell death (58). Notably, it was reported that specific depletion of GS in murine astrocytes in the brain leads to death at postnatal day 3 (59). In addition, deregulated GS activity and/or increased glutamate levels in the cerebrospinal fluid have been implicated in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and ALS (60–63). Our findings that p97 directly controls GS degradation have potential implications for human diseases, in particular ALS, which is characterized by the progressive degeneration of motor neurons. Riluzole, an inhibitor of glutamate release, is the only effective drug for ALS, and it prolongs survival by 3–6 mo (64). Administration of the GS inhibitor MSO to $SOD1^{G93A}$ transgenic mice, which develop a phenotype similar to ALS in humans, decreases both glutamine and glutamate levels in the brain and significantly extends the lifespan of these mice (65, 66). Therefore, it will be of

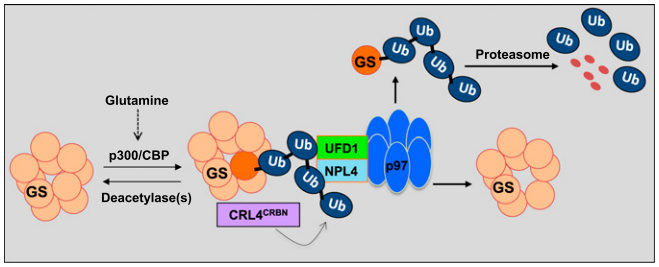

Fig. 7. Proposed model for the role of p97 in mediating regulated degradation of $CRL4^{CRBN}$ substrates, including GS and lenalidomide-induced neosubstrates. For the sake of simplicity, one subunit of GS homodecamer is ubiquitylated by $CRL4^{CRBN}$, segregated by the p97–UFD1–NPL4 complex, and subsequently degraded by the proteasome upon the addition of glutamine. Ub, ubiquitin.

particular interest in future studies to investigate whether possible defects in the degradation of GS and other p97 substrates caused by p97 mutations identified in ALS patients may contribute to pathogenesis or serve as early diagnostic markers of the disease.

Recent work has shown that immunomodulatory drugs trigger CRBN-dependent ubiquitylation of IKZF1, IKZF3, CK1α, and GSPT1, which is followed by their degradation (28–31, 54). Remarkably, all these immunomodulatory drug-induced degradations are dependent on p97. Thus, all five CRBN substrates/neosubstrates examined in this work exhibit striking dependence on p97. We know of no other ubiquitylation pathway, other than ERAD, in which the substrates uniformly exhibit dependence on p97 for their degradation. This unique feature of $CRL4^{CRBN}$ may be a coincidental consequence of the oligomerization status of its known substrates or may point to an obligate functional relationship between these proteins. Further studies are required to illuminate the role of p97 in regulating immunomodulatory drug-induced degradation of these CRBN neosubstrates. GSPT1, in a binary complex with eukaryotic peptide chain release factor subunit 1 (eRF1), functions as a polypeptide chain release factor (67), whereas IKZF1/3 proteins are known to recruit the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex to specific genomic targets (68, 69). By analogy to other degradation pathways in which p97 plays a critical role (70, 71), it is possible that p97 mediates the extraction of ubiquitylated GSPT1 from eRF1 and ubiquitylated IKZF1/3 proteins from chromatin before their degradation by the proteasome.

Our observations point to an unexpected role for p97 in the mechanism of action of immunomodulatory drugs, and this role could have important implications for patient responsiveness to immunomodulatory drug therapy. CRBN expression levels do not correlate with responsiveness to immunomodulatory drugs across MM cell lines (72). Among six cell lines that are resistant to immunomodulatory drugs, only two, RPMI-8226 and KMS11, express low levels of CRBN protein. In striking contrast, the other four intrinsically resistant cell lines (KMS34, LP-1, KMS12BM, and JJN-3) have higher basal levels of CRBN than the immunomodulatory drug-sensitive MM cell lines (72), suggesting that other factors, possibly including p97 and its adaptors, play a critical role in regulating the CRBN-dependent anti-myeloma activity of immunomodulatory drugs.

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