Cullin 2-RBX1 E3 ligase and USP2 regulate antithrombin ubiquitination and stability

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Abstract

Hemophilia A and B are congenital bleeding disorders caused by a deficiency in pro-coagulant factor VIII or IX that is treated by downregulation of antithrombin. However, the molecular mechanisms that regulate antithrombin expression remain poorly understood. Here, we identified Cullin 2 and USP2 (ubiquitin-specific peptidase-2) as novel regulators of antithrombin expression that act by modulating antithrombin ubiquitination. Inhibition of the proteasome caused accumulation of antithrombin and its ubiquitinated forms in HepG2 and SMMC7721 cells. Notably, inhibition of neddylation with MLN4924 suppressed both ubiquitination and degradation of antithrombin, which is recapitulated by silencing of the neddylation enzymes, NAE1, UBA3, and UBE2M, with small interfering RNA (siRNA). We identified Cullin 2 as the interaction partner of antithrombin, and siRNA-mediated Cullin 2 knockdown reduced antithrombin ubiquitination and increased antithrombin protein. We further found that USP2 interacted with antithrombin and regulated antithrombin expression, showing that overexpression of USP2 inhibits the ubiquitination and proteasomal clearance of antithrombin, whereas pharmacological inhibition or siRNA-mediated knockdown of USP2 downregulates antithrombin. Collectively, these results suggest that Cullin 2 E3 ubiquitin ligase and USP2 coordinately regulate antithrombin ubiquitination and degradation. Thus, targeting Cullin 2 and USP2 could be a potential strategy for treatment of hemophilia.
Keywords
coagulation; deubiquitinase; neddylation; ubiquitin

1 | INTRODUCTION

Homeostasis of the coagulation system reflects a balance between bleeding and thrombosis, with pro-coagulant factors promoting thrombosis and anti-coagulant factors inducing bleeding. Thrombotic pathways are divided into intrinsic and extrinsic pathways. The intrinsic pathway sequentially transforms the zymogens, factor FXIII (FXIII), FXI, FIX, and FVIII, into their activated forms, whereas the extrinsic thrombosis pathway sequentially activates FIII and FVII. The two coagulation pathways share the common final pathway involving FX, FV, and FII (ie, thrombin), ultimately promoting clotting. However, anti-coagulant factors, such as antithrombin and alpha-1 antitrypsin, oppose thrombosis. Antithrombin, a serine protease inhibitor, functions by binding and inactivating thrombin, an effect that is enhanced by heparin; thus, antithrombin reverses the clot formation orchestrated by thrombin. Hemophilia A and B are coagulation system diseases that manifest as congenital bleeding owing to a deficiency in pro-coagulant factor VIII or IX. The resulting imbalance between low pro-coagulant factor VIII or IX levels and normal anti-coagulant factor antithrombin levels is responsible for bleeding in hemophilia A and B. The current standard of care is replacement therapy with intravenous infusion of concentrated factor VIII and IX, but an alternative strategy for addressing insufficient thrombin generation in hemophilia patients would be to downregulate antithrombin. Indeed, several studies have suggested that suppression of antithrombin expression using an RNA interference (RNAi) approach could ameliorate hemophilia A and B. Antithrombin mRNA is highly abundant in liver and gallbladder tissues, but is rarely detected in other tissues. Antithrombin is primarily synthesized in hepatocytes and endothelial cells. Given that antithrombin functions as an enzyme to maintain hemostasis in hemophilia, directly targeting its degradation in hepatocytes could potentially be a promising option for reducing antithrombin levels in the circulation. Pharmacological inhibition of the proteasome using bortezomib has been shown to increase antithrombin protein in human hepatoma HepG2 cells and in mice. However, molecular mechanisms that regulate the stability of antithrombin remain poorly understood in any cell type.

The ubiquitin-proteasome system (UPS) is the major molecular machinery responsible for the degradation of most intracellular proteins. UPS-mediated proteolysis requires the labeling of protein substrates with a chain of ubiquitin, a process catalyzed by a trio of enzymes: ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). The largest family of ubiquitin ligases is Cullin-RING ubiquitin ligases (CRL). The Cullin protein family consists of eight members, Cullin 1, 2, 3, 4A, 4B, 5, 7, and 9. In a given CRL, one Cullin family protein serves as a scaffold that tethers a substrate-recognizing receptor (often through an adaptor protein) to the RING finger component (RBX1 or RBX2). The assembly and activation of CRLs is triggered by the attachment of NEDD8, a ubiquitin-like protein, to individual Cullins. Neddylation of Cullins, as well as other protein substrates, is mediated by NEDD8-specific E1 activating
enzyme (NAE, a heterodimer of NAE1 and UBA3), E2 conjugating enzyme (UBE2M or UBE2F), and NEDD8 E3 ligases. MLN4924 is a small molecule compound that acts as a potent and specific inhibitor of the NEDD8-activating enzyme. By doing so, MLN4924 blocks Cullin neddylation and inactivates CRL, resulting in the accumulation of CRL substrates. Among Cullin proteins, Cullin 2 forms a functional E3 ligase complex with elongin B/C, RBX1, as well as the substrate recognition subunit to promote substrate ubiquitination and degradation. To date, there have been no reports on whether CRL, in particular, which specific Cullin, participates in the regulation of antithrombin levels. CRL-mediated ubiquitination and proteolysis can be antagonized by deubiquitinases (DUBs), which reverse ubiquitination and prevent the degradation of ubiquitinated proteins by shortening and removing ubiquitin chains from substrate proteins. USP2, a USP family DUB, is expressed as two isoforms, USP2a and USP2b, which share the same C-terminal catalytic domain but possesses distinct N-termini. Both USP2a and USP2b are expressed in liver cells. USP2a participates in cell-cycle progression, circadian rhythm, and epidermal growth factor receptor (EGFR) recycling, whereas USP2b is involved in host defense.

In the present study, we identified Cullin 2 as an E3 ubiquitin ligase that ubiquitinates antithrombin and promotes its proteasomal degradation, a process that is antagonized by USP2 through deubiquitination and stabilization of antithrombin. Our findings provide mechanistic insights that should aid in the development of new measures to downregulate antithrombin and treat hemophilia.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Anti-USP2 (ab168945), anti-NAE1 (ab187142), anti-UBE2M (ab109507), anti-UBA3 (ab124728), anti-Cullin 1 (ab75817), anti-Cullin 2 (ab166917), anti-Cullin 3 (ab75851), anti-Cullin 4A (ab67035), anti-Cullin 4B (ab67035), anti-Cullin 5 (ab184177), anti-α1 antitrypsin (ab207303), anti-RBX1 (ab133565), and anti-RBX2 (ab181986) were obtained from Abcam (Cambridge, UK). Anti-antithrombin was purchased from Abcam (ab126598) and Novus biologicals (AF1267). Mouse IgG (magnetic bead conjugate) (5873), rabbit (DA1E) mAb IgG XP isotype control (magnetic bead conjugate) (8726), anti-HA (3724), anti-FLAG (14793), anti-USP2 (8036), and anti-K48-linkage–specific polyubiquitin (8081) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-GFP (AP0675M and BS6507) and anti-GAPDH (AP0063) were obtained from Bioworld Technology (Louis Park, MN, USA). Anti-ubiquitin (sc-8017) and horseradish peroxidase (HRP)-conjugated secondary antibodies (sc-2054 and sc-2055) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ML364 (HY-100900), LDN-57444 (HY-18637), SJB-043 (HY-15757), P5091 (HY-15667), DUBs-IN-2 (HY-50737A), PR619 (HY-13814), MLN4924 (HY-70062), bortezomib (HY-10227), and b-AP15 (HY-13989) were purchased from MedChemExpress (Monmouth Junction, NJ, USA).
2.2 | Cell culture

L02, HepG2, SMMC7721, and BEL7402 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The 293FT cell line was obtained from the Cell Resource Center of the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences. L02, HepG2, SMMC7721 and BEL7402 cells were grown in RPMI-1640 medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Bioworld Technology). 293FT cells were cultured in DMEM with high glucose supplemented with 10% FBS.

2.3 | RNA interference

HepG2, SMMC7721, or 293FT cells were seeded in 60 mm dishes for 24 hours, then transfected with siRNA oligonucleotides using Lipofectamine 2000 reagent (Life Technologies, Invitrogen, CA, USA) in RPMI opti-MEM medium, according to the manufacturer’s instructions. Medium was replaced with fresh medium, as appropriate, after transfection for 6 hours and cells were cultured for 48 or 72 hours for further analysis. siRNAs against USP2 (sc-76821), UBA3 (sc-76783), NAE1 (sc-72523), and UBE2M (sc-76786) were purchased from Santa Cruz Biotechnology. Control siRNA (siN0000002–1-5) and Cullin 2 siRNA (stQ0003881–1) were purchased from Ribobio (Guangzhou, Guangdong, China).

2.4 | Plasmids and transfection of cells

GFP-antithrombin and FLAG-antithrombin expression plasmids encode antithrombin as a fusion protein with GFP and FLAG, respectively. The expression plasmids for HA-USP2 wild type (WT) and HA-USP2 catalytic mutant encoded fusion proteins of HA with USP2 WT or C276A mutant, respectively; the USP2 sequence encoded in these plasmids is the USP2a transcript variant (GENE ID: 9099, NM_004205.5) from NCBI (https://www.ncbi.nlm.nih.gov/gene/?term=). FLAG-antithrombin, HA-USP2 WT, and HA-USP2 C276A plasmids were generated in the pcDNA3.1 (+) vector, whereas the GFP-antithrombin plasmid was generated in the pEGFP-N1 vector. All plasmids were purchased from VigeneBio (Shandong, China). Exponentially growing HepG2, SMMC7721, and 293FT cells were transfected with plasmids mixed with Lipofectamine 2000 reagent. Cells were further incubated for 48 hours for subsequent analysis.

2.5 | Western blotting and immunoprecipitation

Immunoblotting was performed as previously described. Briefly, proteins in cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Bedford, MA, USA). The membranes were blocked by incubating with a Tris/saline/0.1% Tween-20 solution (TBS-T) containing 5% nonfat dry milk for 1 hour at room temperature and incubated with a primary antibody overnight at 4°C. Membranes were incubated with species-appropriate HRP-conjugated secondary antibodies for 1 hour and visualized using an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology), with detection by exposure to X-ray film (Kodak, Rochester, NY, USA) or Imagelab system (Bio-rad). Immunoprecipitation assays were performed as previously described. Protein interactions
and ubiquitination modifications were analyzed by incubating anti-antithrombin, anti-GFP, or anti-FLAG antibody with a Dynabeads Antibody Coupling Kit (Life Technologies, Invitrogen, CA) according to the manufacturer’s instructions, after which cell lysates were incubated with beads for 3 h at room temperature. Precipitated proteins were washed three times with PBS containing 0.1% Tween-20 (PBS-T), eluted using Blue Loading Buffer Pack (Cell Signaling Technology), resolved by SDS-PAGE, and immunoblotted.

2.6 | RNA isolation and real-time quantitative PCR

Cells were harvested after treatments and RNA was isolated using TRIzol (Takara). cDNA for quantitative polymerase chain reaction (qPCR) assays was reverse transcribed from mRNA using a reverse transcription kit (AG11711) according to supplier’s protocols (Accurate Biotechnology Co.). All qPCR assays were conducted on a StepOnePlus device (Applied Biosystem) using a SYBR qPCR kit (AG11701, Accurate Biotechnology Co.) and the following primer pairs: antithrombin, 5′-GCTAAACCCCAACAGGGTGAG-3′ (forward) and 5′-ACAAGGGTTGGCTACTCTGC-3′ (reverse); USP2, 5′-GGGCTCCATAACGAGGTGAAC-3′ (forward) and 5′-CTCCACATCTGTCGGCCTTTC-3′ (reverse); and 18S RNA, 5′-AAACGGCTACCACATCCAAG-3′ (forward) and 5′-CCTCCAATGGATCCTCGTTA-3′ (reverse).

2.7 | Detection of antithrombin secretion

Cells were seeded in 6-well plates and treated with different inhibitors in 1 mL serum-free RPMI 1640 medium for 24 hours. Cell medium was first centrifuged at 12 000 g for 10 minutes to remove cell debris. The supernatant was then mixed with four volumes of pre-cooled acetone (−20°C) and kept in −20°C for 20 mins. Secreted proteins were spin down by centrifugation at 15 000 g for 15 minutes at 4°C and subjected to western blot to detect secreted antithrombin.

2.8 | Statistics analysis

All experiments were repeated for at least three times unless specified. All data are presented as means ± standard error of the mean (SEM) of at least three independent assays. Significant differences between two groups were assessed using unpaired t tests. P-values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Antithrombin is degraded by the ubiquitin-proteasome system

Despite a previous report on the subject, it remains unclear whether antithrombin is degraded by the proteasome. Since antithrombin is primarily expressed in the liver before being secreted into blood, where it exerts its anti-coagulation action, we chose the immortalized liver cell line L02 and liver cancer cell lines HepG2, SMMC7721, and BEL7402 to explore the regulatory mechanisms of antithrombin degradation. To this end, we first measured basal antithrombin mRNA levels in L02, HepG2, SMMC7721, and BEL7402 cells. Interestingly, antithrombin mRNA in the immortalized liver cell line, L02, was much higher than that in liver cancer cell lines (Figure 1A). These results are consistent with
the UALCAN cancer database in which antithrombin mRNA levels in liver cancer patients were lower than those in normal controls. Inhibition of the proteasome by treatment with the 20S proteasome inhibitor bortezomib led to a sharp accumulation of antithrombin protein without increasing antithrombin mRNA levels, in L02, HepG2, SMMC7721, and BEL7402 cells (Figures 1B-D and S1A). Bortezomib also induced α1 antitrypsin, another member of the serine protease inhibitor (SERPIN) family, in HepG2 cells (Figure S2A). Interestingly, bortezomib promoted the secretion of antithrombin (Figure S3A,B). Consistent with this, treatment with the 19S proteasome inhibitor b-AP15 also induced a concentration- and time-dependent increase in the accumulation of antithrombin protein in HepG2 and SMMC7721 cells, an effect that was partly attributable to increased mRNA in HepG2 cells (Figures 1E,F and S1B). Similar to bortezomib, b-AP15 also induced the accumulation of α1 antitrypsin in HepG2 cells (Figure S2B) and promoted antithrombin secretion (Figure S3C,D). In the presence of the protein translation inhibitor cycloheximide (CHX), bortezomib markedly extended the half-life of antithrombin in HepG2 and SMMC7721 cells (Figure 1G). Furthermore, immunoprecipitation revealed a remarkable increase in total polyubiquitinated and K48-polyubiquitinated forms of antithrombin in bortezomib-treated HepG2 and SMMC7721 cells (Figure 1H). Collectively, these data suggest that antithrombin is degraded by the ubiquitin-proteasome system.

3.2 | CRL regulates antithrombin ubiquitination

We next sought to determine which E3 mediates the ubiquitination of antithrombin. Given that CRLs are responsible for the degradation of almost 20% of proteins in eukaryotic cells,17 we tested the involvement of CRLs in the degradation of antithrombin by treating HepG2 and SMMC7721 cells with the neddylation inhibitor MLN4924. Indeed, MLN4924 significantly increased antithrombin protein without elevating antithrombin mRNA levels (Figures 2A,B and S1C). MLN4924 administration also prolonged the half-life of antithrombin protein in HepG2 and SMMC7721 cells, as demonstrated by CHX-based pulse-chase assays (Figure 2C), and immunoprecipitation assays revealed markedly suppressed polyubiquitination of antithrombin in these cells (Figure 2D). In addition, MLN4924 upregulated α1 antitrypsin protein in SMMC7721 cells (Figure S2C) and promoted the secretion of antithrombin (Figure S3E,F). To confirm that the effect of MLN4924 on antithrombin expression is dependent on neddylation, we knocked down the neddylation E1s, UBA3, and NAE1, and the E2, UBE2M, in HepG2 and SMMC7721 cells using siRNAs specific for these enzymes. As shown in Figure 2E-G, we confirmed the effectiveness of these siRNAs in downregulating their respective targets, and found that silencing of individual neddylation enzymes resulted in significant accumulation of antithrombin. Together, these findings support the conclusion that CRL regulates the ubiquitination and degradation of antithrombin.

3.3 | Cullin 2 regulates the ubiquitination and degradation of antithrombin

There are eight members of Cullin protein family. To pin-point which Cullin regulates the degradation of antithrombin, we immunoprecipitated endogenous antithrombin in HepG2 cells and immunoblotted for individual Cullins. Our results showed that Cullin 2 interacted with antithrombin (Figure 3A). We also detected interactions of antithrombin with RBX1, a RING finger protein in Cullin 2 ubiquitin ligase, but not RBX2, which is capable of binding
Reciprocal experiments confirmed these results, showing that immunoprecipitation of endogenous Cullin 2 also pulled down antithrombin in HepG2 and SMMC7721 cells (Figure 3C). The interaction between Cullin 2 and antithrombin appears to be functionally significant, because siRNA-mediated Cullin 2 knockdown effectively abolished antithrombin ubiquitination and increased antithrombin protein in both HepG2 and SMMC7721 cells (Figure 3D,E). Based on these findings, we conclude that Cullin 2 ubiquitin ligase mediates the ubiquitination of antithrombin.

### 3.4 Involvement of DUBs in regulation of antithrombin expression

We speculated that ubiquitination of antithrombin might be regulated by DUBs. Accordingly, we tested the effects of several DUB inhibitors on antithrombin expression in HepG2 cells. Specifically, cells were treated with different concentrations of the USP1/UAF inhibitor SJB2–043, the pan-deubiquitinase inhibitor PR-619, the USP7 inhibitor P5091, the USP8 inhibitor DUBs-IN-2, or the UCHL1 and UCHL3 inhibitor LDN-57444. None of these treatments altered the expression of antithrombin protein (Figure 4A,B). Interestingly, the USP2-specific inhibitor ML364 was effective in reducing antithrombin protein in HepG2 cells at higher doses (2.5–10 μM) (Figure 4A-C), and did so without decreasing antithrombin mRNA levels in both HepG2 and SMMC7721 cells (Figure S1D). Antithrombin secretion was also decreased after ML364 treatment (Figure S3G,H). Moreover, ML364 treatment at a lower dose (1 μM) shortened the half-lives of antithrombin in HepG2 and SMMC7721 cells (Figure 4D). Although high concentrations of SJB2–043 and LDN57444 decreased α1 antitrypsin protein levels in HepG2 cells, ML364 did not (Figure S2D). Thus, our data identify ML364 as a small molecule that can potentially selectively downregulate antithrombin.

### 3.5 USP2 deubiquitinates and stabilizes antithrombin

Given that ML364 inhibits USP2, we next investigated the role of USP2 in antithrombin turnover. To determine the potential interaction of USP2 and antithrombin, we performed immunoprecipitation and immunoblotting experiments, which revealed that the endogenous USP2 isoform (USP2a) interacted with antithrombin in HepG2 and SMMC7721 cells (Figure 5A,B). In contrast, no association was detected between antithrombin and USP30, another DUB in the USP family (Figure 5A). Similar to ML364 treatment, siRNA-mediated USP2 knockdown significantly reduced ectopically expressed antithrombin protein in 293FT cells and endogenous antithrombin in HepG2 and SMMC7721 cells, without affecting mRNA levels (Figure 5C-E). Moreover, USP2 knockdown accelerated antithrombin turnover (Figure 5F) and increased total polyubiquitinated and K48-linked polyubiquitinated forms of antithrombin (Figure 5G).

Having found that USP2a interacts with antithrombin in both hepatocyte cell lines (Figure 5B), we further focused on USP2a, asking whether overexpression of USP2a is sufficient to increase antithrombin protein levels. In 293FT cells transfected with antithrombin, overexpression of WT USP2a markedly elevated antithrombin protein, increasing it to an extent comparable to that of bortezomib treatment (Figure 6A). However, overexpression of a dominant-negative USP2a mutant in which the catalytic cysteine residue was replaced with alanine (C276A), had a negligible stabilizing effect on antithrombin protein (Figure...
6A). We also found that the degree of USP2a overexpression was positively correlated with antithrombin accumulation in 293FT cells (Figure 6B). Similarly, overexpression of USP2 increased the levels of endogenous antithrombin in HepG2 and SMMC7721 cells (Figure 6C). Consistent with the results of loss-of-function studies (Figures 4 and 5), overexpression of USP2a slowed antithrombin turnover (Figure 6D). Moreover, overexpression of WT-USP2a effectively reduced total and K48-linked polyubiquitinated antithrombin species in 293FT and HepG2 cells, respectively (Figure 6E,F). Collectively, these data indicate that USP2 stabilizes antithrombin through binding and removal of the ubiquitin chain from antithrombin.

4 | DISCUSSION

Antithrombin, a serine protease inhibitor, is a potential therapeutic target in hemophilia A and B. Here we identified Cullin 2 and USP2 as novel regulators of antithrombin expression that act through modulation of antithrombin degradation (Figure 7). We demonstrated that antithrombin is a short-lived protein in hepatocytes (half-life < 6 hours) that is quickly degraded through the ubiquitin-proteasome system, as evidenced by the accumulation of antithrombin in cells in which either the 20S or 19S proteasome was inhibited (Figure 1D,F). These findings are consistent with previous reports that bortezomib treatment increases antithrombin protein in liver tissues and HepG2 cells. A previous ubiquitylome study showed that antithrombin was ubiquitinated on lysine 209 in mice, but the ubiquitinated sites of antithrombin in humans remain poorly understood and represent an interesting question for future studies. Given the fairly short half-life of antithrombin, targeting antithrombin degradation may be an effective approach for rapidly downregulating circulating levels of antithrombin, which may offer clinical benefits to hemophilia patients.

The specificity of proteasome-mediated proteolysis is generally controlled by E3 ubiquitin ligases. Multiple lines of evidence presented here identify Cullin 2 as the ubiquitin ligase of antithrombin. We showed that inhibition of neddylation via pharmacological or genetic approaches, both of which are known to abrogate CRL activity, prevented the ubiquitination and degradation of antithrombin (Figure 2). We further showed that Cullin 2 and its interacting partner RBX1, but not other Cullin proteins, are associated with antithrombin (Figure 3). Moreover, a loss-of-function study demonstrated an essential role for Cullin 2 in mediating antithrombin ubiquitination and degradation (Figure 3). Because Cullin 2 pairs with a variety of adaptors to regulate the ubiquitination of diverse substrates, additional studies are warranted to identify the adaptor responsible for ubiquitinating antithrombin.

DUBs antagonize the effects of E3 ubiquitin ligases by removing ubiquitin from substrate proteins and stabilizing substrates. In this study, we demonstrated that USP2 is a DUB that stabilizes antithrombin. Our screening of DUB inhibitors identified the USP2 inhibitor ML364 as being capable of promoting antithrombin degradation (Figure 4). ML364 has an IC$_{50}$ of 1.1 μM when inhibiting USP2, a concentration that remains effective in enhancing antithrombin degradation (Figure 4C). Concerning the potential off-target effect of ML364, we further directly determined the role of USP2 in antithrombin expression. We found that USP2, but not USP30, binds to antithrombin, although identification of the domains required for USP2-antithrombin interaction will have to await future studies. Both gain- and loss-of-
function studies have consistently demonstrated that USP2 is necessary and sufficient for removing ubiquitin from antithrombin and preventing its degradation, actions that depend on USP2 deubiquitinase activity (Figures 5 and 6). Our findings thus add antithrombin to the substrate repertoire of USP2, which is reported to stabilize a number of substrates, including MDM2 (a regulator of p53), fatty-acid synthase, cyclin D1, and CRY1. Interestingly, only USP2a is able to interact with antithrombin in HepG2 and SMMC7721 cells. Since the siRNAs we employed to knock down USP2 target both USP2a and USP2b, and over-expression of USP2a is sufficient to stabilize antithrombin, it remains unclear whether USP2b plays a redundant or distinct role in regulating antithrombin expression. Lastly, since Cullin 2-mediated ubiquitination of antithrombin requires the assembly of a functional ubiquitin ligase, it remains technically challenging to downregulate antithrombin by enhancing the activity of Cullin 2 ubiquitin ligase. Directly targeting USP2 could be a feasible strategy for reducing intracellular antithrombin expression, and thereby decreasing its circulating levels. Of note, the effective dose of ML364 in decreasing antithrombin is quite high (μM), which may prevent its potential preclinical application. Thus, development of a more potent and specific USP2 inhibitor is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

| Abbreviation | Definition |
|--------------|------------|
| Bor          | bortezomib |
| CHX          | cycloheximide |
| CRL          | Cullin-RING ubiquitin ligase |
| DUB          | deubiquitinase |
| UPS          | ubiquitin-proteasome system |
| WT           | wild type |
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FIGURE 1.
Antithrombin is degraded by the proteasome. A, Basal antithrombin mRNA levels in L02, HepG2, BEL7402, and SMMC7721 cells, determined by qPCR. B, Western blotting of antithrombin in the indicated cell lines after treatment with bortezomib (Bor) for 24 hours. C,D, HepG2 and SMMC7721 cells were treated with bortezomib at the indicated concentrations for 24 hours or to 100 nM bortezomib for the indicated times. C,D, Representative western blots (C) and the quantification (D) of antithrombin protein expression. E,F, Assessment of antithrombin expression in HepG2 and SMMC7721 cells after exposure to the indicated concentrations of b-AP15 for 24 hours or to 0.5 µM b-AP15 for the indicated times. E, Representative western blots showing antithrombin protein expression. F, Statistical analysis of antithrombin protein expression. G, Turnover of antithrombin protein was assessed by treating HepG2 and SMMC7721 cells with CHX alone or in combination with bortezomib for the indicated times. H, Total and K48-linked
polyubiquitinated antithrombin following bortezomib treatment of HepG2 and SMMC7721 cells was assessed by immunoprecipitation
FIGURE 2.
Inhibition of neddylation stabilizes antithrombin. A, B, Assessment of antithrombin expression in HepG2 and SMMC7721 cells after exposure to the indicated concentrations of MLN4924 for 24 hours or to 1 µM MLN4924 for the indicated times. A, Representative western blots showing antithrombin protein expression. B, Statistical analysis of antithrombin protein expression. C, The half-life of antithrombin was evaluated by exposing HepG2 and SMMC7721 cells to CHX alone or in combination with MLN4924 for the indicated times. D, HepG2 and SMMC7721 cells were treated with MLN4924 for 24 hours, followed by treatment with 100 nM bortezomib for an additional 2 hours. Total polyubiquitinated antithrombin was evaluated by immunoprecipitation. E-G, Western blot analysis of the neddylation proteins, UBA3 (E), NAE1 (F), and UBE2M (G), in HepG2 and SMMC7721 cells 48 hours after transfection with 50 nM siRNAs against the corresponding enzymes.
FIGURE 3.
Cullin 2 regulates the ubiquitination and degradation of antithrombin. A, B. Endogenous antithrombin was immunoprecipitated and immunoblotted for the indicated proteins in HepG2 cells. Cullin 2 (A) and RBX1 (B) were detected in immunoprecipitates. C, Evaluation of the interaction of endogenous Cullin 2 with antithrombin in HepG2 and SMMC7721 cells by immunoprecipitation. D, Western blot analysis of Cullin 2 in HepG2 and SMMC7721 cells 72 hours after transfection with 100 nM siRNAs against Cullin 2. E, Western blot analysis of Cullin 2 in HepG2 and SMMC7721 cells transfected with 100 nM siRNAs against Cullin 2 as in D, followed by treatment with 100 nM bortezomib for an additional 2 hours. Total polyubiquitinated antithrombin was assessed by immunoprecipitation.
FIGURE 4.
Impact of the deubiquitinase inhibitors on antithrombin expression. A, B, Western blot analysis of antithrombin protein expression in HepG2 cells following exposure to different concentrations of the indicated deubiquitinase inhibitors for 24 hours. Representative western blots (A) and corresponding statistical analysis of antithrombin expression (B) following exposure to the indicated inhibitors. C, Western blot analysis of antithrombin protein expression in HepG2 and SMMC7721 cells following exposure to increasing concentrations of ML364 for 24 hours or to 5 µM ML364 for the indicated times. D, Western blot analysis of antithrombin protein expression in HepG2 and SMMC7721 cells following treatment with 50 µg/mL CHX alone or in combination with 1 µM ML364 for 0, 2, 4 or 8 hours.
FIGURE 5.
USP2 regulates antithrombin ubiquitination and degradation. A, Immunoprecipitation followed by western blotting showing the association of antithrombin with endogenous USP2, but not USP30, in HepG2 cells. B, Interaction of endogenous USP2 and antithrombin in HepG2 and SMMC7721 cells, assessed by immunoprecipitation. C, Western blotting of antithrombin and USP2 proteins in 293FT cells transfected with FLAG-antithrombin constructs following siRNA (100 nM)-mediated knockdown of USP2. D, Western blotting of endogenous USP2 and antithrombin in HepG2 and SMMC7721 cells transfected with siRNA against USP2 (or control siRNA). E, RT-qPCR analysis of endogenous (HepG2 and SMMC7721 cells) and exogenous (293FT cells) antithrombin and endogenous USP2 mRNA levels. F, Pulse-chase analysis of antithrombin expression in HepG2 and SMMC7721 cells transfected with siRNA (100 nM) against USP2 (or control siRNA) then treated with CHX (50 µg/mL) for the indicated times. G, 293FT cells were transfected with
FLAG-antithrombin and siRNA against USP2 (or control siRNA) followed by treatment with bortezomib (10 µM) for 6 hours. Total and K48-linked polyubiquitinated antithrombin was detected by immunoprecipitation using an anti-FLAG antibody followed by western blotting.
FIGURE 6.
USP2 overexpression stabilizes antithrombin. A, Western blotting of antithrombin and USP2 expression in 293FT cells transfected with GFP-antithrombin, with or without bortezomib (1 μM) treatment for 6 hours, or co-transfected with HA-USP2 WT or catalytically inactive (C276A) HA-USP2. B, Western blot analysis of antithrombin and USP2 expression in 293FT cells transfected with GFP-antithrombin and increasing concentrations of HA-USP2. C, Western blot analysis of antithrombin and USP2 expression in HepG2 and SMMC7721 cells overexpressing HA-USP2 (or control vector). D, Pulse-chase analysis of antithrombin expression in HepG2 and SMMC7721 cells overexpressing HA-USP2 (or control vector) and treated with CHX (50 μg/mL) for additional indicated times. E, Total and K48-linked polyubiquitinated antithrombin in 293FT cells transfected with GFP-antithrombin and HA-USP2 WT or HA-USP2C276A and treated 48 hours later with bortezomib (10 μM) for 6 hours, detected by immunoprecipitation and subsequent western blotting. F, Total and
K48-linked polyubiquitinated antithrombin in HepG2 cells overexpressing HA-USP2 WT or HA-USP2C276A, treated for 6 hours with bortezomib (10 μM) 48 hours after transfection, followed by immunoprecipitation and subsequent western blotting.
FIGURE 7.
Scheme showing that Cullin 2 ubiquitin ligase and USP2 coordinate antithrombin ubiquitination and degradation