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Review

Laboratory-Based Rationale for Targeting the Protein Homeostasis Network in AL Amyloidosis

Giada Bianchi [1], Maria Moscvin [1] and Raymond L. Comenzo [2,*]

[1] Amyloidosis Program, Division of Hematology, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 4 Blackfan Circle, Boston, MA 02115, USA; gbianchi1@bwh.harvard.edu (G.B.); mmoscvin@bwh.harvard.edu (M.M.)

[2] John C. Davis Myeloma and Amyloid Program, Tufts Medical Center, Tufts University School of Medicine, 800 Washington Street, Boston, MA 02111, USA

* Correspondence: rcomenzo@tuftsmedicalcenter.org

Abstract: AL amyloidosis is an incurable plasma cell dyscrasia with limited therapeutic options. The pathogenetic mechanism in AL amyloidosis is the deposition of insoluble fibrillary aggregates of misfolded immunoglobulin (Ig) free light chains (FLC) and chaperone proteins in target organs. Therefore, AL amyloidosis is the prototypic, protein-toxicity hematologic disorder. Based on laboratory evidence of increased, constitutive proteotoxic stress, PCs are intrinsically vulnerable to agents that target proteins whose function is to guarantee that nascent polypeptides either reach a functional conformation or are disposed of (proteostasis network). The clinical efficacy of proteasome inhibitors (PIs), such as bortezomib, in the treatment of plasma cell (PC) disorders has provided proof of concept that disrupting protein homeostasis is an effective and generally safe therapeutic approach. Therefore, the intrinsic biology of PC offers us the opportunity to rationally develop therapies that target this distinct proteostasis vulnerability of PC dyscrasias. In this manuscript, we will review the laboratory rationale for the effectiveness of FDA-approved and investigational agents targeting protein homeostasis in AL amyloidosis and related PC disorders.

Keywords: AL amyloidosis; proteostasis network; ubiquitin–proteasome system; therapeutic targets; proteotoxicity; protein degradation; protein synthesis; protein folding; unfolded protein response (UPR); endoplasmic reticulum associated degradation (ERAD)

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1. Introduction

AL amyloidosis is the most common form of the family of protein deposition disorders termed amyloidoses [1]. The shared fundamental pathogenetic mechanism of these conditions is the deposition in target organs of polymers of a misfolded protein, organized in fibrils, in association with chaperone proteins [2]. The amyloidogenic precursor protein defines the subtype of amyloidosis, determines the pattern of organ involvement and disease natural history, and ultimately dictates treatment approach [3]. In AL amyloidosis, the clonal Ig free light chain (FLC) is the precursor protein of amyloid fibrils. The underlying hematologic diathesis driving AL amyloidosis is generally a plasma cell (PC) disorder, and less frequently, it is a post-germinal center (GC) B cell lymphoma. In most instances, the PC clone responsible for the production of amyloidogenic FLC is relatively small and would otherwise be classified as a monoclonal gammopathy of undetermined significance (MGUS) in the absence of amyloid-driven end organ damage. However, circa 15% of patients with the PC cancer multiple myeloma (MM) will be diagnosed with overlapping AL amyloidosis during the course of their illness.

While AL amyloidosis and MM shared a common progenitor cell, a mutated, post-GC terminally differentiated B cell, the two conditions diverge in their primary pathogenetic mechanism. In fact, MM primarily causes organ damage as a consequence of the expansion of clonotypic PCs in the bone marrow with only occasional paraprotein-related damage as

in the context of cast nephropathy or hyperviscosity [4]. Vice versa, fibrillogenic deposition of the secreted FLC is the pathogenetic mechanism in AL amyloidosis, making it the quintessential protein toxicity hematologic disorder.

Eukaryotic cells rely on a network of proteins (proteostasis network, PN) to maintain homeostasis and avoid toxicity related to the accumulation of unstable/misfolded or untimely protein species (Figure 1) [5]. In this effort, the PN integrates with adaptive stress responses such as the unfolded protein response (UPR) to restore protein equilibrium in the face of proteotoxicity [6]. Nevertheless, these redundant systems can become saturated if the proteotoxic stress is too overwhelming in duration or intensity and apoptosis then occurs [7]. The work of our groups and others has shown that normal and aberrant PCs depend on an intact PN for their survival, offering the opportunity to develop rationally designed therapies targeting our very own PC biology and turning it against PC disorders [8,9]. Our groups and others have previously shown that both MM and AL-PC suffer from baseline proteotoxic stress related to sustaining and imprecise protein synthesis. It is therefore intuitive to develop therapies that further exacerbate this balance in an effort to induce apoptosis. The classical example of the power of such a strategy is the selective anti-MM activity of proteasome inhibitors (PIs) [10]. Despite proteasomes being ubiquitously expressed in tissues, the first in class PI bortezomib proved selectively toxic against MM- and AL-PC with surprisingly limited side effects, owing to the level of proteotoxic stress experienced by these professional secretory cells [11,12]. The use of bortezomib in AL amyloidosis radically changed the natural history of this disease and profoundly impacted the survival of patients, providing proof that targeting proteostasis is clinically useful in AL amyloidosis.

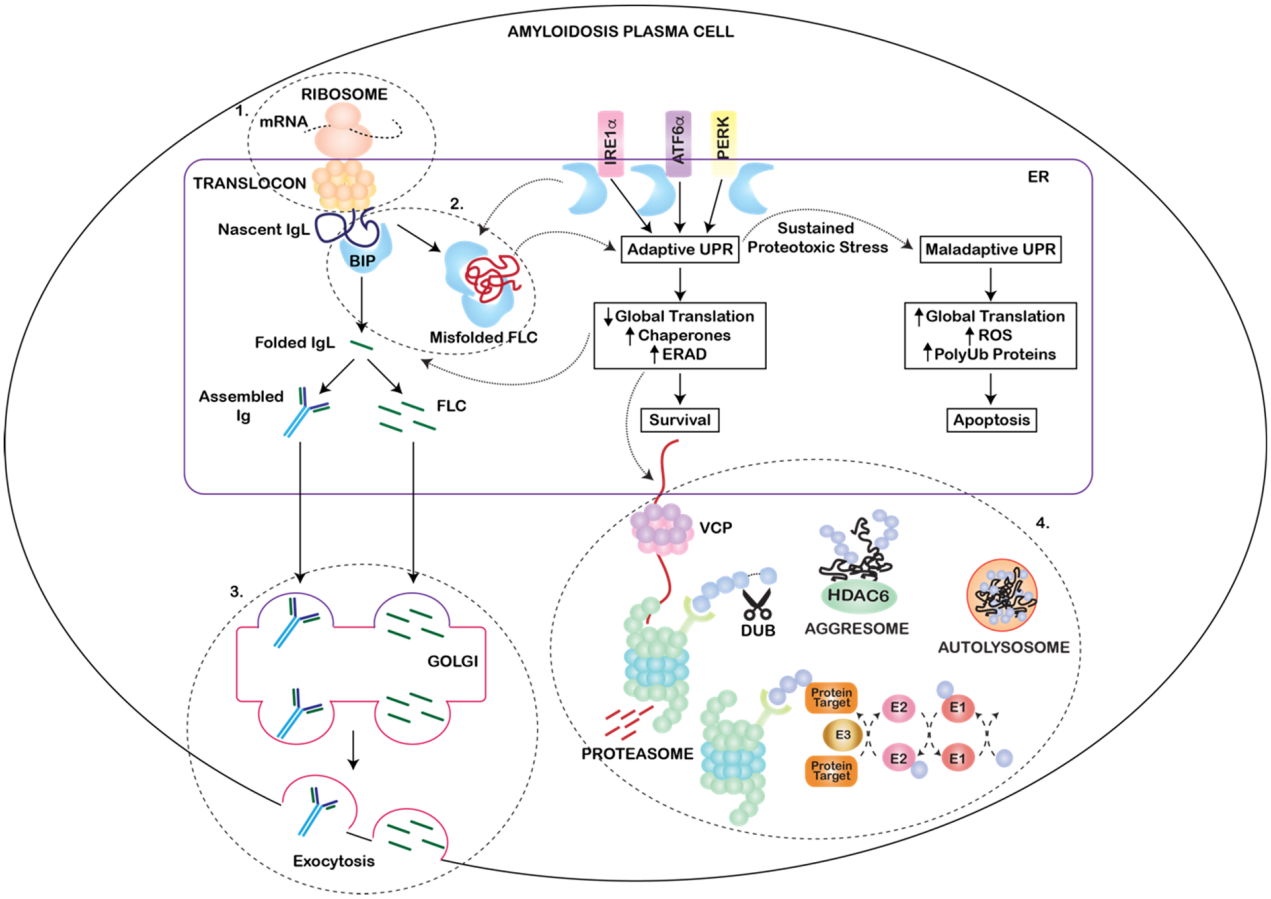

Figure 1. The Proteostasis Network. The schema outlines the complex proteostasis network in an AL-PC following the fate of the immunoglobulin (Ig) free light chain (FLC) as an example of highly

synthesized protein. For secretory proteins, four proteostasis compartments regulating distinct biological functions can be identified (dashed circles): protein synthesis (1), folding (2), secretion (3) and degradation (4). Critical organelles and proteins participating in the ER proteostasis are outlined in capital letters. Crosstalk with the three ER receptors (IRE1α, ATF6α and PERK) mediating the unfolded protein response (UPR) is also outlined. Abbreviations: mRNA: messenger RNA; ER: endoplasmic reticulum; IgL: immunoglobulin light chain; BIP: Ig binding protein, also known as GRP78, glucose regulated protein 78; FLC: free light chain; UPR: unfolded protein response; ERAD: ER-associated degradation; VCP: valosin-containing protein, also known as p97; Ub: ubiquitin; ROS: reactive oxygen species; HDAC6: histone deacetylase 6; DUB: deubiquitylating enzyme; E1: Ub activating enzyme; E2: Ub conjugating enzyme; E3: Ub ligase enzymes.

In this review, we explore the laboratory-based rationale for targeting proteostasis in AL amyloidosis and discussed FDA-approved and investigational agents in advanced clinical development (Table 1). As preclinical models to study AL amyloidosis are scant, the majority of the data herein presented are extrapolated from MM models.

Table 1. Investigational agents targeting protein homeostasis in plasma cell disorders. This table outlines promising agents targeting protein homeostasis in advanced preclinical or early clinical development in MM.

Drug Name	Chemical Structure/Mechanisms of Action	Stage of Development	Notes
Proteasome Inhibitors			
Bortezomib	• Boronic acid • Reversible inhibition of β5 > β1 • Leads to accumulation of polyUb proteins and proteotoxic death • Induces immunogenic cell death	FDA approved as first line in MM and AL amyloidosis	Peripheral neuropathy is an impactful side effect
Carfilzomib	• Epoxyketone • Irreversible inhibition of β5 • Leads to accumulation of polyUb proteins and proteotoxic death	FDA approved as second line in MM	Cardiovascular adverse events
Ixazomib	• Orally bioavailable boronic acid • Reversible inhibition of β5 > β1 • Leads to accumulation of polyUb proteins and proteotoxic death	FDA approved as second line in MM	Gastrointestinal side effects and peripheral neuropathy
Marizomib	• β-lactone-γ-lactam • Irreversible inhibition of β5, β1 and βs	Early phase clinical trials in CNS MM planned	CNS side effects
E3 Ubiquitin ligase modulators			
IMiDs (thalidomide, lenalidomide, pomalidomide	• Cereblon binding and redirection to the selective degradation of IKZF1 and IKZF3	FDA approved across of line of treatment in MM	• Increased incidence of venous thromboembolic events when used in combination • Class X drug in pregnancy • Secondary hematologic neoplasms observed post IMiD + alkylator combination
KPG818	• Small molecule binding to cereblon and modulating E3 ligase activity	Phase 1 clinical trial in hematologic malignancies	
TAS4464, MLN4924	• NEDD8 inhibitors • Inhibits neddylation-mediated activation of Cullin-RING E3 Ub ligases	Phase 1 clinical trials in MM	Terminated due to liver toxicity (TAS4464) or lack of efficacy (MLN4924)

Table 1. *Cont.*

Drug Name	Chemical Structure/Mechanisms of Action	Stage of Development	Notes
DUB Inhibitors			
VLX-1570	• Inhibits UCHL5	Phase 1 clinical trial in combination with dex in RRMM	Terminated due to grade 5 toxicity(lung toxicity).
P5091, XL177A	• Inhibits USP7 activity	Preclinical	
B-AP15	• Inhibits USP14 and UCHL5	Preclinical	
RA190	• Inhibits RPN13 and UCHL37	Preclinical	
ERAD inhibitors			
CB-5083	• Inhibits p97 inhibition • Leads to polyUb protein accumulation • Induces terminal UPR	Phase I terminated	Photophobia and dyschromatopsia reported and probably related to off target effect on PDE6
Autophagy Modulators			
Chloroquine	Alkalinizes lysosomal pH and inhibits autophagosome–lysosome fusion	Phase I/II studies in combination with PI-based backbones in RRMM have been completed	Insufficient clinical activity to warrant further development
Aggresome Inhibitors			
Ricolinostat (ACY-1215)	Inhibits HDAC6 and thus aggresome formation	Phase I and II studies in combination with IMiDs or bortezomib completed or currently ongoing	
Citarinostat (ACY-241)	Inhibits HDAC6 and thus aggresome formation	In phase I study in combination with PVX-410 vaccine and lenalidomide in smoldering MM	
UPR Modulators			
Nelfinavir	• Modulates membrane lipid bilayer composition • Induces UPR	Phase I/II studies in combination with lenalidomide or bortezomib in RRMM completed	Clinical activity shown in lenalidomide or bortezomib refractory MM
PAT-SM6	• Anti-GRP78 monoclonal IgM antibody • Induces UPR • Induces complement dependent cytotoxicity	Phase I Completed in RRMM	Safe with best response SD as single agent
Compound 147	• Covalent modification of PDIs • Reduces secretion of amyloidogenic FLC	Preclinical	
HSP Inhibitors			
KW-2478	• HSP90 inhibitor • Apoptosis	Phase II completed	Further clinical development halted due to severe toxicities and/or lack of clinical benefit
NVP-AUY922	• HSP90 inhibitor • Apoptosis • Downregulation of survival pathways	Phase I/II completed	
IPI-504	• HSP90 inhibitor • Inhibition of UPR	Phase I completed	
Tanespimycin (17-AAG; KOS-953)	• HSP90 inhibitor • Inhibition of downstream signaling pathways • Induction of UPR	Phase II/III completed	

2. Targeting the Ubiquitin–Proteasome System (UPS)

2.1. Proteasome Inhibitors

Based on the very nature of normal and malignant PCs as immunoglobulin (or FLC)-synthesizing factories, these cells are dependent on an intact proteostasis network for survival [8,9]. Given the intrinsically flawed nature of protein synthesis, it is estimated that about one-third of newly synthesized proteins will be unable to reach native conformation and will need to be disposed of through the ubiquitin–proteasome system (UPS) [13]. This figure is significantly increased in cell types characterized by high synthetic function and/or malignant cells, explaining the disproportionate accumulation of polyubiquitinated proteins in AL or MM PCs [14–18]. For secretory and/or transmembrane proteins, this process involves retrotranslocation from the ER through the Sec61 translocon and proteasome-mediated degradation (ER-associated degradation or ERAD) [19]. Proteins along this pathway thus represent potentially relevant therapeutic targets to exacerbate proteotoxic stress in PC disorders. In fact, PIs proved remarkably effective in treating both AL and MM; however, the acquisition of resistance over time is inevitable for most patients, and a suboptimal response to bortezomib has been observed in t(11;14) AL amyloidosis patients, underscoring the need for the development of novel rationally informed therapeutics within the proteostasis network [20].

The proteasome is a 2.4 MDa, ATP-dependent, multicatalytic protein degradation organelle that acts in concert with the K48-ubiquitination of proteins to degrade cell-cycle asynchronous or misfolded proteins. The 20S barrel-shaped catalytic core of the proteasome is composed of one external α ring on each side and two inner β rings containing caspase-like (β1), trypsin-like (β2) and chymotrypsin-like (β5) catalytic activities. Interferon γ-inducible catalytic subunits LMP2 (β1i), MECL-1 (β2i) and LMP7 (β5i) can replace constitutive subunits to form the immunoproteasome in immune cells and facilitate the production of antigenic peptide precursors for MHC class I presentation [21–23]. On one or both sides of the 20S core sits a 19S regulatory cap that is responsible for the critical functions of binding, deubiquitylating, unfolding and delivering of K-48 polyubiquitinated (polyUb) cargo proteins to the catalytic core [24,25]. PIs were initially developed as tools to study the critical function of the proteasome and dissect the activity of each subunit in protein degradation [26,27]. PS-341, later named bortezomib, a reversible, boronic acid inhibitor of the β5 and to a lesser extent the β1 activities of the proteasome [26], proved remarkably active against MM in preclinical and phase I clinical studies, prompting its further development in late-stage clinical trials [28,29]. Bortezomib is thought to mediate MM activity via pleiotropic mechanisms targeting both the cancer cell directly as well as the tumor-supportive bone marrow microenvironment. Based on preclinical data, bortezomib is directly toxic to MM cells and modulates the BM microenvironment with anti-angiogeneic, pro-osteoblastic and anti-osteoclastic effects [30,31]. The initial hypothesis behind the extraordinary activity of bortezomib in MM, alongside the limited side effects observed in clinical trials, was inhibition of the canonical nuclear factor κB (NF-κB) pathway via stabilization of NF-κB inhibitors (IκB proteins). However, the treatment of MM cells with NF-κB inhibitors did not induce the same extent of cytotoxicity as bortezomib, indicating alternative mechanisms [32,33]. Subsequent studies showed that bortezomib impaired the DNA damage response and induced terminal unfolded protein (UPR) and heat shock responses (HSR), causing irreversible proteotoxic stress due to the accumulation of toxic polyUb protein species and intrinsic apoptosis [33–35]. Our group showed that the ratio between polyUb proteins and proteasome activity at baseline can serve as a predictive biomarker of response to PIs in MM [11]. Consequently, an increase in misfolded proteins or a decrease in proteasome capacity sensitizes to PIs, while decreased protein synthesis or increased proteasome capacity causes resistance [36].

In seminal AL amyloidosis preclinical studies, synthesis of the amyloidogenic Ig FLC was shown to be directly responsible for the striking sensitivity of AL PCs to PIs by increasing cellular stress levels and impairing proteostasis [37]. Clinical use of bortezomib-based regimens in AL amyloidosis completely changed the natural history of this disorder

and resulted in high rates of deep responses across all stages of disease, translating into long-term remissions and providing an effective therapeutic strategy for patients ineligible for autologous stem cell transplant as well as effective induction and consolidation therapies for transplant-eligible patients [38–40]. The addition of bortezomib to melphalan and dexamethasone, the historical gold standard treatment for AL amyloidosis, boosted response rates without aggravating side effects [41]. Due to its efficacy and safety profile, the combination of bortezomib, cyclophosphamide, and dexamethasone (CyBorD) rapidly became standard of care in the frontline treatment of AL amyloidosis [42,43]. Bortezomib remains a critical component of AL amyloidosis treatment across all stages of disease with t(11;14) serving as a negative predictive marker of response [20,44]. Most recently, the quadruplet combination of CD38-targeting antibody daratumumab plus CyBorD was approved as frontline therapy of AL amyloidosis, the first (and thus far only) treatment approved for AL amyloidosis [45].

While highly active in MM and AL amyloidosis, acquired resistance to bortezomib is unavoidable over time, and an innate refractory state has been observed both in MM and AL amyloidosis [20]. Clinical resistance to PIs is an adverse prognostic factor in the treatment of PC disorders and a major clinical hurdle, prompting research efforts to develop strategies to overcome it. Carfilzomib, an epoxyketone PI, covalently binds to the β5 subunit of the proteasome, resulting in irreversible inhibition of this catalytic activity [26,46]. In preclinical studies, carfilzomib proved more potent than bortezomib, and its use as a single agent or in combination with other drugs resulted in durable responses in patients with relapsed/refractory MM, including bortezomib-refractory individuals [47–49]. However, a significant signal for carfilzomib-associated cardiovascular adverse events such as hypertension, heart failure, ischemia and arrhythmias emerged during larger clinical studies [50,51]. While the nature of these side effects has not been definitively clarified, there is reasonable concern that it may be related to the on-target effect of irreversible inhibition of the chymotrypsin-like activity of the proteasome. Although effective, a signal for vascular toxicity with renal and cardiac manifestations emerged in early-phase clinical trials of carfilzomib in AL amyloidosis [52,53]. In general, we recommend that carfilzomib administration to patients with AL amyloidosis be carefully pondered and generally reserved for a selected patient population with absent or minimal pre-existing cardiac, renal or small vessel involvement and limited therapeutic options. For instance, carfilzomib was proven to be an effective treatment strategy in patients with AL amyloidosis-related peripheral neuropathy that may preclude the safe use of bortezomib [54]. Finally, prescribers should be considerate in dose-escalating carfilzomib, as the cardiovascular signal in AL was observed for doses exceeding 36 mg/m^2 [51,55].

Marizomib, a β-lactone-γ-lactam irreversible PI, was developed in an attempt to overcome bortezomib resistance by inhibiting all three catalytic activities of the proteasome. Supporting this rationale, preclinical studies in triple negative breast cancer and the concurrent inhibition of β2 and β5 proteasome subunits could overcome resistance to β5-only inhibitors bortezomib and carfilzomib, suggesting that the targeting of multiple proteasome subunits may be an effective strategy to overcome resistance to carfilzomib and bortezomib [56]. In vitro, marizomib proved more potent than bortezomib and effective in bortezomib-resistant MM models; however, central nervous system (CNS) side effects such as gait disturbances and visual hallucinations were observed during early-phase clinical studies in relapsed/refractory MM (RRMM), suggesting that marizomib crosses the blood–brain barrier [57,58]. As a consequence, its development in MM has been halted, but clinical trials are ongoing in primary CNS tumors, particularly glioblastoma multiforme, with a plan to launch a trial of marizomib in combination with pomalidomide and dexamethasone in CNS MM.

These data underscore the trade-off between the potency of proteasome inhibition and clinical tolerability, reminding us of the essential function of the UPS in normal tissues.

Recent seminal work has shown that cells are equipped with a proteasome stress response that leads to the transcription of proteasome subunits in the face of insufficient

proteasome activity [59–61]. NFE2 Like BZIP Transcription Factor 1 (NFE2L1 or NRF1) and its homologue NFE2 Like BZIP Transcription Factor 2 (NFE2L2 or NRF2) are master regulators of baseline and stress-induced proteasome biogenesis. In homeostatic conditions, NRF1 is constitutively transcribed, translated, partially inserted in the endoplasmic reticulum (ER), deglycosylated by N-Glycanase 1 (NGLY1), retro-translocated via Valosin Containing Protein (VCP or p97), and degraded via the ERAD [59,62]. However, when proteasome activity is reduced as in the case of sublethal, partial inhibition via PI, cytosolic, deglycosylated NRF1 is cleaved by the aspartic protease DNA Damage Inducible 1 Homolog 2 (DDI2). Cleaved NRF1 is transcriptionally active, dimerizes with small MAF proteins and binds to antioxidant response elements (ARE) on the DNA, activating a transcriptional program that includes the transcription of proteasome subunits [62]. Recently, knock out (KO) of NGLY1, DDI2 or NRF1 was shown to be cytotoxic in MM cells, including in PI-acquired or innate resistant models, and it could overcome PI resistance, suggesting that this pathway is a promising molecular target in MM [63,64].

Finally, in an attempt to ease administration, there has been interest in developing orally bioavailable PIs. Ixazomib, an orally bioavailable boronate PI, is currently FDA approved as a second-line treatment for patients with relapsed and refractory MM, and it provides a convenient agent with ease of oral administration particularly suited for maintenance therapy and/or treatment of elderly patients [57,65,66]. In a randomized, phase III study of ixazomib plus dexamethasone versus physician's choice in patients with relapsed/refractory AL amyloidosis, the investigational arm failed to improve the overall response rate or depth of response [67]. Nevertheless, a trend was observed in the prolongation of survival/major organ deterioration with the caveat of shorter duration of treatment in the control arm. These data suggest that the long-term administration of ixazomib plus dexamethasone might be a feasible approach to prolong organ and/or patient survival.

2.2. E3 Ub Ligase

The conjugation of multiple Ub moieties on a target protein is accomplished by the sequential activity of Ub activating (E1), conjugating (E2) and ligase (E3) enzymes [68]. There are more than 600 known E3 Ub ligases, and they dictate substrate specificity, thus representing appealing targets for drug development.

Among the substrates of Cullin-RING E3 ligases are tumor suppressor proteins, suggesting that their inhibition may be an effective cancer therapeutic [69]. Cullin-RING E3 ligases undergo NEDD8 Ubiquitin Like Modifier (NEDD8)-mediated neddylation, which is a post-translational modification that is essential for their activation. Consequently, blocking NEDD8 function should result in the blockade of Cullin-RING E3 function [70]. NEDD8-activating enzyme inhibitors showed promising results in preclinical MM models; however, their clinical development has been unsuccessful thus far due to lack of activity (MLN4924) or emergence of dose-limiting toxicities (TAS4464) [71,72].

Recent seminal work unraveled a new mechanism of function of the immunomodulatory drug (IMiD) lenalidomide. Lenalidomide was shown to bind to Cereblon, a component of a E3 ubiquitin ligase complex, and to redirect its function to the selective degradation of transcription factors Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3) [73,74]. This observation paved the way for the development of a novel class of drugs called proteolysis-targeting chimeric molecules (PROTACs) to redirect the physiologic function of E3 Ub ligases for the degradation of specific targets of interest, specifically oncogenes [75]. KPG-818 is a small molecule investigational compound that binds cereblon with high affinity and leads to the reduced production of inflammatory cytokines and levels of IKZF1 and IKZF3 [76]. KPG-818 was effective in preclinical models of lymphoma and multiple myeloma and is currently being evaluated in phase 1 clinical trial in hematologic malignancies.

2.3. Deubiquitylating Enzymes/Ubiquitin Receptors

Deubiquitylating enzymes (DUBs) are a large family of enzymes whose function is the removal of ubiquitin (Ub) moieties from target proteins and its recycling, thus contributing to maintaining proteostasis and guaranteeing the availability of free Ub for the posttranslational modification of proteins participating in critical cellular processes [77,78]. Given the role of Ub in targeting protein substrate for proteasome-mediated degradation and in consideration of the exceptional activity of PIs in PC disorders, the inhibition of DUB may be an appealing therapeutic strategy in these diseases [79]. ADRM1 26S Proteasome Ubiquitin Receptor (ADRM1 or RPN13) is a 19S-associated Ub receptor that on the one end binds polyUb proteins with high affinity and on the other recruits DUBs to hydrolyze Ub moieties from the proteasome substrate, thus allowing engagement by the 20S and downstream degradation [80]. RPN13 is highly expressed in MM, and its inhibition leads to apoptosis in MM cells and animal models in the absence of direct 20S proteasome inhibition [81]. Similarly, the inhibition of proteasome-associated DUBs UCHL5, ubiquitin specific protease 14 (USP14) and Ubiquitin Specific Peptidase 7 (USP7) lead to the accumulation of polyUb proteins in the absence of direct inhibition of the catalytic activity of the proteasome, resulting in apoptosis [82–84].

Unfortunately, VLX1570, a first in human small molecule inhibitor of UCHL5, caused fatal pulmonary toxicity in early-phase clinical studies, with consequent halting of clinical trials [83]. Drug development efforts are ongoing to identify tolerable and effective compounds targeting cancer-associated DUBs.

2.4. ERAD

The cytosolic AAA-ATPase VCP organizes as a homo-hexameric channel that binds and extracts misfolded proteins from the ER and delivers them to the proteasome in the process known as ERAD [19]. Inhibition of VCP in combination with PIs was shown to be cytotoxic in preclinical MM models; however, clinical development of the first in class VCP inhibitor CB-5083 was halted due to the surfacing of retinal toxicity [85,86]. Given the strong biology underlying targeting VCP in MM, research efforts are ongoing to develop inhibitors with improved therapeutic index.

3. Alternative Proteolytic Pathways

Autophagy

Autophagy is an evolutionary conserved mechanism that delivers cytoplasmic content, such as organelles, misfolded proteins and intracellular pathogens, to the lysosome for degradation and recycling [87]. It can therefore be considered an alternative proteolytic pathway to the UPS. Autophagy plays a dual role of guaranteeing energy supply by recycling macromolecules and maintaining protein homeostasis by selective auto-digestion of cellular material. Autophagy was shown to play a pivotal role in the physiologic process of PC differentiation and in ensuring the survival of bone marrow-resident, long-lived PC [37,88,89]. In this context, autophagy is necessary to support ER expansion and Ig synthesis [88]. Extensive research has proven that a close crosstalk exists between UPS, endoplasmic reticulum (ER) stress, heat shock response (HSR), and autophagy that critically contributes to the quality control of newly synthesized proteins and proteostasis in eukaryotic cells [90–93]. Preclinical studies have shown that autophagic selectivity is conferred by specific receptor/adapter proteins that recognize and mediate the engulfment of the substrate in the autophagosome. Recently, the prototypical autophagic receptor, Sequestosome 1 (SQSTM1 or p62), has been implicated in the maintenance of homeostasis in MM by the clearance of redundant misfolded proteins [89]. SQSTM1-deficient PC displayed reduced intracellular ATP, elevated levels of ER stress and died prematurely, in keeping with a role of autophagy in cell homeostasis [88].

Autophagy was originally identified as a pro-survival mechanism in MM by reducing protein-related stress. However, recent studies show that depending on the context, autophagy can promote MM cell death through a non-apoptotic, caspase 10-dependent

mechanism known as autophagy-dependent cell death [94,95]. These seemingly opposite roles of autophagy in determining cell fate suggest that both its pharmacologic inhibition (dysregulation of proteostasis) or its activation (induction of autophagic cell death) could promote anti-MM effect. Consistently, pharmacologic modulation of this pathway in combination with PI resulted in highly variable effects, ranging from synergism to antagonism [96]. For instance, the autophagy inhibitors 3-methyladeninne and chloroquine were shown to induce cytotoxicity in MM cell lines when used alone, but when combined with bortezomib, they resulted in an antagonistic response [95,97,98]. However, in a different study, chloroquine synergistically augmented carfilzomib cytotoxicity and was able to overcome carfilzomib resistance in vitro [99]. Vice versa, the activation of SQSTM1 yielded specific PI resistance by mediating a plastic adaptive response to proteasome stress, thus representing a novel, potential molecular target to overcome PI resistance [89,97]. It is therefore not surprising that the clinical translation of these inconsistent observations has been difficult. In early-phase clinical trials, a modest clinical benefit was observed by combining chloroquine with the PI carfilzomib [100].

Similar to MM, AL amyloidosis cells are exquisitely dependent on autophagy for their survival. AL plasma cells show distinctive organellar features such as ER expansion, perinuclear mitochondria, expression patterns consistent with cellular oxidative stress and reduced autophagic control of organelle homeostasis [37]. This study also reported that AL plasma cells have a unique PI susceptibility that correlates with defective autophagy and altered organelle homeostasis [37].

Adding to the complexity of this pathway, a recent seminal study showed that lysosomal dysfunction and the dysregulation of autophagic flux were evident in cardiac cells from human patients with AL amyloid-cardiomyopathy, implying that the activation of autophagy would be therapeutically beneficial in AL amyloid-cardiomyopathy [101]. In fact, the data show that the activation of autophagy via rapamycin protects against amyloidogenic light chain-associated cardiac proteotoxicity and prolongs survival in a zebrafish model [101].

Altogether, it is likely that the modulation of autophagy would have both favorable and unfavorable effects when used in patients with AL amyloidosis, thus making it extremely difficult to design safe clinical trials [102].

4. Aggresome

In AL amyloidosis, amyloidogenic, misfolded FLCs are secreted and lead to the formation of amyloid deposits extracellularly. From a biological standpoint, this observation is puzzling because quality control mechanisms do not allow misfolded proteins to transit from the ER to the Golgi. Rather, these unstable protein species are retro-translocated from the ER to the cytosol, ubiquitinated and directed for proteasome-mediated degradation via ERAD [103,104]. Aggresomes represent an alternative pathway for the containment of misfolded proteins in perinuclear inclusion bodies [105]. The formation of aggresomes activates autophagy and ultimately lysosomal degradation, which mediates the disposal of aggregated proteins. While a potential therapeutic role for targeting aggresomes has not been evaluated in AL amyloidosis, studies have been conducted in MM, leading to the development of targeted drugs. Histone deacetylase 6 (HDAC6) is a class I HDAC that is necessary for the proper formation of aggresomes by catalyzing the sequestration of polyUb misfolded proteins in perinuclear areas via the dynein–microtubule axis [106–108]. Preclinical studies in MM showed that proteasome inhibition induces aggresome formation, suggesting that aggresomes may represent an escape pathway to survive PI-induced proteotoxic stress. Therefore, HDAC6 represents a promising molecular target to overcome PI resistance [108,109].

Panobinostat, an orally bioavailable, non-selective pan-HDAC inhibitor, is the only FDA-approved HDAC inhibitor in MM. In 2015, panobinostat received accelerated approval by the FDA for its use in combination with bortezomib and dexamethasone in relapsed/refractory MM patients who had received at least two prior regimens, including

bortezomib and an immunomodulatory agent [110,111]. However, in December 2021, a request for withdrawal of this approval was submitted, effectively leading to the discontinuation of approved use of panobinostat in MM.

The therapeutic index of panobinostat was narrow due to the frequent and often severe occurrence of gastrointestinal and hematologic side effects. The hypothesis that the efficacy of pan-HDAC inhibitors is mediated by the blockade of HDAC6-mediated trafficking of polyubiquitinated proteins to the aggresome pathway whereas the adverse events are due to non-specific targeting of gene expression led to the development of selective HDAC6 inhibitors, such as ricolinostat (ACY-1215) and citarinostat (ACY-241). A phase Ib trial of ricolinostat in combination with lenalidomide/dexamethasone reported a 55% overall response rate (ORR) in RRMM and a phase I/II on ricolinostat in combination with bortezomib/dexamethasone reported a 29% ORR in RRMM [112,113]. Ricolinostat in combination with pomalidomide/dexamethasone is currently evaluated in clinical trials (NCT01997840 and NCT02189343). Citarinostat is being evaluated in combination with lenalidomide and PVX-410, a cancer vaccine, in smoldering MM in a phase 1 clinical trial (NCT02886065).

5. Unfolded Protein Response (UPR)

The UPR is an evolutionary conserved, tripartite stress response that is triggered by the accumulation of misfolded proteins in the ER and serves the goal of restoring homeostais [114,115]. It does so by decreasing de novo protein synthesis via translation arrest while selectively inducing chaperones to aid in protein transport and folding and enhancing protein degradation via ERAD. If homeostasis cannot be achieved, persistent UPR activation leads to apoptosis [116]. The signal transductors of the UPR are IRE1, ATF6 and PERK. Activated IRE1 functions as an endonuclease, resulting in XBP1 mRNA splicing [117]. Spliced XBP1 (sXBP1) and ATF6 are master transcriptional regulators, leading to lipid biogenesis to sustain ER expansion, ERAD activation, and induction of chaperones and factors regulating redox balance [118]. Alternatively, PERK causes a repression of de novo protein synthesis via the phosphorylation of eIF2α in an attempt to establish homeostasis. However, if the UPR is sustained over time, PERK selectively induces ATF4 expression, which in turns induces the expression of the pro-apoptotic protein CHOP [35].

Among the distinct UPR pathways, only the IRE1-XBP1 branch mediates bortezomib sensitivity [119]. In fact, in MM, a loss of spliced XBP1 (sXBP1) results in the de-differentiation of plasma cells to plasmablasts with decreased Ig production, decreased proteotoxic stress, and reduced sensitivity to PI [119]. Direct inhibitors of the IRE1 endoribonuclease domain have shown promising preclinical activity in MM, and early-phase clinical trials are anticipated soon [120,121].

Our group and others have shown that drugs capable of inhibiting proper protein folding (ER stressors) such as tunicamycin and thapsigargin synergize with PI in vitro, providing proof-of-principle that combinations of PI with ER stressors may be clinically beneficial [11]. However, ER stressors may have a narrow therapeutic index with potential organ toxicity. Recently, a protease inhibitor used in HIV therapy nelfinavir was shown to induce UPR and overcome PI resistance in MM cells in preclinical models [122]. Importantly, the combination of nelfinavir with bortezomib resulted in synergistic clinical activity even in bortezomib refractory MM, with ORR of 50% in patients with bortezomib-refractory and lenalidomide-resistant MM [123].

A cancer-specific isoform of the HSP70 family of ER chaperone GRP78 (or BIP) has been found to be abundantly expressed on the cell surface of solid tumor and MM cells, representing an appealing target for cancer therapy [124]. Naturally occurring IgM antibodies recognizing BIP have been detected in prostate cancer patients, suggesting this represents a highly antigenic target [125]. PAT-SM6 is a fully humanized, IgM monoclonal antibody directed against surface-expressed GRP78 isoform [126]. Preclinical studies have shown that it binds cell-surfaced expressed GRP78 and induces both direct cytotoxicity

and complement-dependent cytotoxicity. In a single-agent, phase 1 clinical trial, PAT-SM6 appeared well tolerated, but responses were not observed.

Seminal work from the Kelly and Wiseman groups has recently shown that the selective activation of individual UPR pathways differently impacts the fate of misfolded immunoglobulin free light chains in terms of retention, stabilization or degradation [127]. In particular, the activation of ATF6 resulted in a reduced secretion of misfolded secretory proteins without increased degradation overall, leading to attenuated extracellular aggregation [128,129]. In AL compound 147, an investigational ER proteostasis enhancer based on its ATF6 activating properties was shown to reduce the secretion of amyloidogenic FLC in an ATF6-independent mechanism involving the on target, covalent modification of other proteaostasis factors, including protein disulfide isomerases (PDIs) and LCs independent of ATF6 activation. Instead, 147 reduces amyloidogenic LC secretion through the selective, on-target covalent modification of ER proteostasis factors, including PDIs [130]. This is a prime example of how fundamental basic research can inform the development of novel and potentially impactful therapies in AL amyloidosis.

6. Heat Shock Proteins

The heat shock response (HSR) can be activated by proteotoxicity and serves the purpose of inducing the rapid expression of chaperone proteins, which are known as heat shock proteins (HSPs). These constitute a large family of evolutionary highly conserved proteins that are often classified based on their molecular weight: HSP70, HSP 90, HSP10, etc. Chaperones have emerged as critical players in protein quality control with a variety of roles including inhibiting protein aggregation, maintaining the folding and solubility of proteins and facilitating protein trafficking and localization into correct subcellular compartments [131]. Heat shock factor 1 (HSF1) is the "master regulator" of the HSR, controlling the expression of HSP90 and HSP70, which are the two main chaperones that support the redirection of misfolded proteins for degradation and participate in chaperone-mediated autophagy [132,133]. These two chaperones have been extensively studied in cancer by virtue of their role in supporting the folding of proto-oncogenes and promoting the stabilization of inactive mutant forms of onco-suppressors such as p53 [132,134]. In light of their critical role in proteostasis, HSPs have been regarded as promising targets in plasma cell disorders [135]. In preclinical MM models, inhibition of HSF1, HSP70 or HSP90 leads to activation of the terminal UPR and consequent apoptosis [136–138]. HSP90 (e.g., 17-AAG, NVP-HSP990, PU-H71), HSP70 (e.g., PER-16, Ver-155008, MAL3-101) and HSF1 inhibitors (e.g., CCT251236, KRIBB11) showed potent anti-MM activity in preclinical studies [139]. However, phase I/II clinical trials of HSP90 inhibitors luminespib (NVP-AUY922), KW-2478, SNX5422, retaspimycin (IPI-504) and tanespimycin (KOS-953) in RRMM patients revealed a narrow therapeutic index and only modest clinical significance, leading to a discontinuation of further clinical development [140–144].

Extracellular chaperones (EC) stabilize misfolded proteins that escape the intracellular protein quality control and have been implicated in the clearance of unstable protein species via receptor-mediated endocytosis, thereby playing a pivotal role in maintaining extracellular proteostasis [145]. There is significant interest in exploring the role that EC plays in AL amyloidosis and potentially identifying novel therapeutic targets to activate the clearance of misfolded FLC [146].

Human serum amyloid P (SAP) is a plasma protein that is invariably present in all types of amyloid deposits. SAP has shown specific chaperone activity by stabilizing and protecting the fibrillary protein, suggesting a potential role in the pathogenesis of amyloidoses [147,148]. Miridesap, a small molecule investigational compound, triggers the hepatic clearance of circulating SAP but leaves residual SAP in amyloid deposits. This remaining SAP is a specific target for dezamizumab, a fully humanized IgG1 anti-SAP monoclonal antibody, that has been shown to trigger immune-mediated amyloid reabsorption from visceral organs. In a phase 1 dose-escalation study in patients with amyloidosis, including AL, the use of miridesap followed by dezamizumab showed significant organ

responses while having a safe profile. However, a phase 2 trial in patients with cardiac disease was terminated early due to toxicity [149,150].

7. Direct Targeting of FLC Secretion

The pathologic mechanism in AL amyloidosis is the deposition of a misfolded FLC. Therefore, the secretion of amyloidogenic FLC is essential to mediate organ damage. There has been interest in developing therapies directly targeting FLC secretion. An antisense oligonucleotide (ASO) targeting the variable region of lambda free light chain was shown to significantly reduce FLC secretion in λ-secreting MM preclinical models [151]. The major limitation with this approach is the need for patient-specific ASO as the λ-light chain variable region is unique in each individual. Nevertheless, this approach provided proof of concept of the feasibility and potential therapeutic utility of targeting FLC secretion.

Work from the Comenzo lab identified a common sequence in the constant region of λ-light chain, and the group developed an siRNA pool targeting this area [152]. The transfection of the anti λ-light chain siRNA pool in λ-light chain-expressing AL amyloidosis and MM PC caused a rapid and substantial decline in FLC secretion and triggered a terminal UPR presumably due to the retention of orphan Ig heavy chain.

Finally, Moscvin et al. have recently shown that the similar induction of a terminal UPR can be achieved by expressing the catalytic domain of several distinct botulinum neurotoxin serotypes and blocking the SNARE-mediated vesicular secretion of FLC [153]. This approach suggests that direct targeting of the secretory pathway of AL PC may be therapeutically effective, leading not only to the suppression of FLC secretion but also triggering rapid apoptosis presumably due to terminal UPR triggered by the retention of unsecreted FLC.

8. Conclusions and Future Directions

AL amyloidosis is the prototypic, malignant disease of proteotoxic stress based on its high synthetic rate and extracellular release of misfolded, aggregation-prone Ig FLCs. The clinical efficacy of PIs in AL amyloidosis has clearly demonstrated the therapeutic potential of targeting this intrinsic vulnerability of AL-PCs. The notion that AL amyloidosis cells release an improperly folded Ig FLC suggest that quality control mechanisms in the ER are faulty. This characteristic makes AL amyloidosis an even more suitable disease target for therapies that impair protein homeostasis as compared to MM. Nevertheless, innate and/or acquired PI resistance remains an unmet clinical challenge, negatively impacting the prognosis of patients.

In this manuscript, we provided an overview of the laboratory rationale for targeting alternative therapeutic targets to the proteasome catalytic subunits within the proteostasis network (Figure 2). In recent years, the efficiency of the UPS in degrading polyUb protein targets has been leveraged to develop the targeted degradation of specific targets by redirecting E3 Ub ligase activity and facilitating proteasome-mediated degradation [73,74,152,153]. Theoretically, this approach allows the unprecedented opportunity to target previously undruggable proteins, such as MYC [153].

We look forward to an exciting era in AL amyloidosis where a deeper understanding of plasma cell biology can provide a foundation for targeting the Achille's heel of this disorder. We believe that hypothesis-driven, basic science holds the key to develop more effective treatments for AL amyloidosis patients with a potential for cure for most, if not all, our patients. The work and dedication of Professor Merlini in developing novel models for the study of amyloidogenic light chains and in undertaking numerous clinical trials to advance the therapeutic resources available to patients remains the gold standard for physician, investigators and scientists striving in this field.

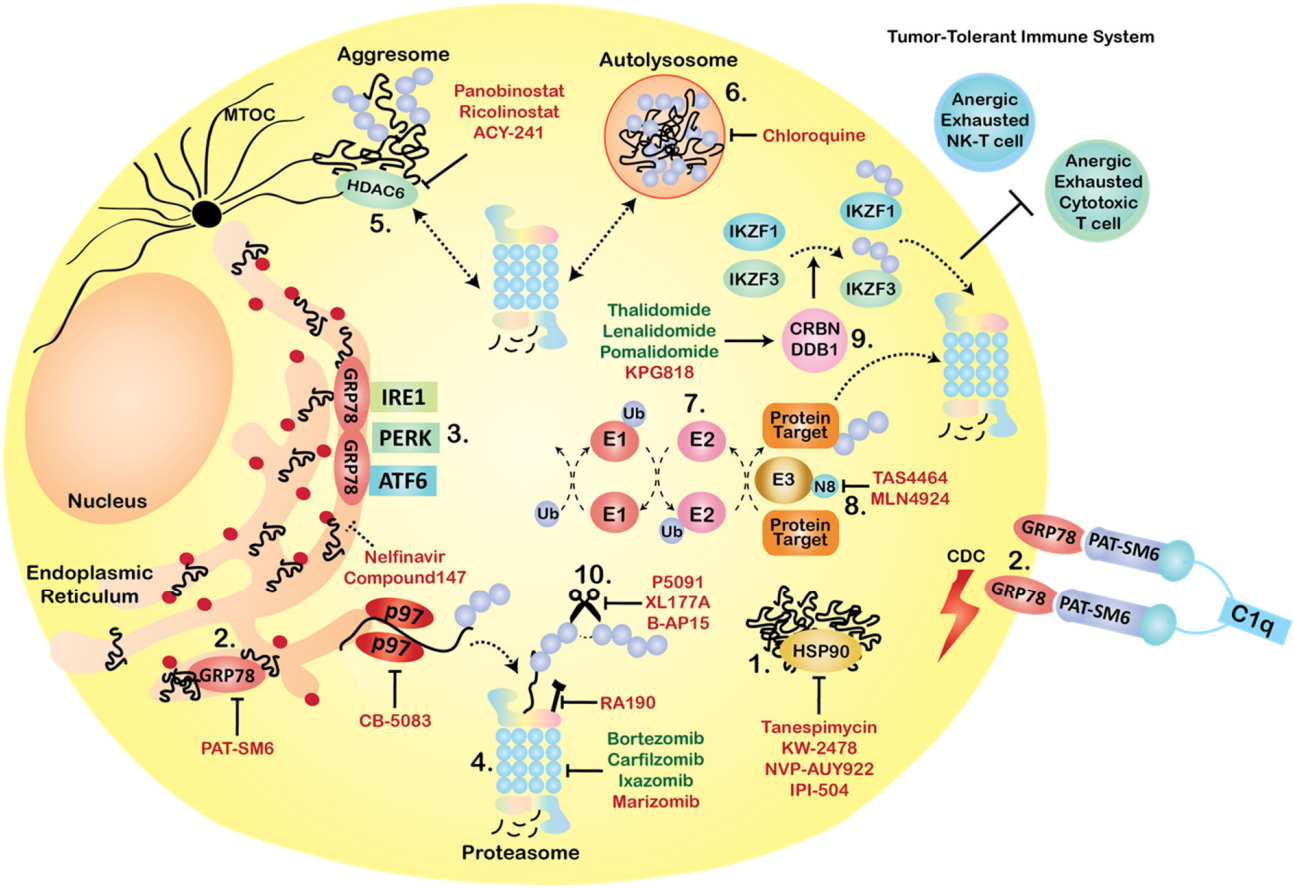

Figure 2. Molecular targets within the proteostasis network for the treatment of MM and AL amyloidosis. In the center of the cartoon is a clonal plasma cells (MM and/or AL amyloidosis plasma cells); also outlined is the nucleus and the endoplasmic reticulum with associated ribosomes (red dots). Elements of the proteostatic network that represent appealing molecular targets for anti-AL amyloidosis/MM therapy are marked by numbers: 1—HSP90 chaperone proteins; 2—ER chaperone GRP78 that is also expressed on the cell surface, representing a target for antibody mediated, complement-induced cytotoxicity (CDC); 3—unfolded protein response sensors (IRE1, PERK and ATF6); 4—the proteasome; 5—aggresome master regulator HDAC6; 6—autolysosome; 7—ubiquitin activating (E1), conjugating (E2) and ligase (E3) enzymes; 8—NEDD8 enzyme (N); 9—cereblon (CRBN)-mediated IKZF1 and IKZF3 proteolysis; 10—deubiquitylating enzymes (DUBs) and Ub receptors; FDA-approved agents are outlined in green while investigational agents are in red. Key components of the proteostasis network are outlined with associated FDA approved (in green) or investigational agents (in red) listed in Table 1. Arrows signify positive activation/induction while T-shaped lines signify inhibition. Dotted lines signify modulation/interaction. Abbreviations: HSP90: heat shock protein 90; GRP78: glucose-regulated protein 78; C1q: Complement Component 1, Q Subcomponent; HDAC6: histone deacetylase 6; CRBN: cereblon; DDB1: DNA damage-binding 1; IKZF1: Ikaros family zinc finger protein 1; IKZf3: Ikaros family zinc finger protein 3; MTOC: microtubule organizing center.

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