Fluorescent reporters for the ubiquitin–proteasome system

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Abstract

Regulated turnover of proteins in the cytosol and nucleus of eukaryotic cells is primarily performed by the ubiquitin–proteasome system (UPS). The UPS is involved in many essential cellular processes. Alterations in this proteolytic system are associated with a variety of human pathologies, such as neurodegenerative diseases, cancer, immunological disorders and inflammation. The precise role of the UPS in the pathophysiology of these diseases, however, remains poorly understood. Detection of UPS aberrations has been a major challenge because of the complexity of the system. Most studies focus on various aspects of the UPS, such as substrate recognition, ubiquitination, deubiquitination or proteasome activity, and do not provide a complete picture of the UPS as an integral system. To monitor the efficacy of the UPS, a number of reporter substrates have been developed based on fluorescent proteins, such as the green fluorescent protein and its spectral variants. These fluorescent UPS reporters contain specific degradation signals that target them with high efficiency and accuracy for proteasomal degradation. Several studies have shown that these reporters can probe the functionality of the UPS in cellular and animal models and provide us with important information on the status of the UPS under various conditions.

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Moreover, these reporters can aid the identification and development of novel anti-cancer and anti-inflammatory drugs based on UPS inhibition.

**Introduction**

Controlled proteolysis is crucial for regulating a broad range of basic cellular processes, such as cell cycle progression, signal transduction, apoptosis, antigen presentation and protein quality control [1]. In the cytosol and nucleus of eukaryotic cells, these processes are predominantly performed by the ubiquitin–proteasome system (UPS). The UPS generally consists of two consecutive steps: conjugation of a chain of ubiquitin molecules to a substrate protein, and breakdown of the polyubiquitin-tagged protein into small polypeptides by the 26 S proteasome. In the first step, four classes of enzymes are involved, which catalyse the formation of the polyubiquitin chain: the ubiquitin-activating enzyme (E1), ubiquitin conjugating enzymes (E2s), ubiquitin ligases (E3s; see Chapter 2), and occasionally ubiquitin-chain assembly enzymes (E4s; see Chapter 1) [2]. Proteins destined for ubiquitination and degradation are singled out by a large variety of E3 enzymes, which recognize specific degradation signals within these proteins and provide them with ubiquitin chains. Degradation signals come in many forms and can be, for example, specific N-terminal amino acids, small sequence elements (such as the destruction box in cyclins), or structural motifs (such as hydrophobic stretches in misfolded proteins). In many cases, degradation signals are conditional and can be activated or inactivated by post-translational modifications. In the second step, polyubiquitinated proteins are recognized and degraded by the proteasome, a large barrel-shaped complex that harbours several proteolytically active subunits within its inner chamber [3] (see Chapter 3).

Aberrations in the UPS have been associated with a number of clinical pathologies [4], such as neurodegenerative diseases (see Chapter 11), cancer (see chapter 13), muscle wasting (see Chapter 12) and viral infection (see Chapter 10). Depending on the type of alterations, this can either lead to accelerated or delayed degradation of ubiquitinated proteins. In cancer, accelerated degradation of tumour-suppressor proteins by the UPS is a recurrent theme, which allows malignant cells to progress into uncontrolled proliferation [5]. On the other hand, the specific accumulation and deposition of misfolded proteins in neurodegenerative diseases, such as Alzheimer’s, Parkinson’s and Huntington’s diseases, suggests inefficient clearance of these potentially toxic proteins by the UPS [6]. Because assessing the functional status of the UPS in cells and animals has shown to be a technically complicated endeavour, the precise role of the UPS in many of these pathologies remains unclear [7]. During the last few years, several groups developed fluorescent protein (FP) reporters of the UPS that allow monitoring of the UPS in cells and animals [8]. In this review, we will describe the different fluorescent UPS substrates and
summarize their applications in studies on the role of the UPS in diseases, and in identification of drugs that affect the UPS.

**Fluorescent UPS substrates**

Fluorescent UPS reporters are based on fusions between a degradation signal or full-sized constitutive UPS substrate, and either green fluorescent protein (GFP), one of its spectral variants, or other FPs (see Table 1). The FPs that are available at present, which have been isolated from various jellyfishes and sea anemones, are generally very stable proteins, exemplified by GFP of *Aequorea victoria* with a half-life of more than 24 h [9]. Introduction of a degradation signal to FPs often results in a markedly reduced half-life of the FP due to rapid proteasomal degradation. Cells or animals expressing these reporters typically contain low levels of the FP and display low fluorescence as a consequence of the rapid turnover. Obstruction of the UPS, for example by administration of small compound inhibitors of the proteolytic activities of the proteasome, induces a dramatic increase in fluorescence intensities in affected cells and tissues that can be readily monitored by fluorimetry, flow cytometry, or fluorescence microscopy.

Since general disturbances of the UPS are likely to cause an overall accumulation of substrates, a straightforward approach would be to follow the levels of any endogenous UPS substrate. Detection of the substrate-of-interest can be aided by the ectopic overexpression of a substrate–FP fusion at levels that can be conveniently monitored by various means of fluorescence detection. A number of research groups have developed FP-tagged variants of these short-lived proteins to analyse their function and dynamics in real time with cell imaging [10–14]. Although these substrate-specific reporters can inevitably provide us with important information about the functional status of the UPS, there are several drawbacks with the usage of substrate–FP fusions as general reporters for ubiquitin-dependent proteolysis. Firstly, increased cellular levels of endogenous UPS substrates do not necessarily reflect dysfunction of the UPS, but may be due to normal regulatory events resulting in functional stabilization of the substrate–FP fusions. For example, stabilization of p53 under specific conditions is not necessarily a consequence of an impaired UPS, but can be caused by genotoxic stress [14]. Secondly, in order for these substrate–FP fusions truly to reflect the behaviour of the endogenous untagged substrates, the FP moiety should not interfere with the functional activity of the substrate. Because these fusions are biologically active, their overexpression might have unwanted side effects. Thus, for example, overexpression of cyclin–FP fusions may affect cell-cycle progression [10,11], and p53–FP fusions may induce cell-cycle arrest or apoptosis [14]. These two drawbacks with substrate–FP fusions make them less suitable as *bona fide* reporters to monitor UPS activity. A number of FP reporter substrates have been developed in which these problems have been avoided (described below).
List of the currently available FP reporter substrates of the UPS. These reporters are based on FPs provided with a specific degradation signal or fused to full-sized substrate of the UPS. Most of the substrates have been made with GFP and several are also available with FPs with different spectra. The ubiquitin conjugases (E2), ligases (E3) and chain assembly factors (E4) required for degradation of these reporters in yeast (y) and mammalian cells (m) are listed. V. Menéndez-Benito and N.P. Dantuma, unpublished work.
These reporter substrates are targeted for UPS-dependent degradation by constitutively active degradation signals. To avoid gross changes in the cell physiology, fusions have been constructed in such a way that they are expected to lack any biological activity (Table 1).

**N-end rule reporters**

Varshavsky and co-workers identified the first degradation signal by studying the fate of proteins with various N-terminal residues in yeast [15]. When artificial ubiquitin (Ub) fusion proteins, such as Ub–X–β-galactosidase (X stands for any amino acid residue), are expressed in cells, the ubiquitin moieties are rapidly clipped off by deubiquitinating enzymes (DUBs). It was found that the stability of the resulting X–β-galactosidase depended on the nature of the N-terminal amino acid residue exposed after ubiquitin cleavage. Certain N-terminal amino acid residues, for example arginine, are recognized in yeast by the E3 Ubr1. In cooperation with the E2 Ubc2, this marks the protein that contains the destabilizing N-terminal amino acid with a ubiquitin chain, conjugated to the proximal lysine residue, resulting in proteasomal degradation. By changing the X amino acid in Ub–X-protein, the half-lives of proteins can vary from several minutes (for example, when X is an arginine) to several hours (for example, when X is a leucine), to a stable protein that is not degraded by the proteasome (for example, when X is a methionine). Interestingly, the N-end rule was shown to be valid not only in yeast but also in metazoans, including mouse and humans, where the substrates are recognized and targeted by the ubiquitin ligases E3α and Ubr2. Although the physiological relevance of the N-end rule has been obscure for many years, further studies have provided clear evidence for a role of this targeting system in the regulation of cardiovascular development in mice, peptide import in yeast, and chromosome segregation.

Based on the N-end rule, a set of GFP-based reporters was developed for functional analysis of the UPS in mammalian cells (Figure 1) [16]. Insertion of arginine or leucine residues as destabilizing amino acids in Ub–X–GFP resulted in proteasomal degradation of GFP, while methionine at the same position gave rise to a long-lived GFP. Ub–Arg-GFP has a half-life of approx. 10 min ([17]; V. Menéndez-Benito and N.P. Dantuma, unpublished work). Administration of proteasome inhibitors induces a striking accumulation of Ub–Arg-GFP. This reporter has also been used in yeast and mammalian models to study the activity of stabilization signals, which are motifs or domains that can counteract the activity of degradation signals and protect proteins from proteasomal degradation [18].

**Ubiquitin fusion degradation (UFD) reporters**

Another constitutively active degradation signal is the UFD signal. Although these ubiquitin–protein fusions differ only at one amino acid from the N-end rule substrates, they are targeted by another ubiquitin conjugase and ligase pair
and depend differentially on additional factors, such as ubiquitin-chain assembly factors and ubiquitin receptors [19]. In UFD substrates, the N-terminal ubiquitin forms the acceptor for the polyubiquitin chains that target the fusion proteins for degradation. To accomplish this, the C-terminal glycine residue of the ubiquitin moiety has been substituted for a valine, preventing cleavage by DUBs (Figure 2). Alternatively, the amino acid immediately downstream of ubiquitin can be substituted with proline, giving rise to Ub–Pro–FP. Due to the specificity and mode of action of DUBs, cleavage of the ubiquitin moiety is strongly delayed in the resulting Ub-Gly76Val–FP and Ub–Pro–FP reporters. As a consequence, these chimaeric proteins are ubiquitinated at the lysines at position 29 and 48 in their ubiquitin moiety [17]. The level of Ub-Gly76Val–GFP in cells under normal physiological conditions was barely detectable, but increased up to 150-fold after complete proteasome inhibition [16]. Accumulation of this reporter in stable cell lines correlated with the ability of inhibitors to induce cell-cycle arrest and apoptosis, two common effects of UPS dysfunction, suggesting that reporter levels give true information about the functional status of the UPS [16,20].

**Figure 1. Schematic representation of N-end rule reporters**

Ubiquitin (Ub) is cleaved from the Ub–R–FP precursor by DUBs, exposing the destabilizing N-terminal residue, which is in this example an arginine (R). The destabilizing N-terminal residue recruits a ubiquitination enzyme that ubiquitinates the FP close to its N-terminus, resulting in degradation of the FP reporter.

**Figure 2. Schematic representation of UFD reporters**

Cleavage of the N-terminal ubiquitin (Ub) is inhibited due to the Gly76Val substitution in the ubiquitin moiety or the proline residue directly downstream of ubiquitin. Polyubiquitin chains are conjugated to the N-terminal ubiquitin at the lysines at positions 29 and 48, resulting in proteasomal degradation of the fusion protein.
At present, only two models have been developed for monitoring the UPS in animals and both take advantage of UFD reporter substrates [7,21]. One of the models is based on xenotransplantation of human cervix carcinoma cells expressing a UFD-targeted luciferase into nude mice [21]. An advantage of this model is that the bioluminescent signal of luciferase allows reporter levels to be monitored in living animals, a method presently not possible for fluorescent reporters. The bioluminescence was hardly detectable in untreated xenotransplants, but upon treatment of the mice with proteasome inhibitors, an enhancement in bioluminescence was already observed after 4 h. Although this model will be very helpful for studying the effect of inhibitors on cancer cells in vivo, the restricted expression of the reporter in the transplanted cancer cells makes it impossible to follow the UPS in tissues of donor mice during proteasome inhibitor treatment or during disease progression. The recently developed Ub-Gly76Val–GFP mouse model can be used for these purposes, since it is a transgenic mouse model in which expression of the GFP reporter is driven by a powerful promoter in all tissues [22]. Histological analysis of tissues from reporter mice after intraperitoneal injection with proteasome inhibitors revealed clear accumulation of Ub-Gly76Val–GFP in the liver at low inhibitor levels, and at higher inhibitor levels, reporter accumulation was found in multiple tissues.

**Endoplasmic reticulum (ER)-associated degradation (ERAD) reporters**

The 26S proteasome is involved not only in degradation of aberrant cytosolic and nuclear proteins, but also in clearance of improper proteins that reside within the ER. Incorrect folding, or aberrant modifications, of proteins in the ER result in dislocation of these proteins from the ER to the cytosol, where they are recognized by E3 enzymes, ubiquitinated and degraded by the proteasome [23]. This process is known as ERAD. ERAD is part of the protein quality control machinery to which all proteins that pass the ER, such as ER-resident proteins, secretory proteins and cell surface receptors, are subjected.

MHC class I molecules are of importance in the recognition and elimination of virus-infected cells. In their attempts to avoid the cellular defence mechanism, some viruses have developed sophisticated mechanisms to block MHC class I antigen presentation. The human cytomegalovirus (CMV) expresses two proteins, US2 and US11, that interact with MHC class I molecules in the ER and target them for proteasomal degradation [24]. The removal of properly folded MHC class I heavy chain from the ER by these viral proteins resembles that of true ERAD substrates. A cellular model for MHC class I degradation was developed based on co-expression of viral US11 with FP-MHC class I (Figure 3). This model has been used for characterization of novel proteasome inhibitors and high-throughput screens [25,26].

CD3e is a well-established ERAD substrate that has been used in a number of studies dealing with the identification of proteins involved in degradation of ER proteins [27]. CD3e is a subunit of the T-cell receptor and, in the absence of
its binding partners, which is the case in all cells other than T-lymphocytes, this subunit is rapidly dislocated and subjected to proteasomal degradation. FP-tagged CD3ε/H9254 can be used for monitoring the efficacy of ERAD in mammalian cells (V. Menéndez-Benito and N.P. Dantuma, unpublished work). The weak ER staining observed in cells expressing CD3ε/H9254-FP is strongly enhanced on treatment with various proteasome inhibitors in a time- and dose-dependent manner.

Polypeptide motif reporters
Two screens in the budding yeast *Saccharomyces cerevisiae* resulted in the identification of a number of artificial degradation motifs that induced degradation requiring the E2s Ubc6/7. The libraries used in these screens were based on C-terminal fusions of the *lacZ* or *URA3* genes, with random oligopeptide expansions encoding stretches of 16–50 amino acids [28]. The screen was designed to search for sequence elements or structural motifs that could accelerate degradation of the LacZ and URA3 products in a wild-type strain, but were inactive in yeast lacking the Ubc6 and Ubc7 ubiquitin-conjugating (*ubc*) mutants. Interestingly, it has been shown that Ubc6 and Ubc7 also play an important role in degradation of ERAD substrates [23]. The CL1 sequence found in this screen encodes a strongly hydrophobic peptide motif. Characterization of the CL1 degradation suggested that recognition of this hydrophobic patch may be similar to recognition of the hydrophobic
membrane-spanning domains of proteins that have been dislocated from the ER [29]. Despite the fact that the CL1 degradation signal resembles ERAD substrates in some aspects, it is exclusively localized in the cytosol and nucleus and does not require the proteins important for ER dislocation of ERAD substrates [29]. Kopito and co-workers developed an FP-based reporter by introducing the CL1 degradation signal into GFP [30]. The GFP–CL1 fusion, dubbed GFPu, was rapidly degraded by the proteasome in mammalian cells. By introduction of nuclear localization signals or nuclear export signals, this reporter could be adapted to a compartment-specific reporter [31]. No further characterization of this reporter has been performed in mammalian cells and its dependence on ubiquitin, as well as its requirements for specific ubiquitination enzymes, remains to be resolved. However, it is tempting to speculate that in mammalian cells also, this reporter may share some of the targeting system with ERAD substrates.

The Deg1 signal of the yeast transcriptional repressor Matα2 is responsible for proteasomal degradation of this repressor [32]. The Deg1 signal encompasses the first 67 amino acids of Matα2. A hydrophobic patch located within an amphipathic helix is the prominent feature for this degradation signal. Interestingly, Deg1-mediated degradation of Matα2 was most prominent in the nucleus and was much slower in the cytosol. In yeast, a Deg1-GFP reporter provided with a nuclear localization signal was efficiently transported to the nucleus followed by proteasomal degradation (Figure 4). However, when the Deg1-GFP reporter lacked a nuclear localization signal and diffused freely in the cell, the half-life was extended 3-fold, indicating a compartment-specific turnover by the UPS of this reporter.

PEST reporters
In general, the destructive action of the 26 S proteasome is directed towards ubiquitinated proteins. There are, however, a few exceptions of proteins that do not require ubiquitination for proteasomal degradation. The most well-characterized protein displaying ubiquitin-independent degradation is ornithine decarboxylase (ODC). Turnover of ODC is triggered not by ubiquitination, but instead by association with its inhibitor antizyme, which facilitates binding of ODC to the proteasome and degradation. ODC is recognized by antizyme through the presence of a PEST sequence (i.e. a sequence rich in Pro, Glu, Ser and Thr amino acids). The ODC PEST sequence has been used to generate the first FP proteasome substrates. These reporters were not originally intended for monitoring UPS activity, but for accurate analysis of promoter activity. A general problem with the usage of GFP as a reporter for monitoring promoter activity is its long half-life. Due to this, GFP will accumulate over time, giving rise to high fluorescence intensities even in the presence of low promoter activities. Hence spatial and transient fluctuations in promoter activity will be difficult to follow with GFP. To solve this problem, Li and et al. [33] aimed to develop a GFP variant with a reduced half-life. A short-lived GFP fusion was generated by
positioning a small fragment of murine ODC at the N-terminus of GFP (Figure 5) [33]. By mutating the PEST sequence, a set of short-lived GFPs were generated, with half-lives ranging from 1–4 h in mammalian cells. It was shown that the PEST-GFP, also known as dGFP (destabilized GFP) was indeed an excellent transcriptional reporter and could reveal changes in nuclear factor κB (NF-κB) promoter activity with much greater sensitivity than stable GFP [33]. Although these reporters are not intended for monitoring the UPS, there is nothing to argue against their applicability in such an experimental set-up. Recently, PEST-GFP has been used for studying the effect of a disease-associated protein on the UPS [34]. On the other hand, some of the above-mentioned FP reporters originally developed for monitoring the UPS may be excellent transcriptional reporters. Especially when dealing with small changes in reporter levels, it is of great importance with each of these reporters to analyse whether the effects are caused by changes in degradation or synthesis [35]. Notably, the

**Figure 4. Schematic representation of reporters containing polypeptide degradation motifs**

C-terminal insertion in an FP of the artificial CL1 degradation motif results in degradation. The natural Deg1 motif of the Mata2 can induce nuclear degradation of FP.

**Figure 5. Schematic representation of PEST reporters**

PEST sequences are degradation signals that can target FP for ubiquitin-independent degradation by the proteasome, through binding to antizyme.
PEST-GFP reporters are the only reporters that are degraded in a ubiquitin-independent manner, and may provide us with additional information, other than can be obtained with the panel of ubiquitin-dependent reporters.

**Fluorescent reporters to monitor the UPS in diseases**

Alterations in protein degradation by the UPS are believed to be involved in several human pathologies [4]. A number of neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease and polyglutamine disorders, are caused by a change in conformation of the disease-related protein, leading to a non-native structure that has the tendency to form aggregates and inclusions. Reporters have been shown to be of particular use in studying the efficiency of the UPS in neurodegenerative and other conformational diseases [7]. Despite the fact that there are many indications suggesting a role of the UPS in neurodegenerative diseases [6], direct evidence is still lacking. This is largely because functional evaluation of the UPS has proven to be a major challenge, owing to the complexity of the system. Many studies involve biochemical assays, measuring only certain aspects of the UPS, and do not evaluate it as an integrated system. The usage of fluorescent UPS reporters can overcome these problems, since it gives a simple functional readout for the ability of the cells to cope with the continuous supply of substrates of the UPS. Moreover, the availability of several complementary reporters provides the opportunity to monitor a wide range of different ubiquitination pathways, making it possible to access the context of the UPS dysfunction.

Based on the finding that nuclear inclusions in neurodegenerative diseases contain ubiquitin and other components of the UPS, it has been postulated that the proteolytic pathway might be disturbed [6]. Indeed, a correlation between functional impairment of the UPS and inclusion formation was shown in a cellular model based on expression of the GFP-CL1 reporter substrate [30]. GFP-CL1 cell lines expressing the aggregation-prone proteins CFTRΔ508 mutant or mutant huntingtin, the protein responsible for Huntington’s disease, accumulated the reporter exclusively in cells containing inclusions. Accumulation of the GFP-CL1 reporter was also found in a number of similar investigations studying mutant rhodopsin, a protein linked to the inherited form of retinitis pigmentosa [36], mutant α-synuclein, which is associated with Parkinson’s disease [37], and mutant androgen receptor responsible for spinobulbar muscular atrophy [38]. Degradation of another reporter, PEST-GFP, was also inhibited by mutant ataxin-1, which is responsible for spinocerebellar ataxia type 1 (SCA1), suggesting that aggregation-prone proteins have a general inhibitory effect on the UPS [34]. Interestingly, the effect of mutant androgen receptor was strongly enhanced by the presence of transglutaminase, and was abrogated in the presence of a transglutaminase inhibitor, suggesting that not only aggregated proteins, but also primarily cross-linked proteins, may be responsible for UPS dysfunction [38].
mechanism responsible for impairment of the UPS function, however, remains elusive. A recent study suggests that it is not caused by direct inhibition of the proteolytic activity of the proteasome, or proteasome sequestration in inclusions [31]. GFP-CL1 reporters were specifically targeted to either the nucleus or the cytosol and the effect of cytosolic and nuclear inclusions on reporter degradation was investigated [31]. GFP fluorescence was found in both cellular compartments independent of the presence of inclusions, indicating that inclusions are not the direct cause of UPS impairment. Thus the mechanisms causing UPS impairment in these cellular models, as well as the nature of the inhibitory proteins, remain to be resolved.

The aberrant ubiquitin UBB⁺¹ is encoded by an abnormal transcript of the ubiquitin B gene that has a dinucleotide deletion owing to a process known as molecular misreading [39]. Although the transcript can be found in both normal and affected cells, the protein product exclusively accumulates in affected cells in a number of conformational diseases, such as Alzheimer’s disease [40]. In vitro studies showed that UBB⁺¹ can be polyubiquitinated and that the presence of ubiquitinated UBB⁺¹ inhibits in vitro degradation of proteasome substrates [41]. It was found in reporter cell lines expressing Ub-Gly76Val–GFP or Ub-Arg-GFP that UBB⁺¹ causes a general UPS dysfunction that correlates with cell-cycle arrest [17]. Interestingly, UBB⁺¹ itself is a UFD substrate of the UPS that is cleared in many cells by proteasomal degradation, while in some cells it accumulates and causes UPS dysfunction. The molecular mechanism of the inhibitory activity of UBB⁺¹ is unknown.

Recently, the role of the UPS in the pathophysiology of a neurodegenerative disease was addressed for the first time in vivo with the transgenic UPS reporter mouse model [35]. A knock-in mouse model of mutant ataxin-7, which is responsible for SCA7, was crossed with Ub-Gly76Val–GFP reporter mice and the levels of the GFP reporter were followed during the progression of the disease in the retina, which is the primary affected tissue in this pathology. No changes in reporter levels were found in the early phase of the disease, prior to inclusion formation, suggesting that a full blockade of the UPS is not directly responsible for the accumulation of mutant ataxin-7. Late in the pathology, an increase in the levels of the reporter was found in the photoreceptor cells of the retina. However, detailed analysis revealed that this was due not to delayed turnover of the reporter, but to increased transcription of the reporter transgene. This again emphasizes the fact that short-lived reporters are very sensitive not only to changes in degradation, but also to fluctuations in synthesis. Interestingly, it has indeed been reported that SCA7 pathology is accompanied by transcriptional dysregulation [42]. Moreover, an inverse correlation was found between accumulation of the reporter and ataxin-7 inclusions [35]. These data suggest a protective role of inclusions against neuropathology and exclude a full blockade of the UPS as a necessary step in polyglutamine neuropathology. The precise role of the UPS in different
diseases needs further investigation, as subtle changes in the UPS cannot be excluded based on these experiments.

The UPS as a therapeutic target in diseases

In vitro and in vivo studies suggest that inhibition of the proteasome may have a therapeutic effect in several diseases, including cancer and inflammation (see Chapter 14). Small compound inhibitors of the proteasome have been used for many years as experimental tools in laboratories [43]. It is well-established that cancer cells are sensitive to these inhibitory compounds and often induce cell-cycle arrest and apoptosis. Some proteasome inhibitors have originally been identified as compounds with anticancer activity, which was later assigned to their proteasome inhibitory activity [44]. This has inspired researchers to probe into the anticancer potential of proteasome inhibitors, which has resulted in the introduction of the proteasome inhibitor Velcade, also known as bortezomib or PS-341, into the clinics for the treatment of multiple myeloma [45].

Given the fact that the UPS is of crucial importance for most cells, the specific effect on cancer cells is surprising. It is tempting to speculate that inhibitors that are more specific, or target other events in ubiquitin-dependent proteolysis, can be used with greater precision and fewer side effects for the treatment of cancer and other diseases. To develop such new inhibitors, cellular models expressing the fluorescent UPS reporters could be extremely suitable in high-throughput screens of large chemical-compound libraries. Recently, FP-MHC-I/US11 reporter cells have been used to screen 16 320 compounds for inhibition of ERAD [25]. This has led to the identification of two compounds, named eeyarestatin I and II, which blocked dislocation of substrates from the ER membrane without affecting proteasomal degradation of cytosolic substrates. Transgenic-animal reporter models for the UPS can be used to evaluate further the bioavailability and therapeutic effects of compounds in vivo [21,22].

Conclusions

In this review, we have outlined the different FP reporters that are currently used for functional analysis of the UPS in cell lines and animals. With the help of these reporters, questions that were impossible to address only a short while ago can now be studied with great accuracy. The availability of mouse models, for the UPS in particular, opens up several new avenues. These model systems allow us, for the first time, to study the in vivo efficacy of the UPS during disease progression or administration of specific compounds, such as proteasome inhibitors. The collection of characterized FP substrates can be used for the generation of other UPS reporter mice, which will hopefully give us a more complete picture of the behaviour of different classes of UPS substrates in vivo. In order to manipulate and modify this crucial and delicate

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system in such a way that it is likely to impart a therapeutic effect, we have to understand fully the role of the UPS in these pathologies. This is a target that may now be in closer reach with the development of FP reporters for the UPS.

Summary

- **FP-based proteins with degradation signals can be used to monitor the efficacy of the UPS in cellular and mouse models.**
- **The collection of fluorescent reporters allows us to study the behaviour of different UPS substrates in parallel.**
- **FP reporters of the UPS have been used to study the status of the UPS in cellular and animal models for human diseases.**
- **Cellular and animal reporter models can be used for drug development, involving high-throughput screens for specific inhibitors of the UPS, and for studying the bioavailability of inhibitors.**

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