



# Reporter-Based Screens for the Ubiquitin/Proteasome System

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Instant and adequate handling of misfolded or otherwise aberrant proteins is of paramount importance for maintaining protein homeostasis in cells. The ubiquitin/proteasome system (UPS) is a central player in protein quality control as it operates in a seek-and-destroy mode, thereby facilitating elimination of faulty proteins. While proteasome inhibition is in clinical use for the treatment of hematopoietic malignancies, stimulation of the UPS has been proposed as a potential therapeutic strategy for various neurodegenerative disorders. High-throughput screens using genetic approaches or compound libraries are powerful tools to identify therapeutic intervention points and novel drugs. Unlike assays that measure specific activities of components of the UPS, reporter substrates provide us with a more holistic view of the general functional status of the UPS in cells. As such, reporter substrates can reveal new ways to obstruct or stimulate this critical proteolytic pathway. Here, we discuss various reporter substrates for the UPS and their application in the identification of key players and the pursuit for novel therapeutics.

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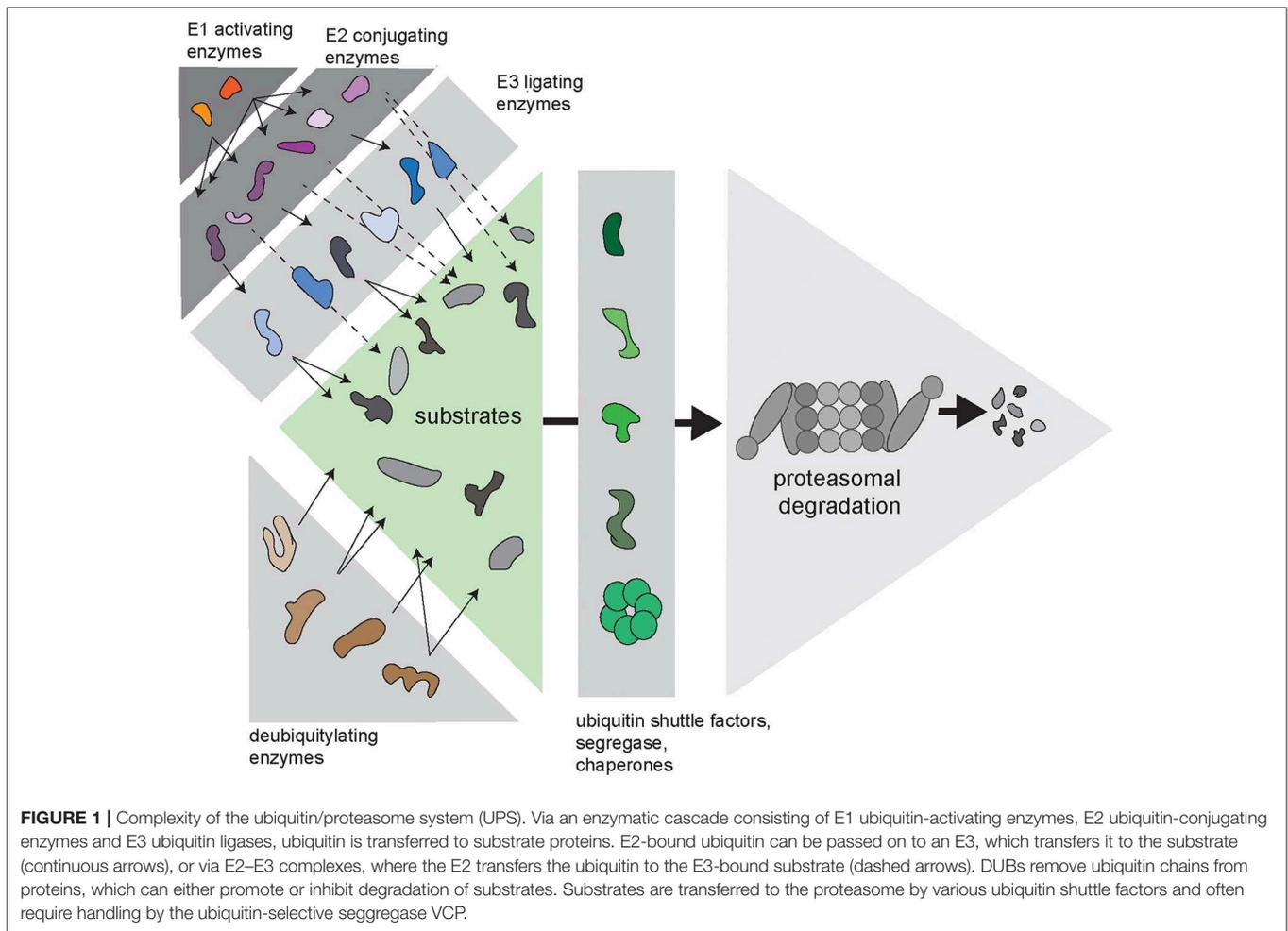
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## TARGETING THE UBIQUITIN/PROTEASOME SYSTEM (UPS)

Cells need to balance production, maintenance and degradation of their proteome throughout their entire lifespan, which may vary depending on the type of cell from hours to decades. Proteins that fail protein quality control are a potential risk for protein homeostasis and are targeted for destruction by the ubiquitin/proteasome system (UPS) (Ciechanover, 2005). Proteasome-mediated degradation is initiated by conjugation of the protein modifier ubiquitin to lysine residues of proteins designated for destruction. This initial ubiquitin moiety can be used for the assembly of ubiquitin chains, which are formed through ubiquitylation of one out of seven internal lysine residues in ubiquitin (Komander and Rape, 2012). Conjugation of ubiquitin to substrates is regulated by an enzymatic cascade consisting of E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (Figure 1). In some cases, specific E4 ubiquitin chain elongators are involved in extending the ubiquitin chains on substrates (Koegl et al., 1999). Subsequently, proteins are degraded into small peptide fragments inside the proteolytic chamber of proteasomes (Bard et al., 2018). Ubiquitylated substrates often require the unfoldase activity of the ubiquitin-targeted segregase valosin-containing protein (VCP), also known as p97, before they can be processed by the proteasome (Twomey et al., 2019). Moreover, several ubiquitin shuttle factors are responsible for the delivery of the substrate to the proteasome (Elsasser and Finley, 2005). To allow efficient degradation, ubiquitin chains have to be removed prior to degradation, which is facilitated by the POH1 deubiquitylating enzyme (DUB) at the entrance of the proteasome



(Verma et al., 2002; Yao and Cohen, 2002). In contrast, the proteasome-associated DUB USP14 can rescue proteins from degradation by removing ubiquitin chains before the proteasome has initiated degradation (Kraut et al., 2007).

Due to their hyperactive state and compromised genome integrity, cancer cells produce elevated levels of aberrant proteins. This phenomenon is believed to make them more susceptible to drugs that restrict the activity of the UPS (Bruning and Juckstock, 2015). Pharmacological inhibition of the UPS is typically accomplished by targeting the proteasome (Kisselev and Goldberg, 2001). Bortezomib was the first FDA-approved proteasome inhibitor and currently serves as a drug for first-line treatment of multiple myeloma and mantle cell lymphoma (Adams, 2004). Despite the fact that the successful introduction of proteasome inhibition for treatment of hematopoietic malignancies has provided the field with an encouraging proof-of-principle, other therapeutic strategies for UPS inhibition remain in an early exploratory stage. The few clinically approved UPS-targeting drugs are rather crude in their action as they all target the main chymotrypsin-like activity of the proteasome (Fricker, 2019). Concerns regarding the observed adverse effects, the development of resistance against proteasome inhibitors and

the poor activity of proteasome inhibitors toward solid tumors are strong arguments for the development of drugs directed against other targets within the UPS.

On the other hand, enhancing UPS activity may be desirable in diseases where accumulation of misfolded proteins is responsible for cellular dysfunction and decay, which is the case for a broad variety of neurodegenerative diseases characterized by accumulation of protein aggregates, such as Alzheimer's, Parkinson's and Huntington's disease as well as amyotrophic lateral sclerosis (Boland et al., 2018). As most compounds block catalytic activities of enzymes, pharmacological stimulation might potentially be more challenging. However, due to the complex nature of the UPS, overall stimulation may be feasible through inhibition of specific enzymes that slow down the process. In line with this notion, it has been shown that the USP14 inhibitor IU1 stimulates the degradation of aggregation-prone proteins, such as tau and TDP-43, both linked to neurodegenerative diseases (Lee et al., 2010).

One of the most daunting tasks in the development of new modulators of the UPS is the identification of proteins and processes that can be targeted. More than 40 E2 conjugation enzymes can pair with over 600 different E3 ligases, while around

100 DUBs are involved in the removal of ubiquitin chains. Moreover, a vast number of proteins is involved in coordinating this process, guiding substrates to the proteasome and prepare them for efficient degradation. It is hard to predict how inhibition of individual players will affect the overall efficacy of the UPS. Screening campaigns are often designed to interrogate the activity of a specific enzymatic target, which requires a preselection of a target-of-interest. An alternative approach are phenotypic assays that are based on the ectopic expression of engineered fluorescent UPS substrates, which lack a biological function but can be readily and quantitatively detected by their fluorescence (Neeffjes and Dantuma, 2004). The latter assays are unbiased and allow the identification of novel ways to modulate UPS activity without requiring *a priori* knowledge on the mode of action of the targets.

## UPS REPORTER SUBSTRATES

UPS reporter substrates are based on targeting an otherwise stable protein for proteasomal degradation through the introduction of a degradation signal (Neeffjes and Dantuma, 2004) (**Figure 2A**). Degradation signals, so-called degrons, are conserved motifs that target proteins for proteasomal degradation. One of the first identified degrons is the N-terminal amino acid of proteins (Bachmair et al., 1986). This was discovered by expressing fusion proteins with an N-terminal ubiquitin moiety, which will be proteolytically cleaved in cells, leaving the C-terminal protein with an amino terminus that corresponds to the sequence following the DUB cleavage site. Depending on the nature of the new N-terminal amino acid, it may function as a degron that recruits ubiquitin ligases and determines the half-life of the protein.

When the DUB cleavage of the N-terminal ubiquitin was prevented by substituting the final glycine of ubiquitin to valine (G76V), proteins were still found to be destabilized, but this time another set of proteins was involved in their recognition and degradation (Johnson et al., 1992). In these fusions, the uncleavable N-terminal ubiquitin is marked with ubiquitin chains that target it for proteasomal degradation (Johnson et al., 1995). This type of engineered proteins are known as ubiquitin fusion degradation (UFD) substrates.

Both the N-end rule and UFD degradation signals are versatile motifs that can be used to target most proteins-of-interest for degradation. Both degrons were used for the development of the first green fluorescent protein (GFP)-based reporter substrates that were expressed in cells (Dantuma et al., 2000) and mice (Lindsten et al., 2003). Expression of a luciferase carrying multiple UFD signals enabled also *in vivo* analysis of the effect of drugs on UPS activity in xenograft transplants in mice (Luker et al., 2003), while a UFD-targeted version of a photoconvertible fluorescent protein allowed determination of the half-lives of UPS substrates in living nematodes (Hamer et al., 2010), illustrating the potential of this approach.

Another engineered degradation signal that has been used for the generation of reporter substrates is a short C-terminal linkage (CL) referred to as CL1. The CL1 peptide was identified in a

yeast screen aimed at identifying peptide extensions that degrade proteins dependent on endoplasmic reticulum (ER)-anchored ubiquitin-conjugating enzymes involved in marking misfolded ER proteins for proteasomal degradation (Gilon et al., 1998, 2000). C-terminal tagging of GFP with CL1 resulted in a short-lived GFP, which has been used for generating cellular (Bence et al., 2001) and mouse UPS models (Bove et al., 2006; Liu et al., 2006). Fluorescent proteins destabilized by CL1 tend to aggregate most probably due to its hydrophobic nature (Menéndez-Benito et al., 2005; Link et al., 2006). As such, GFP-CL1 and related fluorescent reporters may be in particular suited to probe into the ability of cells to eliminate aggregation-prone proteins by proteasomal degradation.

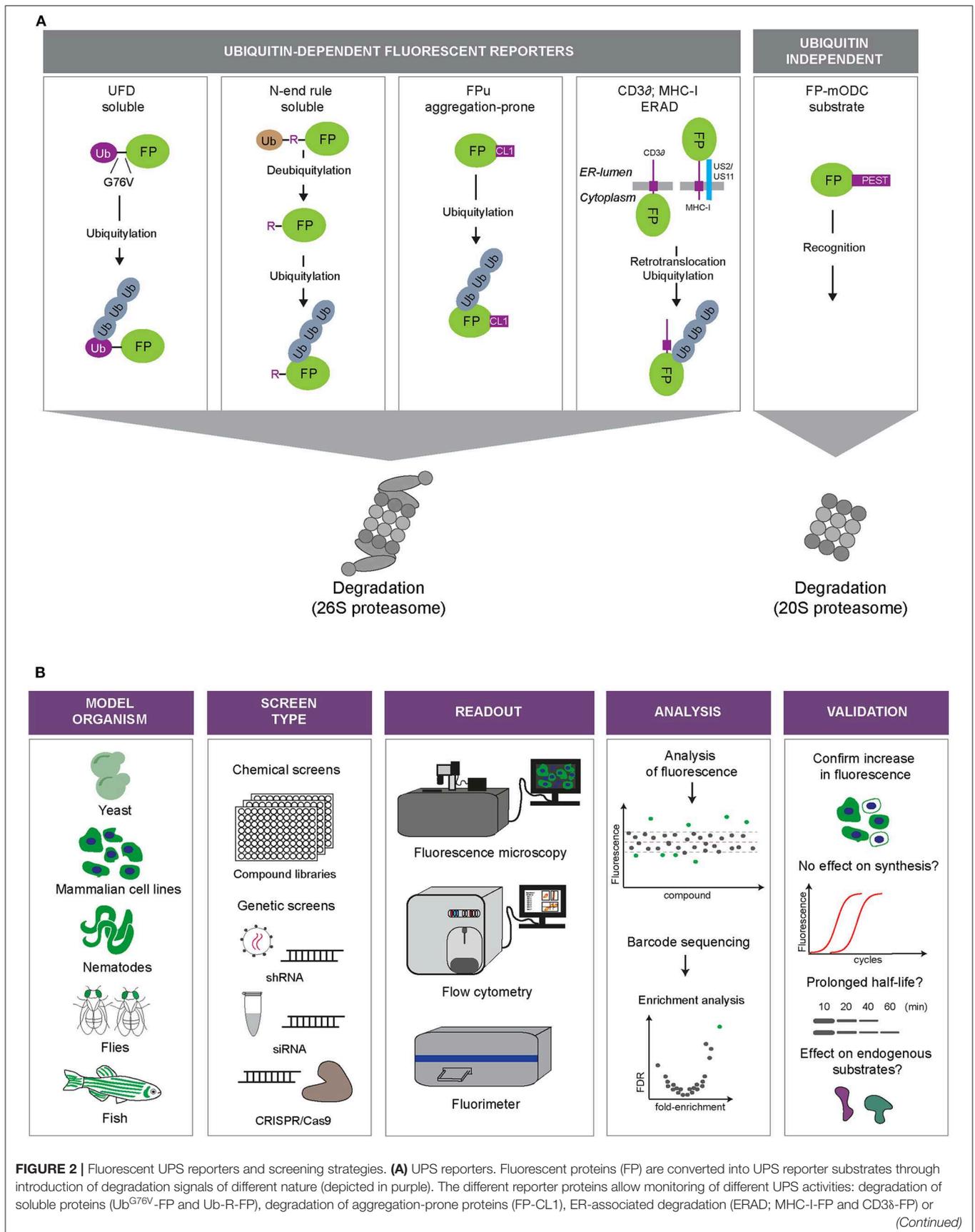
In addition to these engineered motifs, naturally occurring degradation signals have also been exploited to destabilize reporter proteins. Fluorescent proteins have been provided with natural degradation signals or fused to full-length proteasome substrates. A natural motif used for this purpose is the PEST sequence of ornithine decarboxylase (ODC). The ODC degradation signal has the special feature that it can target proteins for ubiquitin-independent proteasomal degradation, thereby bypassing the complex machinery for ubiquitylation (Hoyt et al., 2005).

Other full-length proteins that have been used for the generation of reporter substrates are the heavy chain (HC) of major histocompatibility complex (MHC) class I molecules, which, in the presence of the viral proteins US2 or US11, is rapidly dislocated from the ER into the cytosol, ubiquitylated and disposed by the proteasome (Schust et al., 1998). MHC-HC shares this pathway with other proteins targeted for ER-associated degradation (ERAD) (Bernier et al., 2018). Another commonly used ERAD reporter substrate is the T cell receptor subunit CD3 $\delta$ . When expressed in other cells than T lymphocytes, CD3 $\delta$  fusions are orphan subunits unable to find their binding partner, resulting in targeting of these fusions to the ERAD pathway (Yang et al., 1998).

While MHC class I and CD3 $\delta$  require ERAD proteins that facilitate identification of the reporter as aberrant in the ER and its translocation into the cytosol, the CL1-destabilized reporter substrates, which are cytosolic reporters, will only engage the final steps of the ERAD pathway. Thus, different UPS reporters display differential sensitivities for different branches of the UPS: the N-end rule and UFD substrates behave as soluble, properly folded proteins, CL1 mimics aggregation-prone proteins, CD3 $\delta$  and MHC class I HC are ERAD substrates and ODC-destabilized proteins report on the status of ubiquitin-independent degradation.

## UPS REPORTER-BASED SCREENS

A number of genetic and compound screens have been published in which the usage of UPS reporters played a central role. In these screens, transiently transfected or stably integrated reporters in mammalian cells served as read-outs for global changes in the UPS in high-throughput screens for genetic or chemical modulators (**Figure 2B**).



**FIGURE 2** | ubiquitin-independent degradation (FP-mODC). **(B)** Overview of the steps involved in high-throughput screens using fluorescent reporters of the UPS. Model: Fluorescent reporters are expressed in cell or animal models suited for high-throughput screening. Screening format: Compound libraries or genetic libraries can be used. Modulation of genetic expression can be achieved via siRNA, shRNA, or CRISPR/Cas9 approaches. Readout: Fluorescence microscopy, flow cytometry or fluorimetry can be used as a fluorescence readout or for sorting a specific cell population by fluorescence activated cell sorting (FACS). Analysis: Hits can be identified through readout of fluorescence intensity or sequencing of selected cells. Validation: Examples of various methods that can be used to validate hits.

Phenotypic assays based on reporter substrates have been used for the discovery of novel inhibitory compounds. A ChemBridge library consisting of around 16,000 compounds was screened using a cell line expressing the HC of enhanced green fluorescent protein (EGFP)-tagged MHC-I HC (EGFP-HC) and viral US2, resulting in targeting EGFP-HC for ERAD. This screen resulted in the identification of two structurally-related inhibitory compounds: Eeyarestatin 1 (Eer1) and 2 (Eer2) (Fiebiger et al., 2004). Follow-up studies revealed that Eer1 blocks EGFP-HC degradation by interfering with VCP-mediated segregation and suggested that this may be due to inhibition of the VCP-associated DUB ataxin-3 (Wang et al., 2008). In line with the model that Eer1 interferes with the function of VCP, Eer1 induced accumulation of a VCP-dependent reporter, while it did not interfere in the degradation of a VCP-independent substrate (Chou and Deshaies, 2011).

In another screening campaign for UPS inhibitors, the library of pharmacological active compounds (LOPAC) was tested in a high-throughput format using cells that expressed the ubiquitin-independent substrate ZsGreen-ODC. Surprisingly, disulfiram, an FDA-approved drug for treatment of alcohol addiction, was found to inhibit UPS activity and displayed cytotoxic effects on a myeloma cell line (Rickardson et al., 2007). Disulfiram had been previously found to interfere with NF- $\kappa$ B activity (Wang et al., 2003). This was later pinpointed to copper-dependent inhibition of the proteasome, whose activity is required for NF- $\kappa$ B translocation (Chen et al., 2006). In a more recent study, the ditiocarb-copper complex, a metabolite of disulfiram, was shown to also impair degradation of a UFD reporter substrate via inhibition of the VCP-adaptor protein NPL4 (Skrott et al., 2017), suggesting that disulfiram may modulate several targets within the UPS.

Genetic UPS screens are commonly based on manipulation of the gene expression in reporter cells using siRNA, shRNA or CRISPR/Cas9 technology. Cells expressing a UFD-destabilized reporter were used in a screen aimed at identifying proteins involved in the mammalian UFD pathway (Poulsen et al., 2012). An siRNA-based library targeting 558 genes was used in this screen. This led to the identification of several UFD components including HUWE1, a HECT domain ubiquitin ligase. A natural substrate of HUWE1 is UBB<sup>+1</sup>, an aberrant ubiquitin found in neurological and non-neurological protein misfolding disorders (Van Leeuwen et al., 1998). UBB<sup>+1</sup> has an uncleavable N-terminal ubiquitin moiety and resembles artificial UFD substrates (Lindsten et al., 2002). It is noteworthy that HUWE is overexpressed in lung, breast and colon carcinoma, suggesting also a possible role in tumorigenesis (Adhikary et al., 2005; Yoon et al., 2005; Kao et al., 2018).

A genome-wide CRISPR/Cas9 library was screened using a GFP-CL1-expressing cell line. Fluorescence-activated cell sorting (FACS) was employed to obtain a population enriched for cells with elevated GFP-CL1 levels, which were subsequently analyzed by barcode sequencing (Leto et al., 2019). This resulted in the identification of new genes of the ERAD ubiquitin conjugation machinery, including the ubiquitin ligase RNF139/TRC8 and ubiquitin-conjugating enzyme UBE3C. In a different screen, the near-haploid cell line KBM7 (Carette et al., 2009), stably expressing mCherry-CL1 was used to sort mCherry<sup>High</sup> cells by flow cytometry after insertional mutagenesis with a gene-trapping retrovirus (Stefanovic-Barrett et al., 2018). In addition to RNF139/TRC8, a second ER-resident E3 ligases, MARCH6, was found to function in ubiquitin-dependent degradation of soluble and tail anchored ER proteins.

In a UPS-specific and genome-wide siRNA-based screen, a fluorescently tagged, thermally unstable nuclear reporter was used to identify proteins involved in nuclear protein quality control (Pegoraro et al., 2012). The screen was performed by analyzing 384-well plates with automated fluorescence microscopy. Besides a number of hits that were anticipated, such as proteasome subunits, they found the proteasome assembly chaperone POMP (Burri et al., 2000; Fricke et al., 2007), and eIF3, a translation initiation complex (Abbott and Proud, 2004), to be important for efficient nuclear protein quality control.

Upon construction of two novel shRNA-based libraries, the fluorescent reporter ZsGreen-mODC was used to validate the functionality of these libraries (Paddison et al., 2004; Silva et al., 2005). Cells were transfected with an expression vector for the ZsGreen-mODC reporter together with either a plasmid library consisting of 7,000 unique shRNAs (Paddison et al., 2004) or a sub-library consisting of shRNAs specific for a large number of kinases and proteasome subunits shRNAs (Silva et al., 2005). The performance of the library and setup of the screen was confirmed as shRNAs directed against proteasome subunits were readily identified by accumulation of the reporter substrate. In this particular case, the UPS reporter were used as a fast and robust tool for validation of the screening libraries.

## CONCLUDING REMARKS

With the appearance of advanced techniques and equipment, high-throughput and high-content screenings have become attractive approaches for addressing biological questions and drug development. Many different fluorescent substrate reporters have emerged over the years in parallel with a better

understanding of the UPS and an increased awareness of the UPS as therapeutic target. A number of opportunities for optimizing these reporter assays and tailoring them to specific purposes remain. Although some UPS reporter mouse strains have been generated, it is obvious that mouse models are not suited for large-scale screening efforts. However, other animal reporter models, such as nematodes (Hamer et al., 2010), fruit flies (Pandey et al., 2007), and zebrafish (Imamura et al., 2012), open possibilities to perform genetic and compound screens on a larger scale in *in vivo* models.

In addition to UPS inhibitors, there is an emerging interest for UPS stimulators, which may be harder to identify with the currently available assays. Hence, there is a need for the development of novel reporters that are more suited for detecting an increase in UPS activity. A point of improvement may be the use of internal stable reference proteins, which have been already applied in some screens (Yen and Elledge, 2008; Yen et al., 2008; Wu et al., 2016). These reference proteins can correct for differences in synthesis of the reporter and may give a more robust readout, thereby reducing the number of false hits. Due to the relatively low steady-state levels of the presently available reporter substrates, detection of enhanced degradation in high content screens may be problematic, even in the presence of a stable reference protein. Two recent studies elegantly overcame this limitation by creating a system in which the expression of the reporter protein is repressed by a transcriptional regulator (Zhao et al., 2014; Zeng et al., 2019).

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- Fusion of the transcriptional repressor to a destabilizing signal results in an inverse correlation between the activity of the UPS and the levels of the reporter protein that are regulated by the repressor. However, the applicability of these reporter systems in high-throughput campaigns remains to be validated.
- Genetic screens and compound screens each have their own strengths and weaknesses and can complement each other in screening campaigns. Genetic screens may result in the identification of interesting but poorly druggable candidates, while compound screens may identify powerful drug-like compounds but encounter difficulties in target identification. Upon combination of these complementary approaches and adapting the assays to more disease-relevant settings, fluorescent reporters can be used to their full potential in the pursuit for novel ways of modulating the UPS in human diseases.

## AUTHOR CONTRIBUTIONS

MG, TG, and ND wrote the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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