Emerging drug development technologies targeting ubiquitination for cancer therapeutics

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A B S T R A C T

Development of effective cancer therapeutic strategies relies on our ability to interfere with cellular processes that are dysregulated in tumors. Given the essential role of the ubiquitin proteasome system (UPS) in regulating a myriad of cellular processes, it is not surprising that malfunction of UPS components is implicated in numerous human diseases, including many types of cancer. The clinical success of proteasome inhibitors in treating multiple myeloma has further stimulated enthusiasm for targeting UPS proteins for pharmacological intervention in cancer treatment, particularly in the precision medicine era. Unfortunately, despite tremendous efforts, the paucity of potent and selective UPS inhibitors has severely hampered attempts to exploit the UPS for therapeutic benefits. To tackle this problem, many groups have been working on technology advancement to rapidly and effectively screen for potent and specific UPS modulators as intracellular probes or early-phase therapeutic agents. Here, we review several emerging technologies for developing chemical- and protein-based molecules to manipulate UPS enzymatic activity, with the aim of providing an overview of strategies available to target ubiquitination for cancer therapy.

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Contents

1. Introduction

Ever since the discovery of ubiquitin (Ub) four decades ago (Ciehanover, Hod, & Hershko, 1978), this small protein has been linked to multiple cellular processes, including cell proliferation, development, immune responses, and numerous human diseases including cancer (Calistri, Munegato, Carli, Parolin, & Palu, 2014; Dantuma & Bott, 2014; Senft, Qi, & Ronai, 2018; Zinngrebe, Montinaro, Peltzer, & Walczak, 2014). Indeed, protein ubiquitination, the process of attaching Ub and poly-Ub chains to cellular substrates, is an integral and essential part of the cell machinery, and serves to modify, regulate, and degrade proteins (Fig. 1) (Swatek & Komander, 2016). Ubiquitination initiates through the activation of Ub by a Ub activating enzyme (E1), which adenylates the Ub C-terminus using its catalytic cysteine residue, resulting in a thioester bond. Another thioester reaction is required for the transfer of Ub onto a Ub conjugating enzyme (E2). The Ub-charged E2 enzyme is then recruited by a Ub ligase (E3), which is responsible for the transfer of Ub from its E2-conjugated state to the substrate protein.

To date, two Ub-specific E1 activating enzymes have been identified in humans (Groettrup, Pelzer, Schmidtke, & Hofmann, 2008; McHugh et al., 2018; Schulman & Harper, 2009), and >30 E2s have been found in humans (Morreale & Walden, 2016; Mulder et al., 2016). Humans also harbour approximately 600 E3 Ub ligases, which are classified into the homologous to E6-associated protein carboxy terminus (HECT) family, the really interesting new gene (RING) family and the U-box family (Morreale & Walden, 2016; Mulder, Harari, & Simon, 2008; Zhang & Sidhu, 2014). E3 ligases bind E2 enzymes thioesterified with Ub (E2~Ub) to mediate substrate ubiquitination through different catalytic mechanisms. The HECT E3 ligases form an intermediate thioester bond with Ub to its active site cysteine residue before transferring it to substrates. In contrast, lacking a catalytic cysteine, the RING and U-box E3 ligases facilitate Ub transfer by an allosteric mechanism (Chen et al., 2017; Ozkan, Yu, & Deisenhofer, 2005; Rotin & Kumar, 2009; Zheng & Shabek, 2017). Single or multiple mono-ubiquitination and polyubiquitination at the N-terminus or lysine residues in Ub are all possible modifications of protein substrates and will determine the fate of the protein (Calistri et al., 2014). Ubiquitination at residue K11 of Ub has been linked to endoplasmic reticulum-associated protein degradation (Locke, Toth, & Petroski, 2014); K29-linked ubiquitination has been associated with protein modification (Nucifora Jr. et al., 2016); K48-linked ubiquitination is well studied for its role in proteasomal degradation of cellular substrates (Calistri et al., 2014); and, K63-linked ubiquitination results in protein recruitment, autophagy and degradation through the lysosomal pathway (Erpapazoglou, Walker, & Haguenauer-Tsapis, 2014).

On the other end of the spectrum, the process of ubiquitination can be reversed by deubiquitinating enzymes known as deubiquitinases (DUBs) (Zhang & Sidhu, 2014). Humans encode seven different structural families of DUBs, which comprise almost 100 proteins in total (Hermanns et al., 2018; Wertz & Wang, 2019). Ub-specific proteases (USPs) represent the largest and best studied family, with close to 60 USPs currently described (Yuan et al., 2018). Less research has been reported for the other families, with the next best studied being ovarian
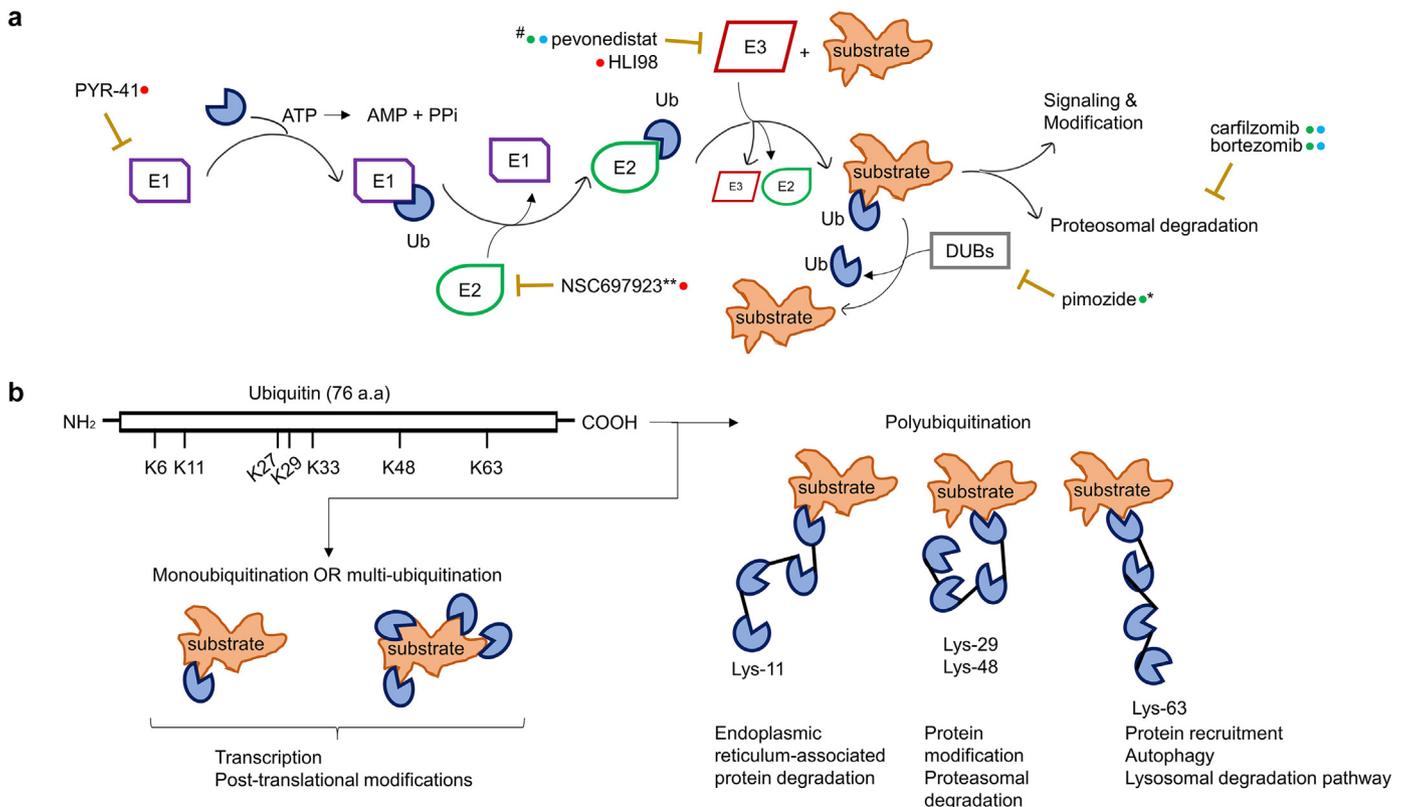

Fig. 1. Drug targets in the ubiquitin proteasome system. (a) Schematic representation of the ubiquitination pathway. Ubiquitination involves three steps. First, E1 activates ubiquitin (Ub) with ATP, forming a thioester bond. Next, activated Ub is conjugated to the E2 enzyme via a transthiolation reaction. Finally, RING and U-box E3 ligases recruit the E2 enzyme and a substrate to facilitate transfer of Ub from the E2 to the substrate by an allosteric mechanism, whereas HECT E3 ligases directly ubiquitinate the substrate followed by the transfer of Ub from E2 to the HECT E3 ligase active site (Chen et al., 2017; Zhang & Sidhu, 2014). The diagram also illustrates drug development targets currently in the market (green dot), in clinical trials (blue dot), or still being tested in industry or academia (red dot). *Pimozide is currently used in the clinic, but not for cancer therapy. # Previously known as MLN294 (Tong et al., 2017). ** Identified within a small-molecule screen as an inhibitor of Ubc13-Uev1A E2 Ub conjugating enzyme (Pulvino et al., 2012). (b) Ub is conjugated through lysines once (monoubiquitination) or multiple times (multiubiquitination), as a single unit or as a chain (polyubiquitination). Monoubiquitination and multiubiquitination processes have been linked to protein function modification, receptor endocytosis, nuclear export, transcription and histone regulation. Lys29- and Lys48-linked polyubiquitination has been shown to lead to proteasomal degradation as part of the UPS. Lys63-linked polyubiquitination has been implicated in the lysosomal pathway, protein recruitment, membrane internalization, endosomal uptake, and autophagy. Deubiquitination may also occur (Swatek & Komander, 2016).

tumor Ub proteases (OTU), followed by JAB1/MPN+/Mov34 domain (JAMM) metallo-enzyme proteases, Ub C-terminal hydrolases (UCHs), and Josephin domain proteases (Yuan et al., 2018).

Targeting the ubiquitination pathway has been an attractive avenue for anti-cancer drug development, especially after the approval and success of proteasome inhibitor drugs (Bortezomib and Carfilzomib) (Fig. 1) for treatment of relapsed or refractory multiple myeloma (Richardson et al., 2003; Richardson, et al., 2005). This has propelled many studies and clinical trials to find new therapeutics targeting individual components in the ubiquitination pathway for the treatment of cancer, with the aim of providing better therapeutics with fewer side effects (Table 1) (Chen, Frezza, Schmitt, Kanwar, & Dou, 2011; Iida et al., 2018; Yazbeck et al., 2018). Another important aspect to consider is the selective killing of cancerous cells rather than normal cells through targeting ubiquitination, as ubiquitination is upregulated due to the error-prone nature of cancerous cells and tissues (Gallo, Ko, & Donoghue, 2017; Ge et al., 2018). Indeed, Bortezomib and Carfilzomib have higher activity in malignant cells than in normal cells and tissues (Berkers et al., 2005; Codony-Servat et al., 2006; Parlati et al., 2009). Other inhibitors of UPS components have also been shown to uniquely target cancerous cells (Fiskus et al., 2014; Li et al., 2013; Pulvino et al., 2012; Tsukamoto et al., 2008). Finally, the resistant, refractive and recurrent traits of cancer have inevitably resulted in the turnover of promising drugs like Bortezomib, due to the development of resistance in response to treatment (Hamouda et al., 2014). Consequently, finding new compounds that can target oncogenic pathways is crucial to establish effective cancer therapies. Therefore, development of innovative strategies to validate and target UPS proteins remains an important aspect of drug discovery in cancer research.

2. Evolution of screening technologies for drug discovery

Before diving into recent advances in technology, we briefly describe the early stage of drug discovery in a historical perspective. Two crucial types of screening methods were pioneered and developed in the 1980s: target-based screens, which focus on a specific protein of interest – reviewed by Croston (2017), and phenotypic screens, which concentrate on the disease state to be treated – reviewed by Wagner (2016). Target-based high-throughput screening (HTS) strategies for drug discovery kicked off in the early 1980s (Pereira & Williams, 2007) (Fig. 2). Around the same time, researchers started to use phenotypic screens and genomic manipulation to paint a picture of cellular replication and the normal state of the cell, mainly in yeast as a model organism (Fraczek, Naseeb, & Delneri, 2018; Strynatka, Gurrola-Gal, Berman, & McMaster, 2018). The combination of these approaches in the late 1980s resulted in the birth of chemical genetics and led to many powerful developments such as directed evolution of enzymes, phage display of peptides and antibodies, and further refinement of target-based HTS (e.g. combinatorial chemistry, split-and-pool synthesis and fragment-based discovery) (Furka, Asgedom, & Diboa, 1988; Smith, 1985; Strynatka et al., 2018) (Fig. 2). From the late 1980s to the beginning of the 1990s, the use of animal models shifted to cell-based screens that minimized the number of reagents required and allowed the output of screens to increase (Strynatka et al., 2018) (Fig. 2). Research during these years also brought forth the generation of the first DNA-encoded compound libraries (DECLs) (Brenner & Lerner, 1992; Kinoshita & Nishigaki, 1995) (Fig. 2).

Within the span of 20 years, high-throughput technologies further advanced to include feats in protein engineering such as proteolysis-targeting chimeras (PROTACs) derived from the ubiquitination pathway (Zhou, Bogacki, McReynolds, & Howley, 2000), streamlined phage display approaches that include Ub variants (UbV) to target protein-protein interactions in the UPS (Brown et al., 2016; Ernst et al., 2013; Ernst & Sidhu, 2013; Gabrielsen et al., 2017; Ordureau et al., 2018; Zhang et al., 2016; Zhang et al., 2017; Zhang et al., 2017; Zhang & Sidhu, 2018) and cell-based pharmacological HTS assays to enhance oncolytic virus cancer-cell-killing efficiency through viral sensitizer screens (Bourgeois-Daigneault et al., 2016; Diallo et al., 2010) (Fig. 2). In less than ten years, from 1999 to 2008, HTS target-based and phenotypic approaches were pivotal for drug discovery, yielding 60% and 69% of new molecular entities and next-generation drugs tested in clinical trials, respectively (Swinney & Anthony, 2011). For instance, E2 Ub conjugating enzyme inhibitors were discovered through a phenotypic and target-based small molecule screen for

Table 1
Advantages and disadvantages of emerging target-based drug development technologies.

| Technology	Advantages	Disadvantages	Developments	Targets	References
Target-based High Throughput Screening (HTS)	■ Can deeply sample chemical space	■ Potential solubility problems ■ Intermediate and by-product reactive and aggregate species ■ Costly ■ Time-consuming	■ DNA-encoded compound libraries (DECLs) ■ Yoctoreactor ■ Rhodamine fluorometric assay	■ USP1 ■ USP9x	(Chen, Dexheimer, et al., 2011; Davydov et al., 2004; Dodge, 2016; Gavory et al., 2018; Gombodorj et al., 2017; Lee et al., 2016; O'Dowd et al., 2018; Tong et al., 2017; Yang et al., 2007; Yang et al., 2005)
Fragment-based HTS	■ More cost-effective ■ Samples chemical space more effectively	■ Potential solubility problems ■ Time-consuming	■ DECLs ■ Chemical synthesis (growing, ligating, merging) ■ Cell lysate-based assays (AlphaLisa) ■ Fluorometric assays ■ Indicator cell line	■ E1 activating enzyme ■ HDM2 E3 ■ CBL E3 ■ Ubc13-Uev1A E2 ■ USP1 ■ USP7 ■ USP9x	
Proteolysis-targeting chimeric molecule (PROTAC)	■ Precise degradation of target	■ Limited activity and complex composition (2nd generation PROTAC) ■ Cumbersome size	■ click-formed proteolysis targeting chimeras (CLIPTACs) offer a decreased size with maintained precision ■ "Greasy tags"	■ 26S proteasome (20S subunit) ■ MDM2	(Lebraud et al., 2016; Sakamoto et al., 2001; Sakamoto et al., 2003; Xie et al., 2014)
Protein design and engineering	■ Ease of manipulation and engineering	■ Lack of effective delivery vectors	■ Ubiquitin variants (UbVs)	■ USP7 ■ USP8 ■ HECT E3 ■ NEDD4L	(Ernst et al., 2013; Gabrielsen et al., 2017; W. Zhang et al., 2016; Y. Zhang et al., 2013)
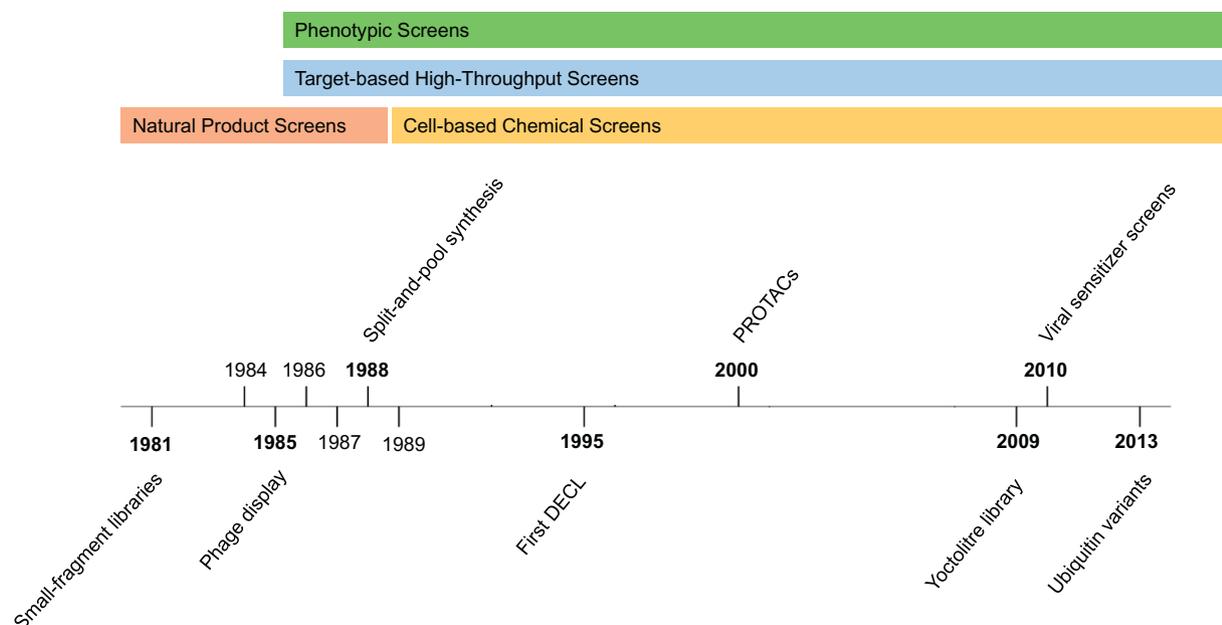
Fig. 2. Timeline of drug development technologies in high-throughput screening. Schematic representation of advances in chemical and biochemical libraries over the past four decades.

tuberculosis therapeutics (Darwin, Ehrt, Gutierrez-Ramos, Weich, & Nathan, 2003) (Fig. 1a and Table 1).

Since the ubiquitination pathways and UPS targets implicated in cancer drug development have been extensively reviewed (Kar, Keskin, Fraternali, & Gursoy, 2013; Li et al., 2018; Pal, Young, & Donato, 2014; Shen, Schmitt, Buac, & Dou, 2013; Wertz & Wang, 2019; Zhang & Sidhu, 2014), here we focus on addressing emerging trends and applications that can rapidly identify modulators for proteins within the ubiquitination pathway. The topics discussed below will examine the use of high-throughput technologies, small molecule approaches, peptide mimetics, and protein engineering. In addition, we will discuss potential applications for these technologies in cancer therapeutic research.

3. Targeting UPS proteins with chemical compounds

3.1. Constructing small molecule libraries

Research on chemical space (Jencks, 1981) stimulated the development of more ambitious target-based HTS approaches (Fig. 2). Drug-protein interactions were simplified into binding events, where the probability of binding would increase with the size of the molecule (Jencks, 1981). Previous HTS relied on assaying natural or synthetic compounds, which typically originated from tedious and time-consuming reactions of individual reagents (Pereira & Williams, 2007) (Fig. 2). With the advent of combinatorial chemistry, mixtures (instead of individual reagents) began to be employed to generate many more different compounds (Furka, 1995) (Figs. 2 and 3a). Later, Furka and colleagues introduced split-and-pool synthesis (Furka et al., 1988), in which the notion of combinatorial chemistry was further exploited to include separation and ligation of compounds. These separation and ligation reactions increased the number of potential combinations (Fig. 3a), which were estimated to total approximately 60 to 120 million chemical compounds globally (Hann & Oprea, 2004). This number has been exceeded now and million- to billion-compound libraries are routinely screened in industry (Davis, Plowright, & Valeur, 2017; Goodnow Jr., Dumelin, & Keefe, 2017; Litovchick et al., 2015). However, since the potential drug-like chemical space is estimated at 10^{63}, these numbers still barely scratch the surface (Davis et al., 2017).

Although combinatorial chemistry and split-and-pool synthesis sampled larger chemical space and revolutionized the way HTS was conducted for target-based drug discovery (Potyrailo et al., 2011), progress remains hampered by significant bottlenecks, including the expense of setting up an HTS system, starting reagents with poor properties, and the presence of reactive and aggregator molecules (Table 1) (Erlanson, 2012). In fact, in the early 2000s, compounds with poor properties like high lipophilicity were responsible for 50% of failed HTS projects (Keseru & Makara, 2009). Starter reagents that lack qualities such as solubility result in inefficient downstream drug candidates that might be impractical in a clinical setting (Keseru & Makara, 2009). Other problems such as reactive molecules and aggregators also delayed progress in target-based HTS (Erlanson, 2012). Reactive molecules or small impurities can oxidize proteins under the conditions used during HTS, resulting in the generation of hydrogen peroxide, which can inactivate proteins and cause false positives. Reactive molecules could arise from the possible combinations in split-and-pool synthesis, or simply as a side product of combinatorial chemistry (Erlanson, 2012). Finally, aggregators are a side effect of high concentration assays. At high concentration, intermolecular interactions of a compound can result in non-specific inhibition of the target protein, but the compound itself might not be a high affinity binder (Erlanson, 2012). This effect has been documented in past studies, where as many as 95% of compounds tested were identified as aggregators (Babaoglu et al., 2008). While these pitfalls remain as intrinsic caveats for small molecule libraries, activity-based small molecule screens have identified UPS inhibitors (Chen, Dexheimer, et al., 2011; Davydov et al., 2004; Gombodorj et al., 2017; Ott et al., 2017; Pulvino et al., 2012; Yang et al., 2007; Yang et al., 2005).

3.2. Activity-based small molecule screens for UPS inhibitors

The cumbersome bottlenecks described above prompted the use of approaches to utilize chemical space in more effective ways (Erlanson, 2012; Yuen & Franzini, 2017). In the activity-based screen approach, small molecules are screened to identify high affinity binders. In these screens, small molecules are added to cells, cell lysates, or in *vitro* reactions with recombinant proteins to test for inhibition of activity of a target protein or a change in phenotype. This can be detected through the presence or absence of fluorescence or luminescence (Janzen, 2014). Some of the most commonly used screening technologies include imaging or detection of: binding- or cleavage-based excitation of fluorescent probe-labeled proteins, fluorescence labeled antibodies targeting a
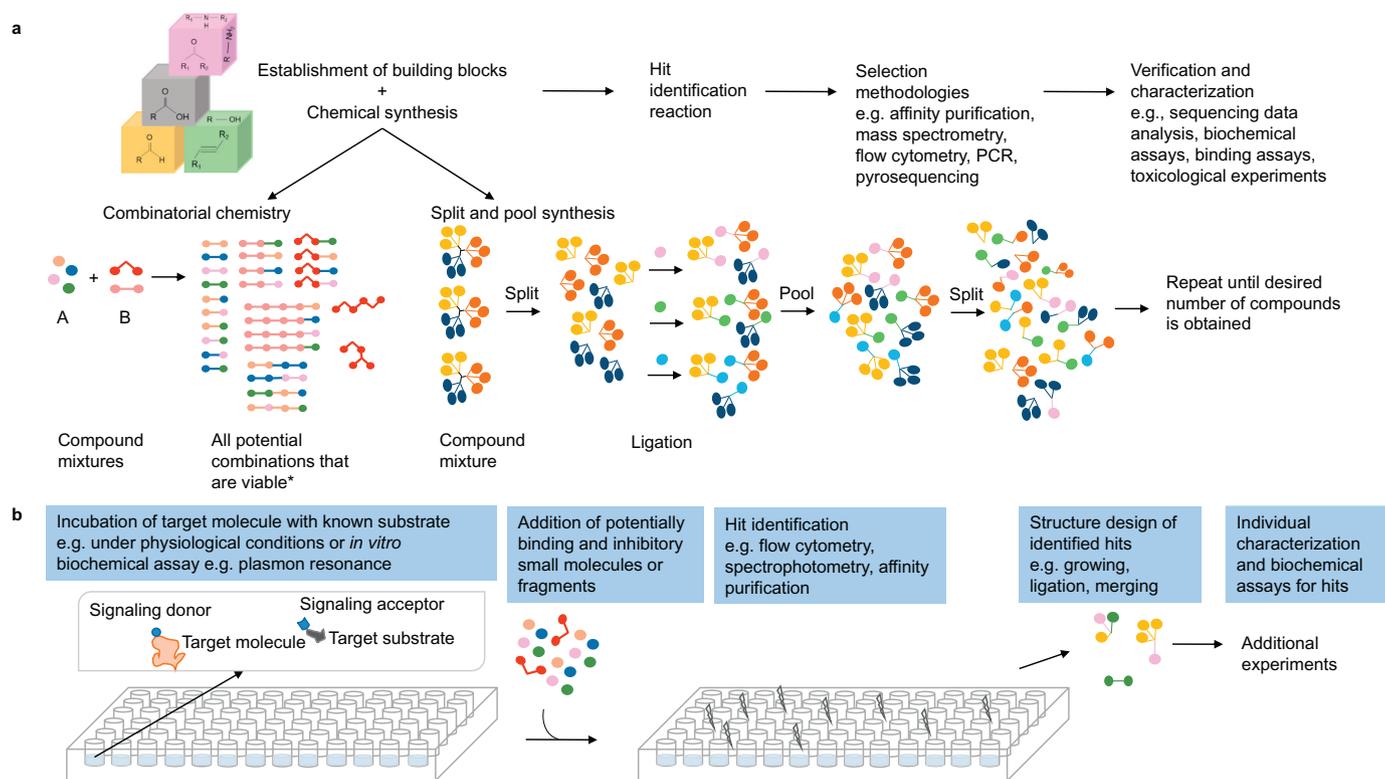
Fig. 3. Workflow of high-throughput screening for drug discovery. (a) Diagram illustrating the typical work required to identify and characterize drug candidates using a target-based high-throughout screening (HTS) or a DNA-encoded compound library (DECL) approach. Different types of chemical synthesis, such as combinatorial chemistry and split-and-pool synthesis, are depicted. (b) Schematic depicting a fragment-based approach to inhibitor development. *Not all potential combinations are represented here.

specific protein, and fluorescence resonance energy transfer (FRET) where one fluorophore emits energy and a proximal one absorbs this energy for excitation. Screens can also be conducted with the use of flow cytometry, which can measure the light scattered through a cell to determine phenotype or expression of fluorescent-labeled proteins within the cell, and with luminescence-based assays, which are similarly designed to the fluorescent imaging assays mentioned above (Janzen, 2014).

Below, we present several representative studies utilizing these screening methods to develop chemical compounds targeting UPS components of different protein families (Table 2). One group was able to identify two molecules, PYR-41 and HLI98 (Fig. 1), which inhibited the E1 activating enzyme Uba1 (Yang et al., 2007) and the RING-E3 ligase HDM2 (Yang et al., 2005), respectively, by first screening a commercial chemical library and then confirming the leads with purchased individual compounds (Table 2). This small-molecule library was previously developed by the Vousden group to target autoubiquitination of E3 ligases (Davydov et al., 2004). In this assay, small molecules were incubated in ubiquitination reactions with recombinant E1 and E2 (UbcH5B), E3 (HDM2), and Ub. An

Table 2
Ubiquitin proteasome system inhibitors identified through small molecule or fragment-based assays described in this review.

Inhibitor	Description	Potency (IC$_{50}$)	Screen	Target (s)	Testing	References
PYR-41	Pyrazone derivative	10 μM	Antibody against ubiquitinated proteins in auto-ubiquitination reaction high-throughput screen (HTS)	Uba1 (E1)	In vitro recombinant protein assay	(Davydov et al., 2004; Yang et al., 2007)
HLI98	7-nitro-5-deazaflavin compound	20 μM		HDM2 (HECT E3)		(Davydov et al., 2004; Yang et al., 2005)
Pevonedistat (MLN-4924)	Adenosine sulfamate mimetic	> 10 nM to <28 μM	Medicinal chemistry-based fine tuning of N6-benzyl adenosine inhibitor identified via HTS	E1 pan inhibitor	Clinical trials	(Chen, Tsu, et al., 2011; Soucy et al., 2009)
NSC697923	nitrofuran	~1 μM	luciferase reporter cell line	UBE2N/Ubc13 (E2)	In vitro cellular assay	(Cheng et al., 2014; Gombodorj et al., 2017; Hodge et al., 2015; Pulvino et al., 2012)
Pimozide	diphenylbutylpiperidine	~2 μM	small-molecule fluorometric assay with rhodamine-labeled Ub substrate	USP1	In vitro cellular assay (cancer) Clinic (Schizophrenia)	(Chen, Dexheimer, et al., 2011; Seeman, 2002)
Bortezomib	Pyrazine and boronic acid derivative	~100 nM	Target and cell-based screen	26S proteasome inhibitor	Clinic	(Adams et al., 1999; Nocentini, Supuran, & Winum, 2018)
N/A (numbered, not named)	Heterocyclic Pyrimidinones	< 10 nM to <30 nM	Small library surface plasmon resonance (SPR) screen followed by medicinal chemistry	USP7	In vitro cellular assays	(Gavory et al., 2018; O'Dowd et al., 2018)

electrochemiluminescence (ECM) labeled antibody targeting ubiquitinated proteins was subsequently added. The authors proposed that reactions with significantly reduced ECM represented small molecule hits inhibiting HDM2 enzymatic activity (Davydov et al., 2004; Yang et al., 2005; Yang et al., 2007). During validation of these hits, PYR-41, a pyrazone derivative (Yang et al., 2007), was found to target the E1 enzyme Uba1, and inhibit its activity with an IC_{50} of approximately 10 μM (Yang et al., 2007). HLI98, a compound from a newly identified 7-nitro-5-deazaflavin family (Davydov et al., 2004; Yang et al., 2005), was shown to target HDM2 E3 ligase activity with an IC_{50} of approximately 20 μM (Yang et al., 2005). To our knowledge, off-target effects and intracellular efficacy have yet to be thoroughly assessed for HLI98. The promiscuous nature of the assay in that it detects ubiquitinated proteins and the high IC_{50} value suggest that other cellular targets of HLI98 may exist.

Another E1-inhibitor, MLN-4924 or pevonedistat (Fig. 1 and Table 2), is an adenosine sulfamate mimetic (Chen, Tsu, et al., 2011). Penovedistat was developed from a medicinal chemistry approach aiming to improve on a previously discovered inhibitor, N6-benzyl adenosine, from a high-throughput screen (Soucy et al., 2009). Pevonedistat was originally identified as an inhibitor of NEDD8 activating E1-ubiquitin activating enzyme 3 (Uba3) complex (Soucy et al., 2009) and was later labeled as a pan-inhibitor of E1 activating enzymes (da Silva et al., 2016; Gavin et al., 2014; Wertz & Wang, 2019). Soucy et al. reported potent inhibition of Uba3 in the single-digit nanomolar range with cross-reactivity against other E1s in the low micromolar range (Soucy et al., 2009). Pevonedistat is currently being tested in clinical trials of patients with acute myeloid leukemia, where the principal side effect seems to be liver toxicity and sepsis due to disruptions in the GTPase RhoA cytoskeleton protein and tumor necrosis factor (TNF)-α (Swords et al., 2017; Swords et al., 2018).

E2 inhibitors were also identified using a luciferase reporter cell line, in which inhibitor-mediated inactivation of the target protein resulted in loss of luciferase expression (Fig. 1 and Table 2). In this study, a small molecule, the nitrofuran NSC697923, inhibited an E2 Ub conjugating enzyme, UBE2N/Ubc13 (in complex with UBE2V1/Uev1A) by preventing thioester bond formation between the E2 and Ub, and consequently, supressed NF-κB activity in active B-cell lymphoma cells (Gombodorj et al., 2017; Pulvino et al., 2012) (Fig. 1). Another study also identified NSC697923 as an inhibitor of Ubc13 in neuroblastoma cells (Cheng et al., 2014) (Fig. 1). NSC697923 has been shown to inhibit Ubc13 at concentrations of ~1 μM (Hodge et al., 2015).

Another group developed the first described fluorometric cell lysate-based assay to mirror physiological conditions for small molecule screens (Ott et al., 2017). They targeted UCHL1, a DUB that is over-expressed in many cancers (Bishop, Rocca, & Henley, 2016), as a proof of principle for developing DUB inhibitors with a cell lysate-based assay that they called AlphaLISA (Ott et al., 2017). HA-tagged UCHL1 was labeled with an alpha-streptavidin donor bead and probed with biotin-tagged Ub. Binding of Ub to the DUB would result in fluorescence, and conversely, no fluorescence would occur in the presence of an inhibitor (Ott et al., 2017). The screen yielded a series of compounds that inhibited DUB activity in the 5–20 μM range, and some had been reported previously, including isogambonic acid, celastrol, mangiferin and rifampicin (Ott et al., 2017).

Inhibitors of the DUB USP1, which has been implicated in the Fanconi anemia signalling pathway in the DNA damage response (Bergink & Jentsch, 2009; Huang et al., 2006; Nijman et al., 2005), were identified with a small-molecule fluorometric assay that employed a Ub substrate modified with rhodamine (Chen, Dexheimer, et al., 2011), which emitted fluorescence upon cleavage (Dang, Melandri, & Stein, 1998). This simple *in vitro* assay could be used in a high-throughput fashion to screen for both small-molecule inhibitors and previously reported larger inhibitors (Dang et al., 1998). The study identified both novel small-molecule compounds as well as approved drugs, such as pimozide (a diphenylbutylpiperidine), as inhibitors of USP1 (Chen, Dexheimer, et al., 2011) (Fig. 1 and Table 2). In non-small cell lung carcinoma cells, pimozide was shown to reverse the chemo-resistance observed with cisplatin (Chen, Dexheimer, et al., 2011), a commonly used chemotherapy drug (Bloemink & Reedijk, 1996; Dasari & Tchounwou, 2014) with known resistance in Fanconi Anemia (Wang, 2007). Interestingly, pimozide is an anti-psychotic drug commonly prescribed to patients suffering from schizophrenia (Seeman, 2002), and although it has been postulated to act as a postsynaptic dopamine receptor blocker, its mechanism remains unknown (Seeman, 2002). Another study confirmed that pimozide could inhibit USP1 and in turn decrease the clonal growth of glioblastoma cells as well as increase their sensitivity to irradiation, though they are commonly resistant to chemotherapeutic and irradiation treatments (Lee et al., 2016). Notwithstanding this success, the potency of pimozide (IC_{50} ~2 μM) is lower than those of clinically approved UPS inhibitor 26S proteasome inhibitor Bortezomib (IC_{50} ~100 nM) (Fig. 1 and Table 2), and another UPS inhibitor VLX1570, which inhibited the DUBs UCHL5 and USP14 (IC_{50} ~100 nM) and was previously studied in clinical trials but terminated due to limiting toxicities (study identifier NCT02372240) (ClinicalTrials.gov, 2018; Wang et al., 2016).

While in general activity- and target-based small molecule screens have provided innovative approaches that make the process of drug discovery more economical and efficient, for most UPS targets, these types of screens yielded compounds with sub-optimal pharmacological properties (e.g. biophysics, solubility, and cellular permeability) and fall short in terms of potency and specificity (Lill & Wertz, 2014). We postulate that the intrinsic structural and catalytic properties of UPS proteins pose an impediment to generation of active-site inhibitors. For example, HECT E3s contains a shallow active site and are subject to dynamic regulation including extreme inter-domain rotations accompanying catalysis (Zheng & Shabek, 2017). On the other hand, the conserved nature of the DUB catalytic pocket probably accounts for the observed low specificity of inhibitors targeting the cysteine active site (Wang et al., 2018). Indeed, almost all of the UPS small molecule inhibitors currently in the clinic or clinical trials function in an allosteric manner. For instance, Nutlins disrupt substrate (p53) binding to the RING-E3 ligase MDM2 to stabilize p53 protein level and transcriptional activity (Vassilev et al., 2004), and Smac mimetics compete with Caspase binding to the BIR domain of RING-E3 IAP (inhibitor of apoptosis protein) to stimulate apoptosis in cancer cells (Liu et al., 2000; Wu et al., 2000). Finally, other than proteasome inhibitors like Bortezomib and Carfilzomib, there is only one family of drugs (Thalidomide/Lenalidomide) targeting UPS components approved for treatment of cancer, specifically for multiple myeloma and other B-cell neoplasms. Thalidomide was once well known for its adverse effects on fetus development before re-purposing for cancer therapy through binding to an E3 ligase Cereblon (CRBN) and stimulating lymphoid transcription factor ubiquitination and degradation (Ito et al., 2010; Kronke et al., 2014; Lu et al., 2014). Therefore, further development of *allosteric* modulators for UPS components will likely lead to more effective and efficacious cancer therapeutics.

3.3. Fragment-based drug discovery of potent and specific USP7 inhibitors

What distinguishes fragment-based approaches from other small-molecule HTS assays is the further fine-tuning of small-molecule screens. Once fragments are identified as binders of the target protein, researchers conduct medicinal chemistry to grow, ligate and merge the fragments into potent and selective inhibitors (Erlanson, 2012; Lamoree & Hubbard, 2017) (Fig. 3b). In this approach, the focus is not on activity, but rather in discovering potential allosteric inhibitors. As such, the approach is not to detect the fragment binders in an environment where the protein might be active, but to first detect whether the fragment binds to the target protein, and customize the fragment via medicinal chemistry based on information gathered through biophysical analyses. In an elegant example, the fragment-based approach was

used to develop inhibitors of USP7 (Gavory et al., 2018) (Table 2). First, surface plasmon resonance (SPR) was used to screen a small library of ~2000 molecules targeting the catalytic domain of USP7, which is responsible for regulating MDM2, the dominant Ub ligase for p53 (Gavory et al., 2018; Iwakuma & Lozano, 2003). What distinguished this work from previous studies, which pioneered the first USP7 inhibitors (Kategaya et al., 2017; Pozhidaeva et al., 2017), was the use of medicinal chemistry to tailor initial screening hits. The inhibitory fragments were used to grow six different compounds that retained the high affinity of the original fragments, setting these results apart from the poor pharmacological properties and low potencies previously obtained (Kategaya et al., 2017; Pozhidaeva et al., 2017). The new USP7 inhibitors were also free from reactive species and were not aggregators. Importantly, this work identified a new allosteric inhibitory site on USP7, implying that affinity-based screens rather than activity-based screens may reveal more druggable sites. Another group also used a similar approach to develop USP7 inhibitors with high potency (Turnbull et al., 2017). The work exemplified in these two studies is particularly relevant for producing compounds that are active against DUBs, where other methods have proven unsuccessful (Wrigley et al., 2011).

The researchers that developed the allosteric USP7 inhibitors followed up with a study in which they described in detail the fragment-based medicinal chemistry approach to identify novel USP7 inhibitors (O'Dowd et al., 2018). This study demonstrated that employing SPR, a commonly used technology to detect binding (Erlanson, 2012), can be first used to identify potential allosteric binders of the protein, before continuing with X-ray crystallography to enable structure-based design (O'Dowd et al., 2018), an approach that has been responsible for many drugs developed to date (Erlanson, 2012; Lamoree & Hubbard, 2017). With both studies, the authors showed that structure-based design can aid with the elucidation of the mechanisms of action of novel inhibitors and can provide new insights into the functions of complex proteins with additional regulatory sites beyond the active site. We believe that this will have important implications for further development of inhibitors of other DUBs.

3.4. DNA-encoded compound libraries (DECLs)

The innovation of DECLs may transform hit identification of allosteric binders of UPS proteins into a faster and more manageable process thanks to the tagging of molecules with oligonucleotides. In HTS assays where mixtures of small molecules are used, or when traditional combinatorial chemistry is employed, the disentanglement of hits is usually arduous and time consuming. However, with DECL technology, inhibitor leads can be identified by rapid and efficient methods, including the polymerase chain reaction (PCR), deep DNA sequencing, and mass spectrometry. Ensemble Therapeutics has presented data describing the use of DECLs to develop macrocycle compounds (Mullard, 2016) that selectively and potently inhibit USP9x (Dodge, 2016), a DUB that is associated with many cancers (Murtaza, Jolly, Gecz, & Wood, 2015). However, the results were presented in a conference proceeding, and without further information, the ultimate outcome of this work remains unclear. Nonetheless, we predict that there will be more screens employing DECLs for inhibition of DUBs and other UPS targets.

DECLs comprise combinatorial molecules, small molecules or fragments that are concatenated to oligonucleotides (Fig. 4) (Mannocci, Leimbacher, Wichert, Scheuermann, & Neri, 2011; Yuen & Franzini, 2017). The oligonucleotides can be DNA or peptide nucleic acid (PNA) (Fig. 4a). The greatest advantage of DNA is its capacity for amplification. Though not amplifiable, PNAs have the advantages over DNA of being more stable and compatible with solid-phase synthesis. PNA libraries are also more amenable to use with combinatorial chemistry and split-and-pool synthesis methods (Zambaldo, Barluenga, & Winssinger, 2015) (Fig. 4b). It has been reported that

DECLs can accommodate up to 10^{16} compounds (Goodnow Jr. et al., 2017). The Hansen group described a yoctoliter (10^{-24})-scale DNA reactor to decrease the amount of material needed to deeply sample chemical space for highly efficient and unbiased molecular evolution (Hansen et al., 2009). The combinatorial chemistry field continues to produce novel strategies for generating DECLs in a cost-effective manner.

Because DECLs are subject to the same processes described above for combinatorial chemistry and split-and-pool synthesis, they are also susceptible to some of the same pitfalls, such as the presence of side reaction intermediates and by-products that can affect interactions and lead to false positives. As such, the generation and downstream purification of these libraries prior to use in HTS is of utmost importance. The chemistries applied in these processes are beyond the scope of this review, but they have been reviewed elsewhere (Favalli, Bassi, Scheuermann, & Neri, 2018; Franzini et al., 2015; Goodnow Jr. et al., 2017; Li, Zheng, Liu, & Li, 2015).

DECLs are typically made as single or dual pharmacophores (Fig. 4b). Single pharmacophores can be synthesized with DNA-recorded or DNA-templated methods. In DNA-recorded methods, compounds are coupled to single-strand (ss) or double-strand (ds) oligonucleotides, and after each coupling, additional DNA barcodes corresponding to each individual compound are ligated, followed by amplification to generate dsDNA. DNA-templating methods involve coupled DNA-compound moieties, which interact with each other via barcode complementarity; once in close proximity, coupling linkers are cleaved to release compounds that react with each other to generate cyclical compounds linked to DNA barcodes. Encoded Self-Assembled Chemical (ESAC) libraries, also known as dual or complex pharmacophore libraries, initiate with compounds coupled to ssDNA; other compounds are coupled to spacers that are used to hybridize two DNA-encoded compounds, followed by amplification that results in the formation of dual pharmacophore compounds coupled to dsDNA.

DECLs are more cost-effective than fragment-based libraries, since many of the methodologies utilized for HTS of DECLs can be performed on the bench without large, dedicated equipment (Yuen & Franzini, 2017). A tagged target protein can be immobilized to a solid support, and following incubation with the DECL, small molecules coupled to DNA or PNA that bind to the target can be enriched via tag-specific methodologies. Following elution, the small molecules can be identified via PCR or sequencing in the case of DNA-encoded libraries, or via mass spectrometry, SPR or NMR, in the case of PNA-encoded libraries (Mannocci et al., 2011) (Fig. 4c). Amplification can also be carried out *in situ* (Yuen & Franzini, 2017).

Regardless of the particular approach, inhibitor development with small-molecule HTS approaches depends on the solubility of chemical moieties (Erlanson, 2012). This has prompted the use of phenotypic screens to initialize screening (Wagner, 2016), and is one of the driving forces behind the field of peptidomimetics, which aims to utilize favorable qualities of peptides, including aqueous solubility, membrane permeability and internalization capacity, while maintaining the ability to interact with proteins and avoiding degradation through proteolysis (Vagner, Qu, & Hruby, 2008). Peptide libraries have also been generated via solid-phase synthesis (Bessette, Rice, & Daugherty, 2004; Kenrick & Daugherty, 2010; Ryvkin et al., 2018; Sidhu, 2000), by covalently linking peptides to a resin and conducting the synthesis in the immobilized form (Fields, 2002). Other than solid-phase peptide synthesis, peptide libraries have been typically generated with the use of bacteria or phage display. For example, antimicrobial peptides continue to be identified through screening peptides expressed in bacteria (Tucker et al., 2018). One study applied phage display to isolate potent competitive peptides for the E2 binding site of HECT E3 ligases that inhibited HECT E3 ligases by oxidizing the active site cysteine (Mund, Lewis, Maslen, & Pelham, 2014). Phage display, in particular, like DECL libraries with fragments, can be harnessed to screen for high-affinity binding polypeptides of immobilized target proteins in a high-
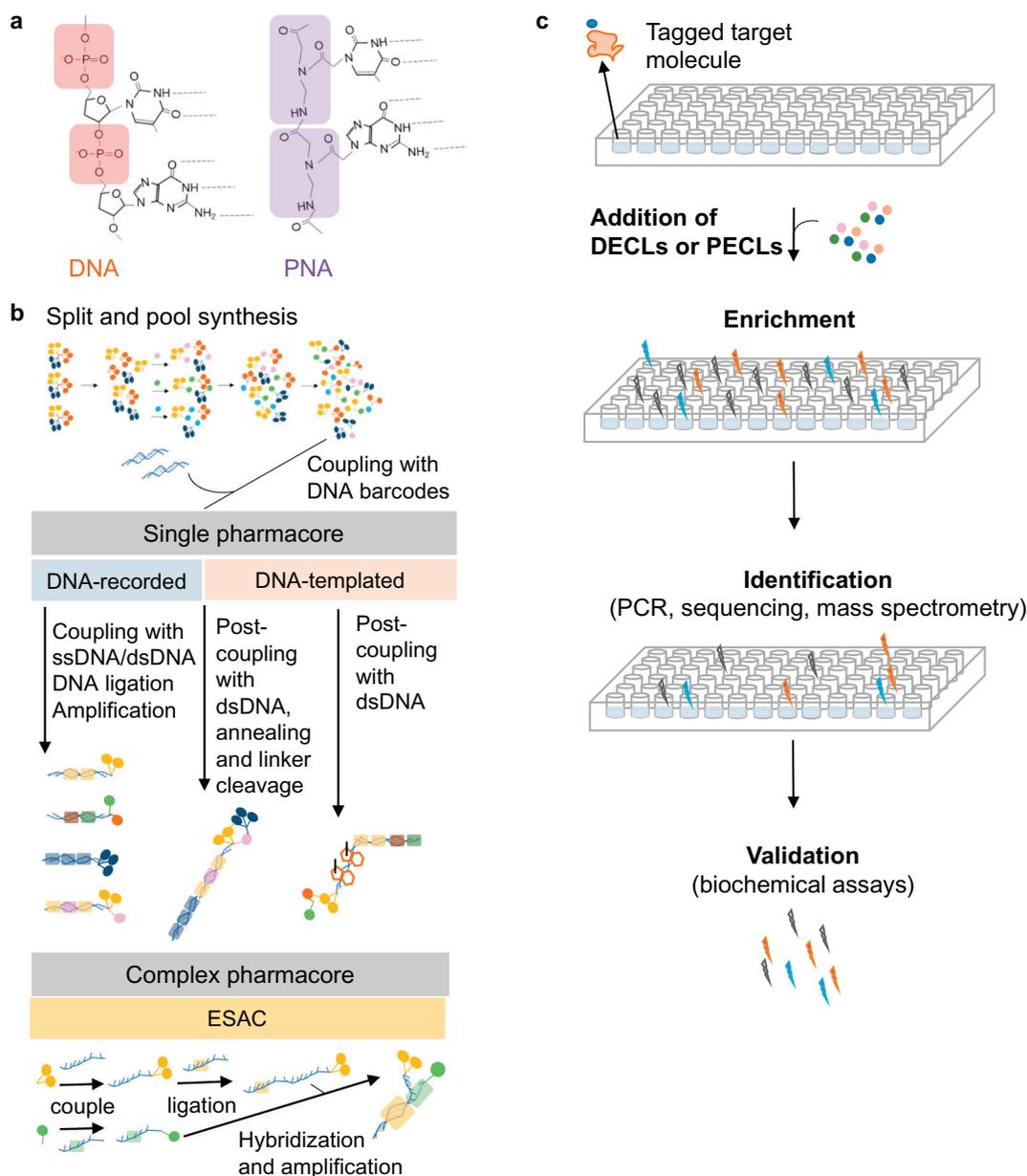
Fig. 4. DNA-encoded compound libraries (DECLs). Approaches for using DECLs for drug discovery are similar to those developed for combinatorial chemistry and split-and-pool synthesis, as reviewed by (Favalli et al., 2018; Franzini et al., 2015; Goodnow Jr. et al., 2017; Li et al., 2015). (a) Compounds are typically developed by combinatorial or other synthesis methods, and then coupled to specific DNA sequences or PNA chains. (b) Schematic representation of DECLs. DECLs typically begin by split-and-pool synthesis and are assembled differently depending on whether they are single pharmacophores (DNA-recorded and -templated) or dual pharmacophores [Encoded Self-Assembled Chemical (ESAC)]. DNA-recording involves the coupling of small molecules to single-stranded (ss) or double-strand (ds) oligonucleotides prior to the ligation of DNA barcodes and amplification. DNA-templated approaches involve similar processes to DNA-recorded, but this time already coupled DNA-compound moieties interact with each other via complementarity; in proximity, linkers coupling the compound to the DNA are cleaved, allowing the compounds to react and generate cyclical moieties. Dual pharmacophore libraries consist of ESAC libraries. In these libraries, like in DNA-recorded, compounds are first coupled to ssDNA. Hybridization occurs thanks to the spacer region of one moiety. Amplification is then used to generate dsDNA. (c) Illustration showing a representative screen where target tagged-proteins are immobilized. Tag-based affinity methods allow for washing off impurities and non-binders to enrich for hits. Screening is conducted via amplification or mass spectrometry of barcoded compounds with DNA or PNA, respectively.

throughput manner (Zhang, Ben-David, & Sidhu, 2017; Zhang & Sidhu, 2014).

4. Targeting UPS proteins with synthetic proteins

A major obstacle to the development of small molecule inhibitors for UPS components is the high structural similarity and functional redundancy among proteins in a particular family (Renatus et al., 2006; VanDemark & Hill, 2002). To overcome this limitation, knowledge of the Ub pathway and phage display technologies were merged to produce Ub variants (UbVs) as potent and highly selective inhibitors and

activators of UPS enzymes (Ernst et al., 2013; Ernst & Sidhu, 2013; Zhang, Sartori, et al., 2017; Zhang & Sidhu, 2014).

Ub interacts weakly but specifically with thousands of structurally similar proteins, via a common binding surface (Husnjak & Dikic, 2012) and its intrinsic conformational heterogeneity (Lange et al., 2008). In addition, Ub is a highly stable protein that lacks cysteine residues and can be easily produced recombinantly, making it an ideal scaffold for generating inhibitors of proteins in the UPS (Job et al., 2015).

Via protein engineering, several groups have generated UbVs with remarkable potency, specificity and cellular activity for the modulation of the enzymatic activities of UPS proteins. Indeed, given its

small size, lack of disulfide bonds, and structural stability, Ub represents an ideal scaffold for the next generation of protein therapeutics (Job et al., 2015).

4.1. UbV inhibitors of deubiquitinases

UbVs are capable of specifically inhibiting DUBs *in vitro* and *in vivo*. By mutating residues in the Ub core and on the surface, researchers at Genentech developed a UbV that bound tightly to USP7 and inhibited activity in cells, resulting in enhanced MDM2 ubiquitination and p53 stabilization (Zhang et al., 2013). Our group also developed an inhibitor of USP7 (UbV.7.2, IC_{50} values in the low nM range) by employing surface mutations only. Notably, UbV.7.2 proved to be more potent, and most importantly, much more selective than Genentech's UbV, showing that core mutations may reduce specificity and thus should be employed with great care. UbV.7.2 inhibited USP7 in cancer cells, and

consequently, caused dramatic reductions of MDM2 levels and induction of apoptosis, in synergy with cisplatin, suggesting that UbVs could be used as enhancers of chemotherapeutic drugs (W. Zhang, Sartori, et al., 2017). We also developed a UbV targeting USP10, a second DUB that deubiquitinates p53, and showed that the UbV promoted export of p53 from the nucleus to the cytoplasm (Zhang, Sartori, et al., 2017).

Recently, we developed UbVs for all four Ub-binding domains of USP15 and engineered potent bivalent inhibitors of the enzyme by linking a UbV targeting the DUSP domain to a UbV targeting the catalytic domain (Teyra et al., 2019) (Fig. 5a). These dimeric UbVs exhibited enhanced specificity and inhibition of USP15 catalytic activity, and acted as inhibitors of the transforming growth factor β (TGF- β) signalling pathway in cells (Teyra et al., 2019). We also utilized UbVs to reduce the abundance and activity of cell surface receptors by inhibiting USP8, an enzyme that regulates levels of epidermal growth factor receptor (EGFR) and other receptors (Fig. 5b) (Ernst et al., 2013). Moreover,
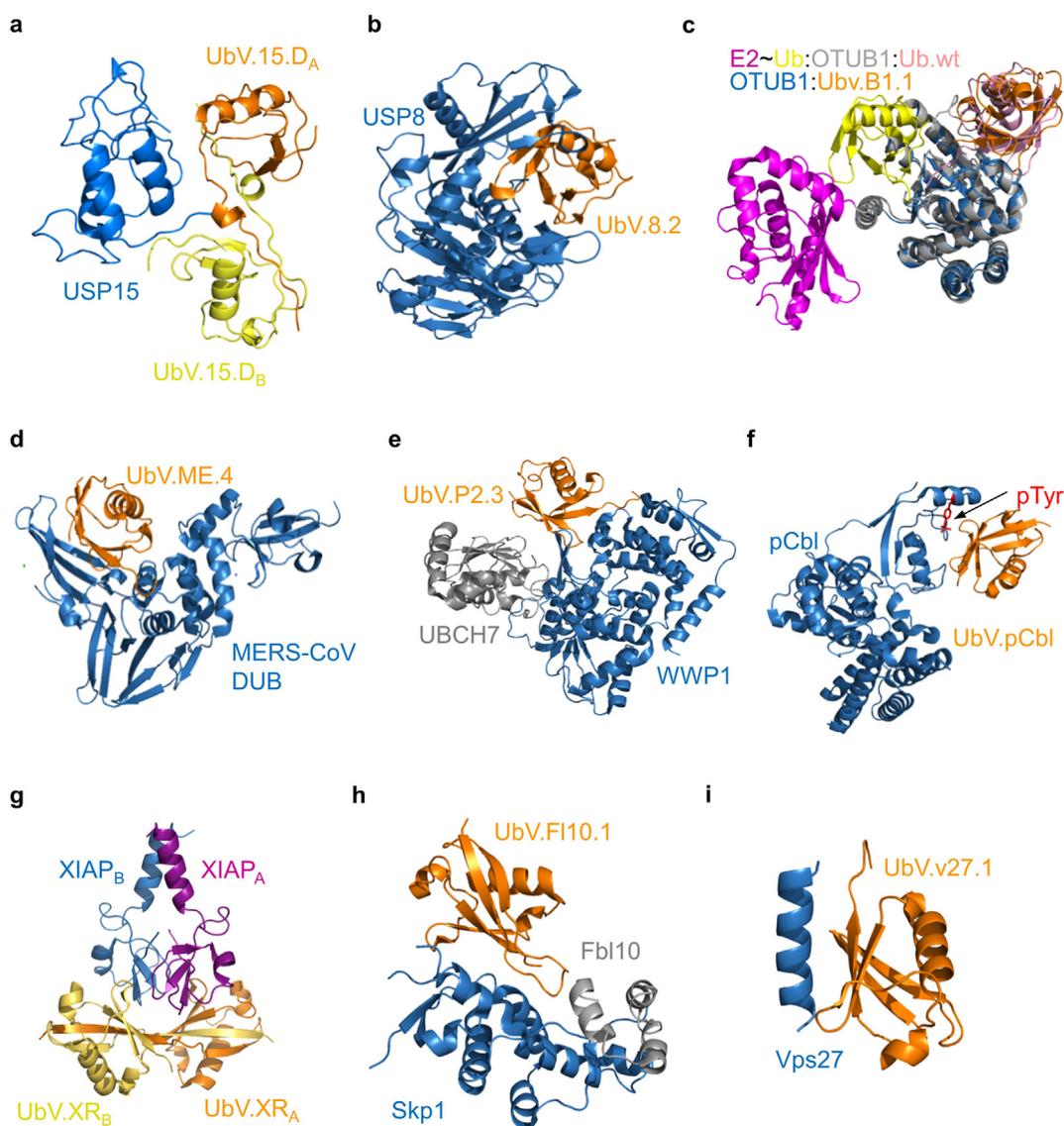

Fig. 5. Ubiquitin variants as modulators of the ubiquitin proteasome system (UPS). The cartoons depict crystal structures of UPS proteins in complex with UbVs. (a) Dimeric UbV.15.D (orange and yellow) complexed with the DUSP domain of USP15 (blue) (PDB entry 6DJ9). (b) USP8 (blue) in complex with the inhibitor UbV.8.2 (orange) (PDB entry 3N3K). (c) OTUB1 (blue) in complex with Ubv.B1.1 (orange) (PDB entry 4I6L). The structure is superposed with OTUB1 (gray) in complex with distal wild-type Ub (Ub.wt, pink) and UbcH5b E2 ligase (purple) covalently conjugated to Ub (yellow) (PDB entry 4DDG). (d) The Middle East Respiratory Syndrome (MERS) virus' USP (blue) in complex with the inhibitor UbV. ME.4 (orange) (PDB entry 5 V69). (e) HECT E3 ligase WWP1 (blue) in complex with the inhibitor UbV.P2.3 (orange) and E2 enzyme UbcH7 (gray) (PDB entry 5HPT). (f) Phosphorylated Cbl (pCbl, blue) in complex with the inhibitor UbV.pCbl (orange) (PDB entry 5O76). The phosphorylated tyrosine residue in pCbl is represented as red sticks. (g) The XIAP RING domain dimer (blue and purple) in complex with the dimeric activator UbV.XR (orange and yellow) (PDB entry 5OT). (h) Fbl10-Skp1 (gray and blue, respectively) in complex with the inhibitor UbV.Fl10.1 (orange) (PDB entry 6BVA). (i) UIM-1 of yeast Vps27 (blue) in complex with UbV.v27.1 (orange) (PDB entry 5UCL).

we recently established a Yeast-Two-Hybrid (Y2H) screening platform (Bruckner, Polge, Lentze, Auerbach, & Schlattner, 2009) for the *in vivo* selection of UbVs able to inhibit the cellular catalytic activity of USP2 (Pascoe et al., 2019), a DUB involved in the protection of prostate cancer from apoptosis (Priolo et al., 2006). Extending beyond USP DUBs, we targeted OTUB1, a member of the OTU DUB family, with a UbV that bound distal to the active site (Fig. 5c), but nonetheless, potently inhibited K48-linked di-Ub cleavage (Ernst et al., 2013).

In the past, we have also employed UbVs as inhibitors of pathogenic DUBs. In order to promote infection and replication in host cells, while evading the immune response, many human pathogenic viruses have evolved the ability to hijack and manipulate the UPS (Luo, 2016). For example, the Ub-specific protease of the herpes simplex virus 1 antagonizes NF-κB activation, whereas the E6 oncoprotein of the human papillomavirus recruits host E3 ligases to promote Ub-dependent degradation of p53 and consequent carcinogenesis (Hoppe-Seyler, Bossler, Braun, Herrmann, & Hoppe-Seyler, 2018; Ye, Su, Xu, & Zheng, 2017). We developed highly selective and potent UbVs targeting the DUBs of the Middle East respiratory syndrome coronavirus (MERS-CoV) and the Crimean-Congo hemorrhagic fever virus (CCHFV), two emergent and globally important viruses (W. Zhang, Bailey-Elkin, et al., 2017). UbV.ME.4 bound with subnanomolar affinity to the MERS-CoV DUB by establishing a wide network of hydrophobic packing and hydrogen bond interactions (Fig. 5d). Cell-based assays showed that UbV.ME.4 efficiently suppressed the DUB-induced activation of IFN-β promoter, thereby providing efficient blockade of viral replication and reducing MERS-CoV progeny titer by ~10,000-fold (W. Zhang, Bailey-Elkin, et al., 2017). Moreover, Han et al. recently highlighted the interplay between the HECT E3 ligase WWP1 and the VP40 matrix protein of Ebola virus in promoting viral budding from the host cell (Han et al., 2017). Therefore, Ub-based modulators of the UPS may enable the generation of novel antiviral drugs.

Saturation mutagenesis scanning experiments (Maynard, Chen, Georgiou, & Iverson, 2002) performed with distinct UbVs targeting the structurally similar catalytic domains of USP2 or USP21 enabled elucidation of the interaction landscape of Ub with the USP family (Leung, Dekel, Shifman, & Sidhu, 2016; Pascoe et al., 2019). Thus, UbVs can be used not only as inhibitors of DUBs, but also, as model molecules to identify alternative target sites for the development of small-molecule inhibitors that can target structurally conserved proteins with high specificity.

4.2. UbV inhibitors and activators of E3 ligases

HECT E3 ligases, which play crucial roles in cancer, neurological disorders and viral infections (Scheffner & Kumar, 2014), contain a HECT domain that forms a covalent bond with Ub (through a catalytic cysteine) before transferring it to a substrate protein. Understanding of the diverse HECT family has been limited by a paucity of selective and potent modulators. Despite enormous efforts, development of synthetic molecules targeting HECT E3s has been hampered by large conformational changes accompanying catalysis, a shallow active site, and dynamic regulation of activity. To overcome this limitation, we systematically developed UbV inhibitors and/or activators for 20 of the 28 human HECT E3s (Zhang et al., 2016). Structural analysis of UbVs targeting HECT E3 ligases revealed that UbV inhibitors acted by blocking the binding site for E2 ligase rather than by blocking the active site (Fig. 5e) (Zhang et al., 2016), further demonstrating that the plasticity of the Ub fold is amenable for the development of UbVs with novel biochemical properties. Surprisingly, a number of UbVs enhanced catalytic activity of HECT E3 ligases (Ernst et al., 2013; Zhang et al., 2016). For example, UbVs that bound tightly and selectively to NEDD4 increased intracellular ubiquitination of the NEDD4 substrate Ying-Yang1 (Ernst et al., 2013) and inhibited the migration of cancer cells by promoting the ubiquitination of Ras homolog gene family member B (RhoB)

(Zhang et al., 2016). UbVs also modulated the activity of membrane-bound ion channels by either activating or inhibiting NEDD4L (Zhang et al., 2016). UbVs that activated or inhibited NEDD4L reduced or increased cell-surface levels of the epithelial sodium channel (ENaC), respectively (Zhang et al., 2016). Since elevated ENaC levels are associated with hypertension (Ronzaud et al., 2013), and suppression of this ion channel has been linked to Liddle's syndrome, novel therapies for hypertension could be developed by targeting NEDD4L with molecules that modulate ENaC levels at the cell surface (Aziz, Memon, Rahman, & Ali, 2016).

With ~600 members, the RING family accounts for >90% of human E3 ligases. In contrast to HECT E3s with an active catalytic cysteine, the RING domain acts as an inert scaffold that recruits E2 enzymes thioesterified with Ub (E2~Ub) and facilitates Ub transfer to substrates. Due to the lack of a catalytic cysteine, inhibition of RING function has been challenging. We identified a UbV targeting the RING domain of the E3 ligase Cbl (UbV.pCbl), which reduced the internalization of EGFR while sustaining Akt pathway activation (Fig. 5f) (Gabrielsen et al., 2017). Remarkably, UbV.pCbl bound tightly to phosphorylated Cbl (pCbl), but not to non-phosphorylated Cbl, demonstrating that UbVs can recognize post-translationally modified proteins with high specificity (Gabrielsen et al., 2017). Unexpectedly, we also isolated a dimeric UbV (UbV.XR_D) that recognized the RING domain of the E3 ligase XIAP with high specificity and stimulated ligase activity *in vitro* and in cells (Gabrielsen et al., 2017). The structure of UbV.XR_D in complex with XIAP revealed that UbV.XR_D forms a domain-swapped dimer that stabilizes the E3-E2~Ub conformation, resulting in enhanced activity of the E3 ligase (Fig. 5g) (Gabrielsen et al., 2017). Additionally, we utilized the UbV technology to target a Ub-binding exosite on the RING domain of the anaphase-promoting complex subunit 11 (APC11) (Brown et al., 2016; Yamaguchi et al., 2016) to probe the biochemical mechanisms of the large, 13-subunit cell-cycle regulatory anaphase-promoting complex (APC/C). In sum, this work provided a general strategy to inhibit or activate RING E3 ligases to modulate these enzymes in order to understand their catalytic mechanisms and biological functions.

UbV inhibitors have also been developed for members of the multisubunit Skp1-Cul1-F-box (SCF) E3 ligase family, and these have been shown to affect the mitotic cell cycle progression of cancer cells (Fig. 5h) (Gorelik et al., 2016; Gorelik et al., 2018). Structural analysis of a UbV in complex with its target Skp1-Fbw7 revealed extensive contacts with both Skp1 and the F-box and showed that the UbV inhibited activity by blocking the binding site for Cul1 (Gorelik et al., 2016). Structure-based engineering of a loop on the UbV enabled the development of specific inhibitors for 17 different members of the SCF E3 ligase family (Gorelik et al., 2018). Finally, the UbV technology has been extended to target non-catalytic Ub-binding domains, exemplified by the small, helical Ub-interacting motif of the yeast protein Vps27 (Fig. 5i) (Manczyk et al., 2017).

4.3. UbV inhibitors of non-UPS proteins

Engineered UbVs have also been developed to target proteins that are not known to associate with Ub. Hoffmann et al. used ribosome display (Zahnd, Amstutz, & Pluckthun, 2007) to develop a UbV that bound to and inhibited the activity of tumor necrosis factor alpha (Hoffmann et al., 2012). Similarly, we recently isolated UbVs that bound specifically to the human epidermal growth factor receptor 3 (HER3) and the growth factor receptor-bound protein 2 (Grb2) and showed that UbVs acted as potent antagonists of Grb2-mediated cell signalling (Leung, Jarvik, & Sidhu, 2017).

We also developed a potent and highly selective UbV inhibitor of the tumor suppressor p53-binding protein 1 (53BP1), which recognizes ubiquitinated histone H2A and acts as a central regulator of the double-strand break repair pathway (Fradet-Turcotte et al., 2013). Expression of the UbV in either human or mouse cells prevented the

accumulation of 53BP1 at double-strand break sites, stimulating gene conversion and enhancing the efficiency of CRISPR-Cas9 dependent gene editing (Canny et al., 2018). This work highlighted the robustness of protein design and engineering with the Ub scaffold and the potential for targeting DNA damage signalling proteins for applications beyond the UPS.

In summary, UbV technology represents a robust platform for the rapid development of intracellular probes for target discovery and validation. Delivery of UbVs to cells represents a major obstacle for their direct use as therapeutics, but the constant advancements in delivery technologies for folded proteins has the capacity to unlock the potential of UbVs as drug candidates.

5. Harnessing the power of ubiquitination for selective target degradation

5.1. Proteolysis-targeting chimera (PROTAC)

Drug discovery campaigns traditionally focus on the development of tight and specific small molecules that act as inhibitors by blocking binding pockets or active sites of disease-associated proteins. Although

this approach has proven successful (Salami & Crews, 2017), it has been limited by the number of druggable protein targets, accounting for only ~3% of the human proteome (Dixon & Stockwell, 2009; Landry & Gies, 2008; Rask-Andersen, Almen, & Schioth, 2011; Santos et al., 2017; Uhlen et al., 2015). In fact, while nuclear receptors, enzymes, ion channels and G-protein-coupled receptors represent the majority of current drug targets, the human proteome is mainly composed of proteins involved in the organization, maintenance and assembly/disassembly of protein complexes that regulate signal transduction events and other critical functions. Therefore, to expand the druggable protein space, researchers have employed alternative approaches that allow targeting of transient protein-protein interactions rather than deep grooves to which small molecules can bind.

This paradigm shift focuses on the use of small molecules to control the cellular levels of drug targets through their precise degradation rather than the inhibition of their activity. This shift from target inhibition to induced target destruction is exemplified by proteolysis-targeting chimeras (PROTACs), small molecules that recruit a ubiquitin E3 ligase for protein degradation (Lucas & Ciulli, 2017; Neklesa, Winkler, & Crews, 2017; Toure & Crews, 2016). In fact, PROTACs are hetero-bifunctional molecules composed of a ligand for a target protein
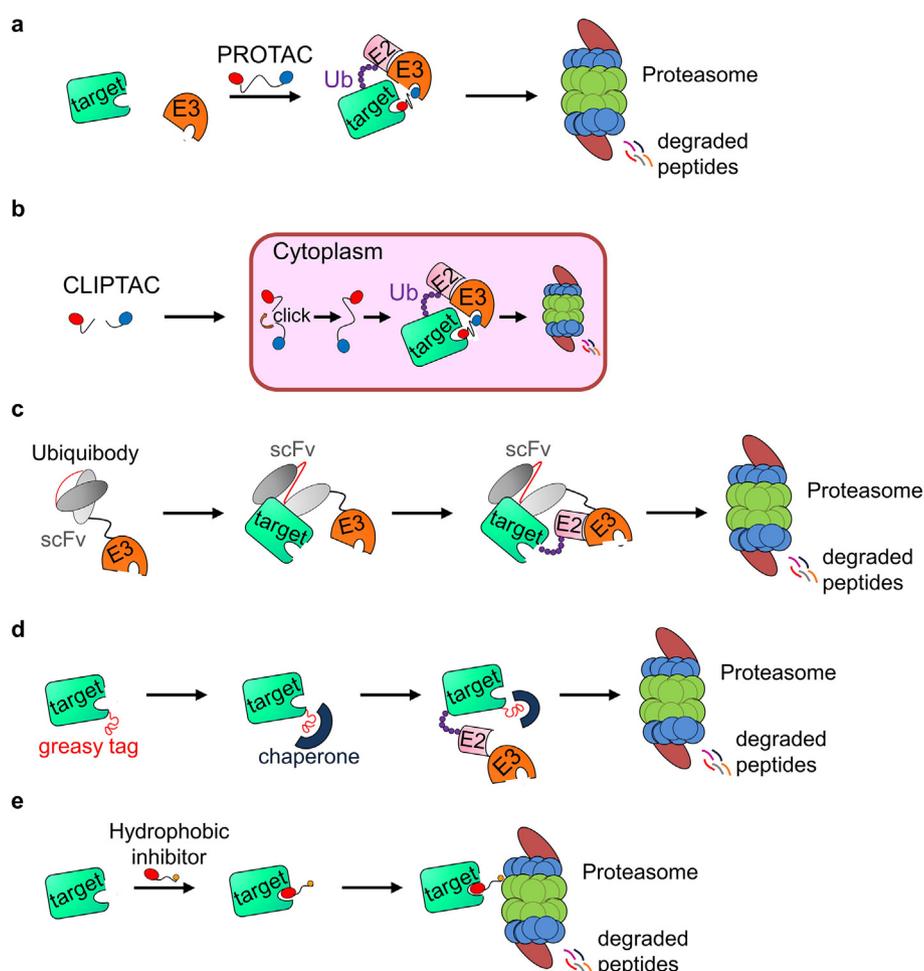

Fig. 6. Methods for targeted protein degradation. (a) Representation of the proteolysis-targeting chimera (PROTAC) technology. PROTACs are heterodimeric molecules composed of a protein-targeting warhead connected to an E3 Ub ligase binder via a chemical linker. PROTACs mediate the formation of a stable complex between the target protein and an E3 ligase, triggering ubiquitination and proteosomal degradation of the target protein. (b) Click-formed proteolysis targeting chimeras (CLIPTACs) exploit the bio-orthogonal click reaction between a trans-cyclooctene-tagged binder for a target protein and a tetrazine-containing E3 Ub ligase binder. The two molecules are administered individually and, upon cell penetration, form a complete PROTAC that is able to induce protein degradation. (c) Ubiquitibodies are constructed by fusing a scFv to the CHIP E3 Ub ligase. The scFv enables specific recognition of a target protein and the E3 ligase facilitates ubiquitination to trigger degradation. (d) A target protein was fused to a HaloTag to achieve hydrophobic protein tagging via site-specific and covalent conjugation with a HaloTagged-adamantyl moiety (England, Luo, & Cai, 2015). The adamantyl moiety displayed on the target protein mimics the partially unfolded state of proteins, triggering the activity of cellular quality control machinery, which recruits the UPS for protein degradation. (e) A hydrophobic tag was developed by modifying an inhibitor of a target protein with an hydrophobic (Boc$_3$Arg) moiety (Long et al., 2012). Upon binding to the target protein, the modified inhibitor enabled recruitment to the proteasome and subsequent degradation.

(warhead) and a ligand for an E3 Ub ligase (Toure & Crews, 2016) that mediate the formation of a complex between the drug target and an E3 ligase promoting ubiquitination and subsequent degradation of the target (Fig. 6a).

The first PROTAC was built by linking target protein inhibitors to peptides that recruited the $SCF^{\beta-TRCP}$ E3 ligase (Sakamoto et al., 2001). Although these protein degraders induced rapid depletion of targets such as the methionine aminopeptidase-2, the estrogen receptor and androgen receptors (Sakamoto et al., 2001; Sakamoto et al., 2003), the peptidic nature of the E3 ligase engager and its intrinsic instability posed limits for the application of the technology. A breakthrough in the PROTAC technology occurred with the use of the non-peptidic inhibitor Nutlin as the engager of the E3 ligase MDM2, resulting in partial degradation of androgen receptors in cells (Schneekloth, Pucheault, Tae, & Crews, 2008). However, due to the limited activity and complex chemical composition of this second generation of PROTACs, researchers were motivated to further improve stability and pharmacokinetic properties by decreasing size and identifying novel E3 ligase recruiters (Buckley et al., 2012). These efforts led to the development of current PROTAC technologies.

PROTACs rely on degradation rather than inhibition of target proteins, showing a remarkable efficacy at doses 1000 times lower than conventional drugs (Bondeson et al., 2015), a feature that has the potential to reduce systemic toxicity in vivo. Recently, a systematic analysis of the mode of action of PROTACs revealed further benefits of this technology (Bondeson et al., 2018). This study showed that the stability of the target:PROTAC:E3 ternary complex rather than the affinity of the target:PROTAC interaction is the main factor responsible for activity (Bondeson et al., 2018). This finding opens the possibility of turning low affinity small molecules into potent protein degraders, potentially allowing the rapid development of new drugs.

Comparison between the degrading activities of PROTACs with different E3 ligase ligands demonstrated that the Ub ligase engager not only dictates the efficacy of these molecules, but also controls the target specificity (Bondeson et al., 2018). In fact, PROTACs based on promiscuous kinase inhibitors (Bondeson et al., 2018; H. T. Huang et al., 2018) were rendered more selective simply by engaging different E3 ligases, suggesting that the specificity of degradation induced by a PROTAC is mainly driven by the endogenous ligand specificity of the E3 ligase. Therefore, PROTACs hold promise for the development of highly specific protein degraders even when using non-specific warheads to target proteins of interest. Thus far, only a limited number of E3 ligases have been exploited for the generation of PROTACs. The technology can be further advanced by the development of novel ligands to recruit other Ub ligases among the ~600 human E3 enzymes (Nakayama & Nakayama, 2006).

The importance of each PROTAC component - warhead, linker and E3 engager - for achieving optimal target degradation has been described (Bondeson et al., 2015; Lai et al., 2016; Maniaci et al., 2017; Zengerle, Chan, & Ciulli, 2015), but the prominent role played by the linker geometry has emerged only recently (Burslem et al., 2018; Gadd et al., 2017). For example, the short linker connecting the promiscuous bromo- and extra-terminal (BET) JQ1 ligand and the potent VH032 von Hippel–Lindau target's E3 ligase (VHL) engager, allowed for the formation of a stable target:VHL interface, ensuring the efficient and specific degradation of Brd4 over the highly homologous Brd2 and Brd3 proteins (Gadd et al., 2017).

PROTACs offer numerous advantages over conventional small molecules and provide an opportunity to expand the druggable protein target space. However, a number of questions remain to be addressed. Despite the modular nature of PROTACs, the efficacy and specificity of new PROTACs needs to be assessed empirically, as changes in the PROTAC chemical composition and geometry might affect activity and specificity. Therefore, the development of high throughput methods for synthesis and characterization of PROTAC molecules will accelerate the development of new inhibitors. Limited in vivo data are available

(Qin et al., 2018; Saenz et al., 2017); toxicity, pharmacokinetics, bioavailability, tissue distribution and metabolism of PROTAC molecules will require extensive investigation. The large size of PROTACs (~1000 Da) might affect cell permeability, potentially resulting in suboptimal presentation of a target to the E3 ligase; smaller PROTACs could improve cellular uptake and potency. To overcome this obstacle, Lebraud et al. developed in-cell click-formed proteolysis targeting chimeras (CLIPTACs): PROTACs that are formed intracellularly upon click chemistry–mediated self assembly of the target warhead and an E3 ligase engager (Lebraud, Wright, Johnson, & Heightman, 2016) (Fig. 6b).

5.2. Other strategies for targeted degradation

Similar to the PROTAC technology, chimeric proteins formed by a single-chain variable fragment (scFv) of an antibody or a fibronectin type III domain monobody fused to the E3 Ub ligase carboxyl terminus of Hsc70-interacting protein (CHIP) have also been used to degrade target proteins (Portnoff, Stephens, Varner, & DeLisa, 2014). Although this strategy enabled the efficient depletion of target proteins in cells, its application has been hindered by the correct folding of disulfide bond-containing scFvs in the reducing environment of the cell cytoplasm (Fig. 6c).

Another method to trigger selective protein degradation via the UPS is represented by the modification of proteins with hydrophobic tags (Neklesa & Crews, 2012). Covalent modification of proteins with hydrophobic tags induces the recruitment of molecular chaperones, which promote the ubiquitination and proteolytic removal of the target by the proteasome 26S (Neklesa et al., 2011) (Fig. 6d). This approach achieved selective depletion of HER3 and reduced cancer cell proliferation (Xie et al., 2014). Similarly, linkage of small molecule inhibitors to a hydrophobic tert-butyl carbamate-protected arginine (Boc_3Arg) moiety induced selective degradation of target proteins (Long, Gollapalli, & Hedstrom, 2012) (Fig. 6e). In contrast to hydrophobic tags, the (Boc_3Arg) moiety enabled the efficient depletion of both glutathione S-transferase (GST) and bacterial dihydrofolate reductase (DHFR) in a manner independent of Ub and ATP, suggesting that degradation was mediated by a direct interaction between the (Boc_3Arg) moiety and the proteasome subunit 20S (Long et al., 2012), without requiring target ubiquitination. These recent developments demonstrate that induced protein degradation has been significantly improved over recent years and is now well positioned to expand the druggable protein space.

6. Summary

The UPS provides a wide canvas for developing next-generation cancer therapeutic agents. While advances in HTS technologies have decreased the time and cost needed to carry out drug discovery studies, activity-based small molecule screens have not yielded a satisfying number of viable drug leads. Recent developments in fragment-based medicinal chemistry methodologies offer a promising approach to tackle the small molecule-based screen problems. The advent of UbVs also represents an exciting new technology that can rapidly provide highly potent and selective inhibitors and activators of enzymes that have resisted conventional approaches. Combined with fragment-based drug discovery methods and recent development in DECLs, small molecule screens for chemical compounds that can displace UbVs from target proteins could enable deep and effective sampling of chemical space to modulate the UPS. Finally, PROTAC technology represents another promising means for cancer therapy. Indeed, it seems likely that the most effective cancer therapies in the future could well rely on the combination of these diverse innovations to develop drug-like entities with activities well beyond the scope of current drugs.

Disclosure statement

The authors declare no conflicts of interests.

Acknowledgments

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