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Research review paper

Synthetic biology approaches for targeted protein degradation

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ABSTRACT

Protein degradation is an effective native mechanism used in modulating intracellular information, and thus it plays an essential role in maintaining cellular homeostasis. Repurposing native protein degradation in a synthetic context is gaining attention as a new strategy to manipulate cellular behavior rapidly for a wide range of applications including disease detection and therapy. This review examines the native mechanisms and machineries by which mammalian cells degrade their own proteins including the sequence of events from identifying a candidate for degradation to the protein's destruction. Next, it explores engineering efforts to degrade both exogenous and native proteins with high specificity and control by targeting proteins into the degradation cascade. A complete understanding of design rules with an ability to use cellular information as signals will allow control over the cellular behavior in a well-defined manner.

1. Introduction

Proteins play a diverse role in controlling cell physiology, responsible for all cellular metabolism, cell division, and apoptosis. In many cases, the proper coordination and synchronization of protein functions depend on precise control of their degradation in a spatial and temporal manner (Davey and Morgan, 2016; Harashima et al., 2013; Satyanarayana and Kaldis, 2009). Recent insights into the underlying degradation machineries are crucial in enabling their synthetic applications towards targeted degradation of a protein of interest (POI). These studies provided insights on how the protein quality control machineries recognize sequences (usually referred to as degrons) on native or misfolded proteins in order to guide their rapid degradation either by using the 26S proteasome or by autophagy (Budenholzer et al., 2017; Collins and Goldberg, 2017; Dikic and Elazar, 2018; Levine and Kroemer, 2019; Yu et al., 2018).

As synthetic biology becomes more advanced, there is a pressing need to develop proteins with half-lives that can be controlled precisely to enable construction of more complicated protein-based logic systems. Engineered protein systems that could autonomously stabilize productive proteins and degrade those with functions that are not germane would enable huge leaps in areas such as medicine and metabolic engineering. While there are several short reviews highlighting the recent progress in the area (Natsume and Kanemaki, 2017; Yu et al., 2015), there is a need to provide a more comprehensive summary of the emerging synthetic biology toolkits for conditional and targeted protein degradation in mammalian systems. In this review, we will focus on engineering efforts exploiting the proteasomal pathway as it is well characterized and the primary mechanism responsible for protein degradation in most eukaryotic systems (Collins and Goldberg, 2017).

2. Proteasomal protein degradation

Proteasomal degradation is an imperative process in maintaining cellular homeostasis and ensuring that the cell cycle is properly coordinated (Harper et al., 2002; Morgan, 1997; Sherr and Roberts, 1999). As such, many key regulatory proteins have specific chaperones responsible for their controlled degradation (Saeki, 2017; Schrader et al., 2009; Yu and Matouschek, 2017). In general, degradation requires a means of trafficking the target protein to the 26S proteasome and an unstructured region to initiate its destruction (Prakash et al., 2009). Two major mechanisms, ubiquitination-dependent or ubiquitination-independent pathways, are primarily responsible for proteasomal degradation (Erales and Coffino, 2014; Schrader et al., 2009).

2.1. Ubiquitination-dependent pathway

Ubiquitination is a central mechanism of targeting proteins to the proteasome (Chau et al., 1989; Komander and Rape, 2012; Wilkinson, 2000). Typically, multiple ubiquitin (Ub) units are attached to a lysine residue on the target protein via the sequential action of a three-enzyme cascade: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating

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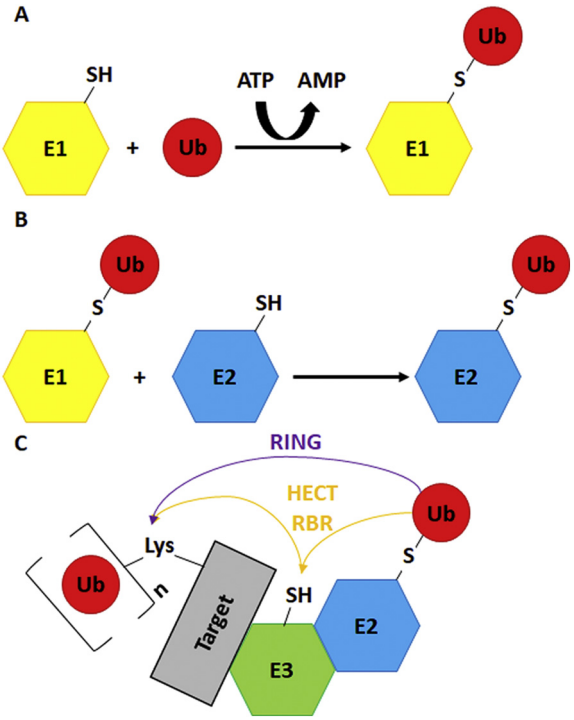

Fig. 1. The ubiquitination enzyme cascade. A) Ubiquitin activating enzyme (E1) forms a thioester bond with ubiquitin at the expense of ATP. B) Ubiquitin conjugating enzyme (E2) accepts the activated ubiquitin from E1 in a trans-thioesterification reaction. C) Ubiquitin ligase (E3) is responsible for both target recognition and the transfer of Ub from the active site of E2 to a lysine on the target or growing polyubiquitin chain. RING E3s catalyze the transfer of Ub directly without first accepting it (purple arrow). Alternatively, HECT and RBR E3s first form a thioester with Ub before transferring it to the target (gold arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enzyme (E2), and a ubiquitin ligase (E3) (Fig. 1), creating a poly-ubiquitin chain (Komander and Rape, 2012). This tagging process leads to recognition of the polyubiquitin chain by the 26S proteasome to degrade the target proteins to small peptides (Deshaies and Joazeiro, 2009).

Briefly, Ub is activated by an E1 ubiquitin-activating enzyme, which forms a thioester bond with the C-terminus of Ub in an ATP-dependent reaction (Fig. 1A). Activated Ub is next transferred to a cysteine residue in the active site of an E2 ubiquitin-conjugating enzyme, yielding an E2-Ub thioester intermediate (Fig. 1B) (Berndsen and Wolberger, 2014). Though E1 and E2 are essential to the ubiquitin-dependent protein degradation pathway, there has been little effort to engineer them. Since E3 contains all of the targeting and Ub-tagging capabilities as will be discussed in the remainder of this section, all of the engineering has been focused there.

Generally, E3 ligase falls into two broad structural classes – either the monomeric homology to E6-AP C-terminus (HECT) domain or the larger really interesting new gene (RING) finger family (Lorick et al., 1999; Metzger et al., 2012). HECT domain proteins consist of two functionally distinct regions that enable transfer of an activated Ub from E2—bound to N-terminus of the HECT E3—to the POI (Huang et al., 1999; Kamadurai et al., 2009). The RING finger E3 ligase family contains a canonical RING finger domain that is responsible for facilitating E2-dependent ubiquitylation (Zheng et al., 2000). The largest class of multi-subunit RING finger E3 ligases is the cullin (CUL) RING ligase (CRL) (Feldman et al., 1997; Skowyra et al., 1997), which contains the SKP1-cullin-F-box protein (SCF) complex. The F-box protein is responsible for substrate binding and is attached to one end of CUL via the adaptor protein, Skp1 (Jin et al., 2004; Skaar et al., 2009a, 2009b, 2013). To the other end of CUL, a RING finger E3 ligase binds to an ubiquitin-charged E2 to catalyze the transfer of ubiquitin to the target substrates. The ability to interchange F box proteins within the same cullin E3 framework allows for great flexibility in proteome management (Lydeard et al., 2013).

This proximity-driven ubiquitylation strategy provides a simple framework to hijack the native E3 ligase machinery for non-natural POIs for targeted degradation (Clift et al., 2017; Fulcher et al., 2016; Nishimura et al., 2009). This idea has inspired the development of bi-functional chemical linkers called proteolysis targeting Chimeras (PROTACs) containing distinct substrate-binding and E3 ligase-binding groups for hijacking the native E3 ligase machinery (Collins et al., 2017; Gu et al., 2018; Lai and Crews, 2017; Sakamoto et al., 2001; Toure and Crews, 2016). The conjugate molecule serves to assemble a ternary complex between the E3 ligase, target protein, and probe molecule, allowing the E3 ligase complex to ubiquitinate the non-natural substrate and promote proteasome-dependent degradation. While these drug-like PROTAC molecules allows targeted degradation of native proteins, it is often challenging to identify and synthesize the target-specific binding moiety (Collins et al., 2017). However, their ease of delivery makes them a powerful drug treatment option, and PROTACS have begun to enter clinical trials this year (Mullard, 2019). Alternatively, purely protein-based strategies for targeted protein degradation based on proximity control have gained traction due to the ease and flexibility of design (Fulcher et al., 2017).

A minor category of E3, the RING-between-RING (RBR) family, shares features of both HECT and RING E3s. The name is derived from the presence of two RING domains (RING1 and RING2) that sandwich an in-between-ring (IBR) domain (Lydeard et al., 2013). The first RING domain serves to recruit E2 as might be expected (Wenzel et al., 2011), but the second RING domain contains a catalytic cysteine that complexes with Ub before being transferred to the target, similar to HECT E3s (Duda et al., 2013). While no synthetic biology has been attempted using the RBR E3 ligases, the hybrid properties of this system might allow for future, novel applications.

2.2. The N-end rule degrons

The N-terminal amino acid residue of a protein plays a central role to its half-life by acting as a N-degron (Bachmair et al., 1986; Hwang et al., 2010) that is recognized by specialized E3 ligases, *N*-recognins (Hwang and Varshavsky, 2008; Tasaki et al., 2009; Varshavsky, 1996; Xia et al., 2008; Xie and Varshavsky, 1999). Upon *N*-recognin binding, the target is polyubiquitinated at an internal lysine and targeted for degradation (Bachmair and Varshavsky, 1989; Chau et al., 1989). The in vivo stability of a protein is directly correlated to the identity of its N-terminal residue and can vary from a half-life of less than 1 h (Arg) to longer than 100 h (Val) (Varshavsky, 2011). This simple rule set provides a new strategy to engineer the half-life of POIs by artificially exposing the desired N-terminal residue (Bachmair et al., 1986; Varshavsky, 2005).

2.3. Ubiquitin-independent proteasomal degradation

Not all proteins targeted to the proteasome are first ubiquitinated. The proteasomal degradation of ornithine decarboxylase (ODC), a well-folded protein, takes place without ubiquitination with the help of a C-terminal degradation tag (C-degron) (Murakami et al., 1992). A second protein, antizyme 1 (AZ1), mediates the interaction between ODC and the 26S proteasome by exposing a stretch of amino acids at the C-terminus from which degradation begins (Zhang et al., 2003a, 2004). Because the initiation sequence is relatively short and well-characterized, this C-terminal portion of ODC has been used extensively as a reliable, facile C-degron tag for a wide of protein targets (Hsieh et al., 2009; Joshi et al., 2015; Li et al., 1998; Zhao et al., 2018).

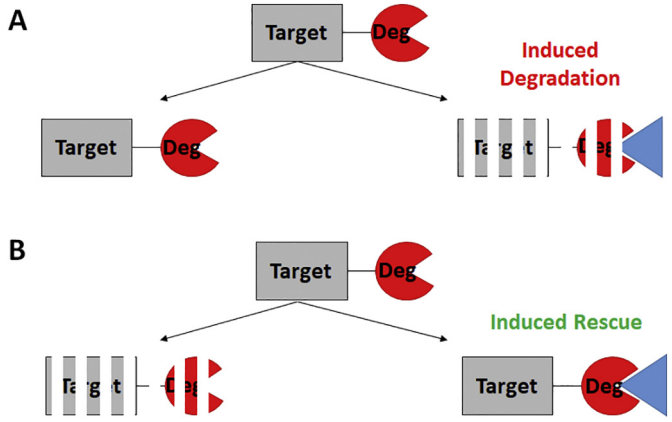

Fig. 2. General scheme of controlled protein fate by a conditional degron. A) By default, the conditional degron (red circle with missing wedge) is stable (left), but upon addition of a small molecule cue (blue triangle), the degron becomes unstable and the target (grey rectangle) is degraded (right). B) By default, the conditional degron is unstable and the target is degraded (left). Upon addition of the small molecule, the degron is stabilized and the target is rescued from degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Engineering protein degradation based on stimuli-responsive degrons

A common strategy to modulate protein stability is to insert a conditional degron tag either to induce degradation or to rescue the target from degradation (Fig. 2). A wide range of external stimuli can be used to activate the desired phenotypes, making this a highly flexible and adaptable strategy for a wide array of POIs.

3.1. Small-molecule induced degradation

Small molecules are frequently used to activate degron activities. The ligand-induced degradation (LID) system, a mutant of the rapamycin-binding protein FKBP, was first identified as a cryptic inactive degron (Bonger et al., 2011). Upon the addition of the synthetic small molecule Shield-1 (Shld1), a derivative of rapamycin that has no reported biological activity (Banaszynski et al., 2006), the cryptic degron is displaced and exposed for interaction, thereby inducing degradation of the corresponding fusion POI. While the LID system is able to degrade proteins of interest rapidly when activated, the stable version still necessarily contains a bulky FKBP fusion protein, which might interfere with the biology activity of the POI. To minimize this issue, small molecule-assisted shutoff (SMASh) was developed (Chung et al., 2015). In this configuration, the POI contains a C-terminal fusion to the following components in order: a specific viral protease cut site, the viral protease for that cut site, and a degron tag. With no additional cues, the protease cuts at its recognition site, releasing the target protein from the degron, and therefore the target is stable by default. Unlike the LID system, the POI does not contain a bulky fusion after protease cleavage, and therefore it is more likely that native activity will remain unimpaired. Upon the addition of a specific protease inhibitor, the degron is able to act upon the entire protein, including the target. SMASh demonstrated a strong signal-to-noise ratio, and spacers can be configured to allow SMASh to function from either the N- or C-terminus, offering flexibility to the end-user. These strategies allow easy deactivation of cellular phenotypes by using a small molecule and have been successfully applied in mammalian cell culture, transgenic mice, plants, and virus studies (Lemmens et al., 2018; Yan et al., 2015).

3.2. Small-molecule induced rescue

In contrast to induced degradation, induced protein recue has also been made possible using a small molecule. A degron tag that is inherently unstable is fused to a POI to induce degradation. However, when bound to a highly specific small molecule ligand, stability is restored and the POI is rescued. Emphasis has been placed on developing degrons whose ligands are inexpensive, active at low concentrations, commercially available, and cell membrane permeable. One of the earliest examples was based on FKBP12. Rescue of various proteins could be induced by Shld1. An orthogonal strategy involved a mutated *E. coli* dihydrofolate reductase (ecDHFR), which is rescued specifically by trimethoprim (TMP). It was demonstrated that two different proteins could be rescued on cue by introducing either Shld1 or TMP (Iwamoto et al., 2010), expanding the toolkit and protein space that could be studied. The ability to execute orthogonal and conditional protein rescue lends itself to the possibility to create synthetic logical circuits to modulate protein functions and cellular activities.

3.3. Small-molecule induced rescue by removable degrons

To eliminate possible negative effects on protein activity caused by the degron fusion, technologies have been developed to cleave the degron tag once rescue is induced by the small molecule. The first such technology, termed split ubiquitin for rescue of function (SURF), innovated the use of a split ubiquitin domain, which induces endogenous cleavage by the de-ubiquitination enzyme (DUB) after reconstitution (Pratt et al., 2007). The degron was an engineered FKBP12-rapamycin-binding protein (FRB) with point mutations that fated FRB and its fusions to degradation (Fig. 3). Upon the addition of rapamycin, FRB is stabilized by its interaction with native FKBP, situated on a separate construct containing the second half of the split ubiquitin. Simultaneously, the two split ubiquitins are forced into proximity, allowing the POI to be cleaved from the degron. Different FRB mutants or native FRB fused to a separate degron could be used to offer varying kinetic properties.

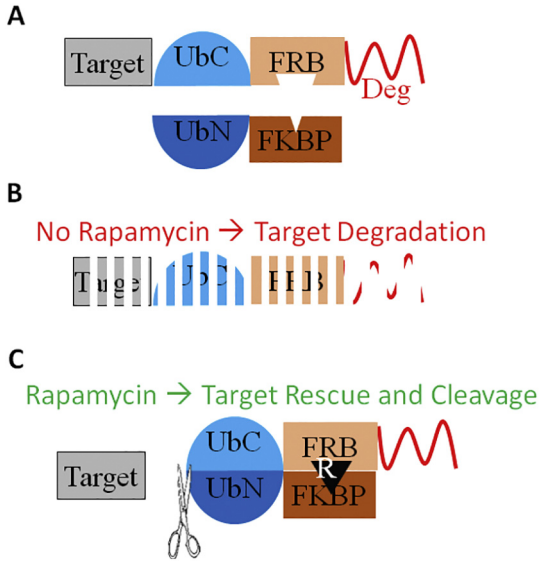

Fig. 3. Split ubiquitin for rescue of function (SURF). A) SURF is composed of a target protein fused to the C-terminus of ubiquitin (UbC), FRB, and a degron tag. A second component is composed of the N terminus of ubiquitin (UbN) and FKBP. B) The default state in the absence of rapamycin is the degron leads to the complete proteolysis of the component to which it is fused, including the target. C) In the presence of rapamycin (black triangle), FRB and FKBP are drawn into proximity by binding to rapamycin. Consequently, UbC and UbN are complimented, leading to the rescue and cleavage of the target protein from SURF.

An improved version of SURF was developed by using a mutant FKBP called FKBP*, and the native FRB was used to reconstitute the split ubiquitin. Both proteins were expressed under a single promoter using a viral "self-cleaving" 2A site (Holst et al., 2006; Szymczak et al., 2004). This Traceless Shielding (TShld) method offers improvements over its predecessor both in terms of dynamic range and ease of use (Lau et al., 2010). The same group placed TShld under a tetracycline inducible promotor, and showed that in this manner, they could completely eliminate any system background by adding a layer of transcriptional control as well (Lin and Pratt, 2014). In our own group, we used TShld to control the prodrug converting enzyme, yCD, and found HeLa cell viability was affected only in cells that received both the prodrug and activating molecule (Gaynor and Chen, 2017). This demonstration shows the promise of engineered controlled degradation in therapeutic applications.

3.4. Temperature- and light-responsive degrons

Conditional control on protein degradation has also been reported without the use of exogenous small molecules. One simple way is to take advantage of changes in culture conditions such as temperature. One such example capitalized on the slow kinetics of the N-end rule pathway at low temperatures. When arginine is the leading residue of DHFR fused to a POI, the complex is rapidly degraded at 37 °C, yet it is much more stable at lower temperatures (23 °C) (Dohmen et al., 1994). This temperature-sensitive DHFR degron has been shown to work in yeast and vertebrate cells cultures (Kearsey and Gregan, 2009; Su et al., 2008). This principle was further extended to multicellular organisms such as plants and fruit flies with temperatures in a lower, more-permissive operating range using a newly designed low temperature (lt) N-degron system (Faden et al., 2016).

Light is a popular optogenetic tool used in the field of synthetic biology because it offers the unparalleled combination of spatial and temporal control (Goglia and Toettcher, 2019; Kim et al., 2017; Liu et al., 2018; Zhang and Cohen, 2017). The light oxygen voltage (LOV2) domain is a light-sensitive protein that can reversibly interact with its C-terminus Jα-helix in the light (unbound) versus dark (bound) state (Harper et al., 2003). By fusing an ODC degron to the C-terminus of the Jα-helix, researchers developed a degron tag that is hidden within the LOV2 domain in the dark state. Upon the application of blue light, release of the hidden ODC degron tag led to degradation of a POI fused to the N-terminus of LOV2 (Renicke et al., 2013). The use of light for conditional degradation is particularly attractive as it has no reported impact on cell physiology. However, delivering blue light in a developing embryo or a living animal is not trivial, and other alternative light-sensitive domains that are responsive to red or far red light (Kawano et al., 2017; Ochoa-Fernandez et al., 2016) may be repurposed to broaden the use of light-responsive degrons.

4. Proximity-based targeted degradation

Similar to PROTACs, proximity-based protein adapters have also been developed for targeted protein degradation (Fig. 4). Recruitment of the target protein to E3 ligase occurs either using a specific protein binder such as nanobodies or by a small molecule-induced dimerization domain. Nanobody-based strategies are particularly attractive, as they do not require genetic modification of the protein of interest for targeted degradation. Since nanobodies can be engineered to bind almost any protein targets (Hassanzadeh-Ghassabeh et al., 2013; Van Audenhove et al., 2013), protein adaptors can be easily engineered to target most "undruggable" proteins for degradation. While these protein-based strategies are still in their infancy and their therapeutic efficiency remains unexplored, they serve as valuable tools for studying protein loss-of-function as well as developing synthetic protein circuits for rapid modulation of cellular phenotypes.

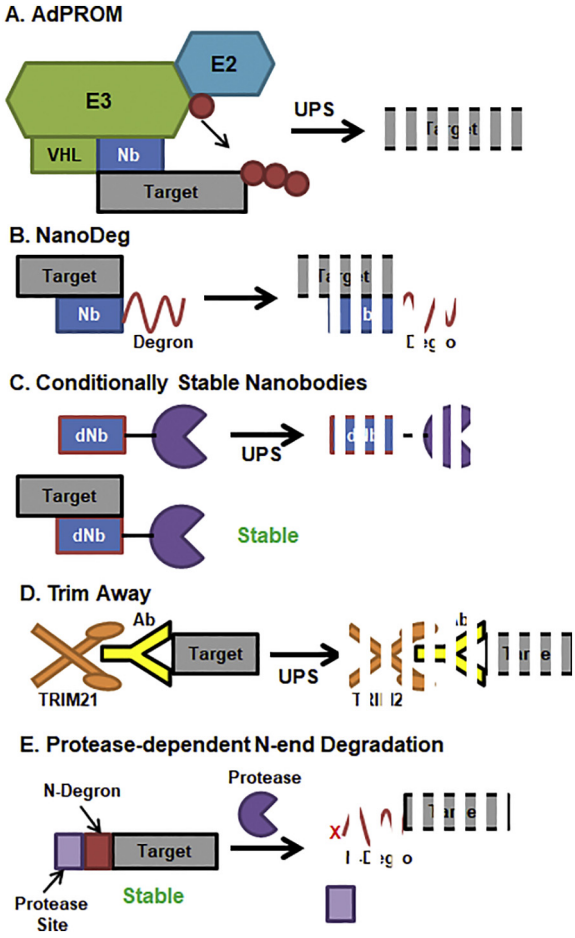

Fig. 4. Protein-based targeted degradation systems. A.) AdPROM utilizes a VHL-nanobody fusion to recruit target proteins for ubiquitination and degradation. B.) NanoDeg fuses a nanobody to a degron domain so that upon nanobody binding the target protein the entire complex is degraded. C.) Conditionally stable nanobodies contain destabilizing mutations that render the nanobody unstable until bound to its antigen. This allows for antigen-dependent rescue of destabilized nanobody fusion proteins with useful effector domains. D.) Trim-Away involves harnessing TRIM21 which recognizes the FC region of antibodies to direct antibody-bound target proteins for degradation. E.) Protease-dependent N-end degradation is achieved by using protease cleavage to create a newly exposed N-terminal residue that makes the target protein vulnerable to rapid degradation by the N-end rule.

4.1. Re-targeting the native degradation machinery

In plants, the auxin family hormones are responsible for inducing interaction between the F box protein TIR1 and proteins containing an auxin inducible degron (AID) (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). Due to homology found between plant cullin RING E3 complexes and those of other eukaryotes, ectopically expressed TIR1 has been demonstrated to target a variety of proteins tagged with AID for degradation in the presence of auxin (Fig. 5) (Holland et al., 2012; Nishimura et al., 2009). This strategy induces the interaction between a target protein and the innate ubiquitin-dependent degradation pathway in a manner similar to the PROTAC approach using only a single molecule, auxin. Further engineering of the auxin hormone itself has led to a light-inducible version in which auxin does not interact with TIR1 and AID-tagged proteins except in the presence of light (Delacour et al., 2015). This innovation provides for more stringent spatiotemporal control of protein degradation. AID brings together advantages of the specificity afforded by protein engineering with the control and reversibility granted by the use of small molecules.

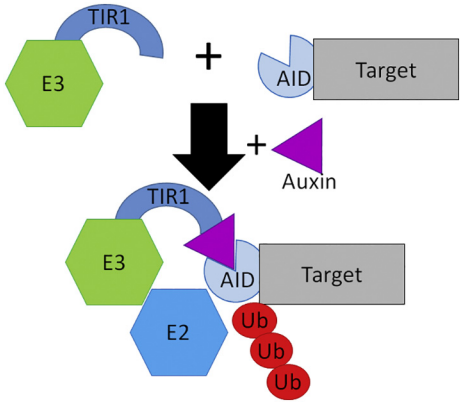

Fig. 5. Auxin inducible degron (AID) system. TIR1 acts as an F-box protein that recognizes targets with an AID tag in the presence of the plant hormone auxin. When all of these components are present, E3 ligase is able to recruit E2 to polyubiquinate the AID tag. This results in complete degradation of the target protein in an auxin-dependent, reversible manner.

However, as mentioned above, small molecules are difficult to be integrated into healthcare strategies because of the inability to localize them to specific tissues within the body and to control the local concentration.

4.2. Protein degradation mediated by nanobodies

F-box re-engineering has proved a powerful technique for harnessing the natural degradation machinery towards specific cellular targets, and it has provided a mechanism to target modified proteins specific to different cellular states (Kong et al., 2014; Zhang et al., 2003b; Zhou et al., 2000). In this spirit, one of the early attempts to eliminate the need for a small molecule trigger while maintaining modular targeting ability involved fusing a single-domain nanobody targeting GFP directly to a F-box protein in a technique termed "degrade green fluorescent protein" (deGradFP) (Caussinus et al., 2011). In this configuration, any GFP-containing fusions are targeted for degradation through the native ubiquitin-mediated protein degradation pathway without the need for a small molecule. The fusion of endogenous proteins to GFP allowed for simple, visual tracking of this system that could be correlated to phenotypic outcomes of protein depletion. However, surprisingly, deGradFP is unable to process GFP on its own, pointing out limitations in the pool of potential target candidates likely due to shape, size, and the availability of an unstructured handle for the proteasome. Similar strategies have also been successfully implemented using other smaller "stand-alone" E3 ligases such as CHIP and XIAP—including recently their bacterial mimics—in mammalian cells, plants, yeast, and neuron cells (Baltz et al., 2018; Gross et al., 2016; Hatakeyama et al., 2005; Kanner et al., 2017; Ludwicki et al., 2019; Portnoff et al., 2014). For applications in which genetic engineering to produce a GFP fusion (*ex.* human health) are undesirable, this technique also falls short. While in theory one could substitute a different nanobody, this would require a complete re-engineering of the F-box protein. To circumvent these limitations, several bifunctional adaptor systems have been reported to provide a more modular approach for targeted protein degradation.

4.3. Affinity-directed protein missile (AdPROM)

The VHL E3 ligase natively binds hydroxy-proline modified HIF1α to direct HIF1α for ubiquitin-dependent proteasomal degradation. In the affinity-directed protein missile (AdPROM) system, VHL is redirected towards binding a protein of interest by fusing a camelid-type nanobody to VHL (Fig. 4A) (Fulcher et al., 2017, 2016). The bifunctional VHL-nanobody recruits the POI towards the rest of the CUL2 E3 machinery for degradation. To demonstrate this principle, an anti-GFP nanobody (aGFP) was fused to VHL and constitutively expressed in modified cell lines which endogenously expressed GFP-tagged proteins (Fulcher et al., 2016). The VHL-aGFP construct was found to efficiently degrade GFP-tagged proteins to undetectable levels. Interestingly, the aGFP-VHL version of AdPROM yielded no degradation of the VHL-nanobody fusion proteins, suggesting that AdPROM must properly orient the POI with respect to the E3 complex in order to undergo ubiquitination of the POI while avoiding ubiquitination of the AdPROM components. The same group also successfully designed AdPROM constructs to target untagged endogenous proteins using nanobodies that directly recognize these POIs. In addition to nanobodies, AdPROM was shown to be successful when using FN3-type monobodies, thus demonstrating that two different engineered binding proteins could be used in addition to the multiple POIs (Fulcher et al., 2017). Compared to small-molecule therapies, gene delivery technology for therapeutics is still limited. Thus, AdPROM systems are more limited than PROTACs towards immediate therapeutic applicability. Recently, however, fusions of monobody-VHL fusions have successfully been delivered to cells via a bacterial toxin delivery system with similar degradation efficacy (Schmit et al., 2019).

4.4. Bifunctional recognition system (NanoDeg)

A similar strategy to AdPROM called NanoDeg was developed by Zhao et al. as a bifunctional recognition system for targeted protein degradation (Zhao et al., 2018). NanoDeg contains a nanobody recognition motif fused to a degron domain (Fig. 4B). When the target protein binds the nanobody, it is recruited for degradation by the degron tag. As an initial demonstration, an anti-GFP nanobody (VHH) was fused to the ubiquitin-independent ODC degron and transiently transfected in a HEK293T cell line stably expressing GFP. The VHH-ODC fusion reduced GFP levels by 65%. The study also successfully replaced ODC with an ubiquitin-dependent degron, CL1, and explored different variants of both degrons to tune the half-life of the VHH-degron and thus the amount of GFP degradation. The fusion of a nanobody targeting β-actin with an ODC variant resulted in NanoDeg construct capable of degrading 90% of endogenous β-actin in HEK293T cells. NanoDeg offers a plug-and-play framework for designing nanobody-degron fusions to finely control target protein levels within cells. Furthermore, unlike AdPROM, NanoDeg does not require the fusion of an endogenous E3 ligase to the nanobody and thus circumvents the need to properly orient the target with the E3 ligase.

4.5. Antibody binding E3 ligase for targeted degradation (Trim-Away)

Another adaptor strategy is to utilize TRIM21, an E3 ligase that recognizes the FC fragment of antibodies (James et al., 2007). TRIM21 natively recruits the proteasome to antibody-bound pathogens within the cytosol (Mallery et al., 2010). Clift et al. sought to utilize TRIM21 to redirect unmodified target proteins for degradation in a scheme termed Trim-Away (Clift et al., 2017). TRIM21 and the targeting antibody are exogenously delivered, which leads to the TRIM21 directed degradation of the antibody bound protein of interest (Fig. 4D). Trim-Away was successfully targeted towards 9 different endogenous proteins in 10 different cell types to achieve rapid (half-life on order of minutes) and specific degradation. In some cell lines, endogenous TRIM21 alone was sufficient to achieve degradation effects. However, in most cases, exogenous TRIM21 was necessary to achieve maximum POI depletion. This is unsurprising since this is a single turnover system, and the entire TRIM21, antibody, and target protein complex are directed to degradation. Another drawback to Trim-Away is that the antibody must be delivered through microinjection or electroporation, thus limiting its application beyond laboratory contexts. However, nanobodies were successfully adopted for Trim-Away by co-expressing TRIM21 and a FC-nanobody fusion, which offers a promising avenue to avoid

microinjection/electroporation (Clift et al., 2017).

5. Conditional degradation using other cellular signals

5.1. Conditionally stable nanobodies

The development of conditionally stable nanobodies by Tang et al. also serves as an intriguing strategy for targeted protein degradation (Fig. 4C) (Tang et al., 2016). In the study, an anti-GFP nanobody (GBP1) was screened for conditionally destabilizing mutations. The resulting destabilized mutant, dGBP1, fused to BFP was found to be undetectable in the absence of GFP/YFP, but strong BFP fluorescence was recovered in the presence of GFP/YFP. Most importantly, the destabilizing mutations lay in conserved regions of the nanobody sequence, and thus were shown to be transferred successfully and generalizable to other nanobodies. The fusion of effector proteins, such as site-specific recombinases Cre and Flpo, to the destabilized nanobodies (dNbs) was also found to render the effectors antigen-specific. Furthermore, the fusion of tandem dNbs was explored to reduce background signal (multiple copies of same dNb) and to create AND-gated antigen rescue (orthogonal dNbs). Unlike previous degradation strategies which utilize heterobifunctional designs, the target recognition motif (nanobody) here has been engineered directly for switch-like degradation behavior (Ariotti et al., 2018; Tang et al., 2016). Such elegant integration of degradation behavior allows this strategy to realistically inspire more complex architectures using endogenous signals.

5.2. Protease-dependent N-end degradation

Conditional degradation can also be achieved by using protease cleavage to reveal N-degrons. Protease cleavage sites are placed upstream of the N-end residue of a POI. Prior to cleavage the protein is stable, but upon cleavage a new N-end residue is exposed and degraded at a rate accordingly to the N-end rule (Fig. 4E). This concept was first explored by Taxis et al. in a system termed TEV protease induced protein inactivation (TIPI) (Taxis et al., 2009). They designed a TDegX component that is fused to the N-terminus of the target protein. The TDegX contains a TEV protease cut site in which cleavage exposed a new N-terminal "X" residue of an N-degron. The implementation of TIPI in yeast resulted in rapid TEV-dependent degradation of target proteins fused to TDegD and TDegF. Although this strategy requires the fusion of synthetic components to the POI, it is powerful in establishing a framework for creating conditional protein modules. Subsequent works have utilized this concept with orthogonal and split proteases to establish post-translational control of genetic circuits (Fernandez-Rodriguez and Voigt, 2016), and most recently programmable protein circuits capable of complex logic behavior (Gao et al., 2018).

A more complicated design using TEV has also been demonstrated (Jungbluth et al., 2010). Site-directed cleavage by the TEV protease deprotected two degrons fused to two separate POIs, resulting in degradation of both parts of the processed protein. In this design, both target proteins act as a steric shield, blocking the degrons from interacting with the proteasomal degradation machinery. The deprotection of two orthogonal degrons provides the feasibility to execute Boolean logic gates for conditional degradation. These developments represent crucial progress towards repurposing artificial degrons from tools for the study of protein effects to useful components in synthetic biology platforms.

Recently in prokaryotes, N-terminal NEDD8 fusions to an HdiR-derived degron generated proteins that are stable until NEDD8-specific endopeptidase NEDP1 is heterologously expressed. Similar to the previous strategies, with the degron now exposed, the POI is rapidly depleted in an SsrA-independent fashion (Liu et al., 2017). This work represents the first targeted prokaryotic N-terminal proteolysis system, and the choice of NEDD8 and NEDP1 over other proteases and recognition sequences (*ex*.TEV) grants the user extremely high specificity. Importantly, since HdiR is derived from the highly conserved LexA-like regulator superfamily common to most bacteria, it should be relatively facile to generate a degron appropriate for a host species of choice.

6. Conclusion and future outlooks

For cells to function efficiently and adapt to environmental signals, they must quickly revise their protein composition. Cells have evolved to do so through fast-acting protein degradation. Our understanding of protein degradation mechanisms and pathways has opened up an exciting new paradigm for rapid and specific protein control through engineered protein degradation systems.

Both ubiquitin-dependent and –independent degradation mechanisms have been exploited successfully within synthetic architectures for targeted degradation of non-native substrates. One popular strategy is tagging proteins of interest with degron domains and using an inducer to unblock or remove the degron. For example, recently a degron library of various strengths was developed and successfully employed to generate and tune a protein-based pulse generator circuit (Chassin et al., 2019). Another strategy is to highjack degradation machinery and redirect their specificity towards proteins of interest (*ex*. E3 ligases within PROTACs and AdPROM schemes). Further understanding of degradation machinery will enable us to harness these components for more complex synthetic degradation schemes. For example, the N-end rule has served as an important design principle for tuning protein half-lives that extend beyond simple mapping of native degron domains onto POIs. Future elucidation of mechanistic rules such as this will enable modular and rational design of more synthetic protein circuits.

Especially of interest is the use of endogenous proteins within synthetic degradation schemes, whether to be targeted for degradation themselves or as potential inducers of degradation. Real-time autonomous interrogation and subsequent decision-making within cellular environments has tremendous applications for disease therapeutics and metabolic engineering. Small-molecule PROTACs have thus far seen the greatest therapeutic potential as not only more potent than their analogous inhibitors, but also capable of targeting previously "undruggable" proteins.

While these strategies provide different levels of conditional control on protein degradation, their practical implementation remains difficult. First, protein therapies produced in eukaryotic systems will likely be targeted by the expression host themselves. Secondly, introducing many of the stabilizing or destabilizing small molecules to the patient may prove toxic. Thirdly, the targeted delivery of proteins still requires further technological advancement, though recent advances in the field demonstrate promise (Lieser et al., 2019). However, the specificity in targeting different POIs and spatiotemporal control offered by the technologies discussed in this review are promising, and they may provide an avenue towards the rapid development of patient-specific personalized medicine. We remain hopeful that novel advancements in synthetic biology will continue to drive the technology forward towards other practical metabolic engineering and medical applications.

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