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Protein degradation for drug discovery

Ligand-induced genetic degradation as a tool for target validation

Aisha Yesbolatova[1], Yusuke Tominari[2], Masato T. Kanemaki[1,*]

[1]National Institute of Genetics, Research Organization of Information and Systems (ROIS), and Department of Genetics, The Graduate University for Advanced Studies (SOKENDAI), Yata 1111, Mishima, Shizuoka 411-8540, Japan
[2]FIMECS, Inc., 26-1, Muraoka-Higashi 2-chome, Fujisawa, Kanagawa 251-0012, Japan

Targeted protein degraders, known as proteolysis targeting chimeras (PROTACs), are drawing more attention as next-generation drugs to target currently undruggable proteins. As drug discovery of functional degraders involves time- and cost-consuming laborious processes, we propose employing a ligand-induced genetic degradation system to validate candidate proteins before degrader development. Genetic degradation mimics degrader treatment by depleting a degron-fused protein in the presence of a defined ligand. All genetic systems use a combination of a degron and defined ligand that enables a protein of interest fused with the degron to be recruited to an E3 ubiquitin ligase for ubiquitylation and subsequent degradation by the proteasome. However, these events are based on different principles and have different features. We review the dTAG, HaloTag-based, auxin-inducible degron (AID), and destabilizing domain (DD) systems and discuss a strategy for degrader discovery against novel target proteins.

Section editors:
Alessio Ciulli, FRSC – Professor of Chemical & Structural Biology, School of Life Sciences, University of Dundee, Division of Biological Chemistry and Drug Discovery, James Black Centre, Dow Street, Dundee DD1 5EH, United Kingdom.
William Farnaby – Professor of Chemical & Structural Biology, School of Life Sciences, University of Dundee, Division of Biological Chemistry and Drug Discovery, University of Dundee, James Black Centre, Dow Street, Dundee DD1 5EH, United Kingdom.

Introduction

The current main approach to drug discovery is to design occupancy-based protein inhibitors. Due to the lack of a binding site for inhibition, many proteins playing an important role in disease development, such as transcription factors having no enzymatic activity, are undruggable with this strategy. Genetic knockdown using nucleic acid (e.g., siRNA) was expected to solve the problem. However, off-target effects, the instability of nucleic acids, and slow depletion due to a long half-life of the target protein are major challenges [1]. Alternatively, protein expression can be controlled by an E3 ubiquitin ligase. Thalidomide and its analogs (known as immunomodulatory drugs or IMiDs) change substrate specificities of a CUL4–RING E3 ligase containing cereblon (CRBN) so that the neo-substrates such as IKZF1 and IKZF3 transcription factors are degraded [2–4]. Similarly, anticancer sulfonamides, such as indisulam, bind to DCAF15, another component of a CUL4–RING E3 ligase, and promote degradation of a neo-substrate RBM39 [5]. These drugs have been successfully used for cancer treatment showing that controlling protein degradation by an E3 ubiquitin ligase is a promising strategy for anticancer drug discovery.

*Corresponding author: M.T. Kanemaki (mkanemak@nig.ac.jp)

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Targeted protein degraders known as proteolysis targeting chimeras (PROTACs) are drawing attention as a new strategy to induce protein knockdown [6–9]. These heterobifunctional degraders bridge a protein of interest (POI) and an E3 ubiquitin ligase, and the POI is ubiquitylated for proteasomal degradation (Fig. 1A). As small molecules directly binding an E3 ligase have already been identified [6], it would be possible to design a heterobifunctional degrader by identifying a compound with affinity for the POI. A problem is that designing and identifying an optimized degrader is relatively time- and cost-consuming, and length and variety of linker and orientation of each binding moiety affect its efficacy [10]. Therefore, it is important to identify a validated target before designing degraders to determine an optimized one.

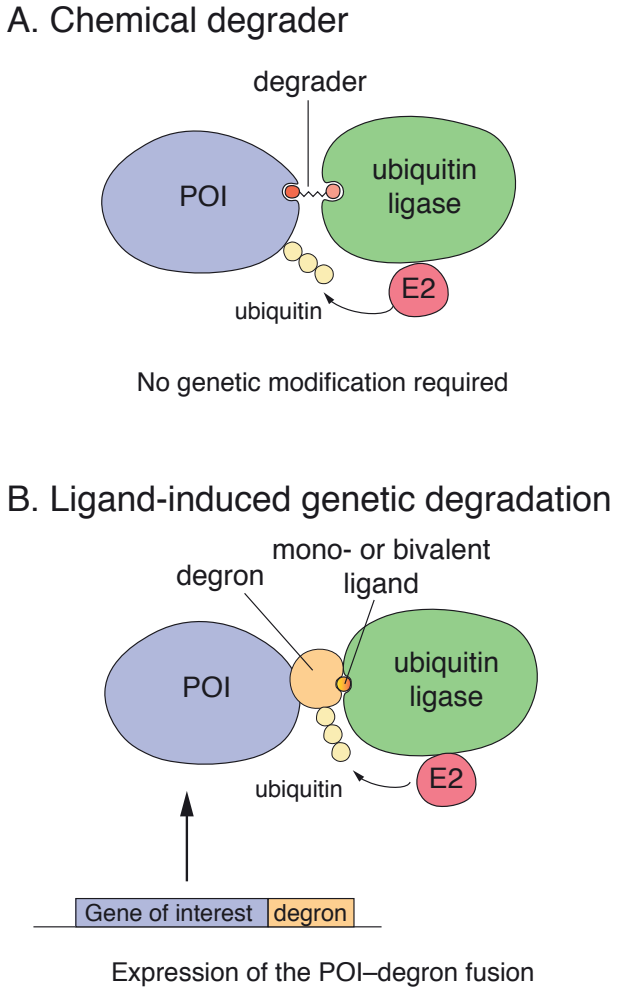

Fig. 1. Comparison of degrader and ligand-induced degradation. (**A**) A portion of the chemical degrader has a known binding ability to E3 ubiquitin ligase and is linked to a moiety that binds to the target protein. Rational design of the target protein binding moiety and linker length makes it possible to develop chemical degraders specific to previously undruggable proteins. (**B**) Though chemical degradation presents a new facet of drug discovery, the design and synthesis of chemical degraders may be a tedious process. In this sense, genetic tagging of the target protein with a degron tag developed to bind a defined ligand may provide an efficient solution. Because the degron–E3 ubiquitin ligase interaction mediated by the ligand is constant, the only manipulation required is genetic introduction of the degron to the target protein.

In this review, we propose utilizing ligand-induced genetic degradation technology for target validation. Ligand-independent genetic degradation systems utilizing a nanobody are not discussed in this review [11–13]. All degron tags discussed in this review are activated (or inactivated) in the presence of a defined ligand, so that degron-fused proteins are degraded via the ubiquitin-proteasome system (UPS). Some of the genetic degradation systems function through direct recruitment of degron-fused proteins to an E3 ubiquitin ligase analogous to heterobifunctional degraders (Fig. 1B). As genetic modification to introduce a degron tag is now feasible using genome editing (e.g. CRISPR–Cas9) [14], it is possible to screen multiple candidate proteins for verification before designing chemical degraders.

dTAG

dFKBP-1 is a heterobifunctional degrader composed of two binding moieties, a synthetic ligand for FKBP12 (SLF) and thalidomide, which bind FKBP12 and CRBN, respectively [9]. CRBN is a component of the CUL4–RING E3 ligase (CRL4), forming a CRL4–CRBN complex. In the presence of dFKBP-1, FKBP12 is recruited to CRL4–CRBN for rapid degradation by the UPS. Interestingly, both endogenous FKPB12 and other proteins fused with FKPB12 are efficiently and selectively depleted by dFKBP-1; thus, FKBP12 can be used as a ligand-induced degron. To improve the dFKBP-1 system by avoiding depletion of endogenous FKBP12, Gray, Bradner and co-workers employed a 'bump-and-hole' strategy previously demonstrated for FKBP12 [15]. $FKBP12^{F36V}$, a mutant version of FKBP12 with a molecular weight of 12 kDa, has a hole in the SLF binding site at which a bumped SLF analog called AP1867 can bind, but the original SLF does not [16]. They used $FKBP12^{F36V}$ as a degron to fuse with BRD4 and other proteins and successfully depleted them by adding bivalent ligands called dTAGs, which comprise AP1867 and thalidomide moieties (Fig. 2A and Table 1). The most potent dTAG-13 induced depletion of $FKBP12^{F36V}$–BRD4 at 50 nM to 1 μM in cultured cells. Importantly, $FKBP12^{F36V}$–BRD4 was rapidly and selectively depleted without affecting other BRD paralogs. Furthermore, $FKBP12^{F36V}$ fused with an oncogenic KRAS mutant ($FKBP12^{F36V}$–$KRAS^{G12V}$) was quickly depleted to follow changes in the transcriptome and proteome. The dTAG technology can be applied to mice to control protein expression [15]. This is advantageous for the systematic analysis of xenograft tumors for anti-tumor drug discovery.

dTAG has already been used in functional studies. Taking advantage of rapid depletion, an effect on the transcriptome has been reported after depletion of YY1 and ENL [17,18]. We expect that dTAG will be used frequently. However, whether a component of a large complex can be degraded by dTAG

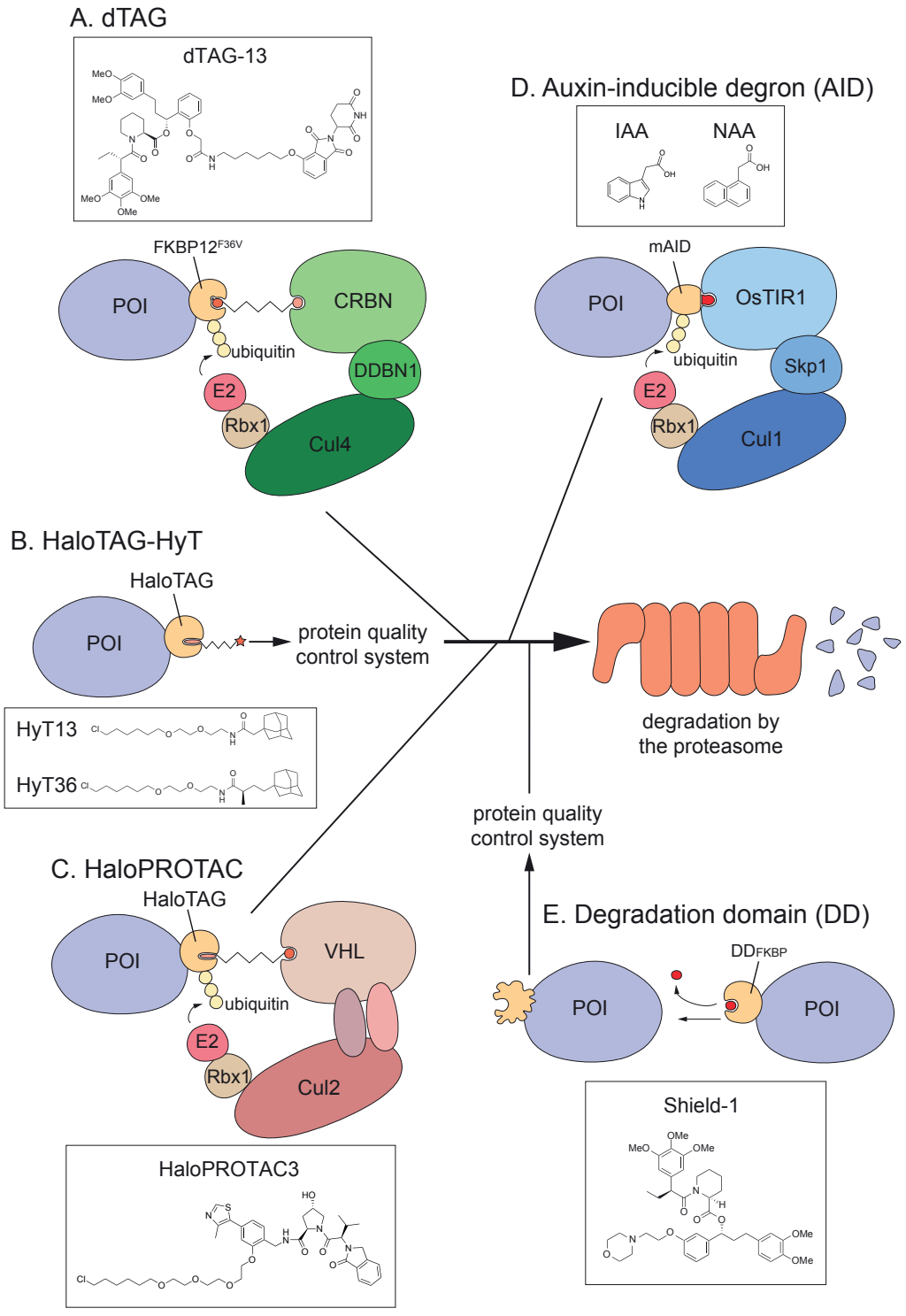

Fig. 2. Overview of ligand-induced genetic degradation systems. The ligand structure is shown in the box in each panel. (**A**) The dTAG system is a technique based on a bivalent ligand that exclusively links the genetically introduced FKBP12^{F36V} degron to a CRL4–CRBN E3 ubiquitin ligase. (**B**) In the HaloTag-HyT system, an HyT ligand (e.g., Hyt13 and Hy36) contains a hydrophobic moiety that recruits the HaloTag-fused POI to the protein quality control system. (**C**) The HaloPROTAC system uses a ligand designed to link the HaloTag-fused POI to a CRL2–VHL E3 ubiquitin ligase for ubiquitylation and degradation. (**D**) The AID system requires two genetic modifications to introduce an mAID degron to the POI and OsTIR1 that forms a CRL1–TIR1 E3 ubiquitin ligase. Auxin works as molecular glue binding TIR1 and promoting the association of TIR1 with mAID. (**E**) In the DD system, a DD tag-fused POI is stable in the presence of the specific ligand, whereas removal of this ligand destabilizes the tag-fused POI and subjects it to protein quality control, resulting in the degradation of POI–DD.

Table 1. Comparison of ligand-dependent genetic degradation technologies.

	Inducer	Nature of degron	Number of protein components	Ligand concentrations	Half-life in mammalian cells[a]	Tested organisms	Others
dTAG	dTAG13	$FKBP12^{F36V}$ (12 kDa)	1	50 nM–1 μM	Less than 1 h	Mouse	
HaloTag-HyT	HyT13, 36	HaloTag (33 kDa)	1	500 nM–10 μM	More than 1.5 h	Mouse, zebrafish	HyT13 does not work with HaloTag7
HaloPROTAC	HaloPROTAC3	HaloTag (33 kDa)	1	500 nM–1 μM	More than 4 h		
AID	Auxin (e.g., IAA, NAA)	mAID (7 kDa)	2	100–500 μM	Less than 30 min	Zebrafish, *C. elegans*, fruit fly, and yeast	
DD_{FKBP}	Shield-1	DD_{FKBP} (12 kDa)	1	100 nM–1 μM	More than 45 min	Mouse, rat and *C. elegans*	Ligand binding induces stabilization
LID	Shield-1	FKBP12–degron (13 kDa)	1	100 nM–2 μM	About 1 h		Works only at the C-terminus

[a] The half-life is a representative value. Given for general comparison.

remains to be addressed. Notably, dTAG is likely not functional in non-vertebrates, as CRBN is not conserved in these organisms.

HaloTag-HyT and HaloPROTACs

The bacterial dehalogenase derived HaloTag is a versatile protein tag with a molecular weight of 33 kDa. HaloTag forms a covalent bond with compounds containing alkyl chloride and has been used for protein purification, microscopy, and other purposes [19,20]. Crews and co-workers initially aimed to degrade proteins tagged with HaloTag by using a HaloTag binding ligand called hydrophobic tag (e.g., HyT13), which has a hydrophobic moiety mimicking protein misfolding or denaturation (Fig. 2B and Table 1) [21]. A POI-HaloTag fusion bound with HyT13 is degraded by the proteasome through a protein quality control system. HyT13 successfully induces degradation of HaloTag-fused proteins at 500 nM–10 μM in cultured cells and works in zebrafish and mice. However, HyT13 is not effective in a stable HaloTag variant called HaloTag7 [22]. To improve degradation efficacy, the same group improved the system by developing HyT36 (Fig. 2B), which induces better degradation when bound to the original HaloTag (also known as HaloTag2) and HaloTag7 [23]. A shortcoming of HaloTag-HyT is that depletion is relatively slow in cell culture ($t_{1/2}$ = 1.5 h) and may not be very efficient, especially when HaloTag7 is used.

Taking the above proof-of-concept results that HaloTag can be converted to a degron, Crews and co-workers subsequently developed a series of bivalent ligands called HaloPROTACs to degrade a POI fused with HaloTag7. HaloPROTACs bridge HaloTag7 and the von Hippel-Lindau protein (VHL) that forms a CUL2–RING E3 ubiquitin ligase complex (CRL2–VHL), so that the POI–HaloTag7 fusion recruited to CRL2–VHL is degraded via the UPS (Fig. 2C and Table 1) [24]. When treated with 625 nM of the most potent ligand, HaloPROTAC3, 90% of GFP–HaloTag7 is depleted. In contrast, only 50% of GFP–HaloTag7 was depleted in cells treated with HyT36 in a similar experimental setting [24], suggesting that HaloPROTAC is a better system for depleting HaloTag-fused proteins. However, the depletion kinetics are relatively slow ($t_{1/2}$ = 4 to 8 h) compared to other genetic degradation technologies. As is the case with the other systems, GFP–HaloTag7 was successfully re-expressed after removal of HaloPROTAC3. Whether HaloPROTACs can be used in animals has to be tested in the future. As HaloTag is commercially available and has been used in many fields, HaloPROTAC3 and its variants will be useful for target validation and functional studies in the future. Similarly, another bivalent ligand that bridges HaloTag and cellular inhibitor of apoptosis protein 1 (cIAP1) has been reported to deplete HaloTag-fused proteins [25].

Recently, bivalent ligands were reported that bridge a CH6 tag (a modified hexa-histidine tag [26]) and cIAP1 [27]. Proof-of-concept experiments showed that a bivalent ligand named

SNIPER(CH6) induced degradation of transgene derived proteins tagged with CH6. A shortcoming of this strategy is that SNIPER(CH6) is not cell permeable, so that a carrier peptide conjugated with a hexa-histidine tag is required for introducing SNIPER(CH6) to cells. Following the strategy used for HaloTag and CH6 tag, it should be possible to use another affinity tag to induce degradation by making bivalent ligands.

Auxin-inducible degron (AID)

Plants have evolved unique degradation pathways that are controlled by CUL1–RING E3 ubiquitin ligases (CRL1), possibly to cope with environmental changes without translocation. The genome of *Arabidopsis thaliana* encodes almost 700 F-box proteins, which can form a CRL1 E3 ubiquitin ligase complex and recognize specific substrates for degradation [28]. In contrast, the human genome encodes only ~70 F-box proteins [29]. Interestingly, phytohormone auxin (indole-3-acetic acid; IAA) binds the plant-specific TIR1 F-box protein and promotes an association with the AUX/IAA family of transcriptional repressors for ubiquitylation by CRL1–TIR1 [30–32]. Taking advantage of the conserved CRL1 subunits, other than TIR1, in all eukaryotic cells, we transferred the auxin-dependent degradation pathway to other eukaryotic species to develop the auxin-inducible degron (AID) technology (Fig. 2D and Table 1) [33]. We initially found that TIR1 derived from *A. thaliana* (AtTIR1) is incorporated to form CRL1–TIR1 in budding yeast. The *Arabidopsis* IAA17 protein was used as an AID. In yeast cells expressing AtTIR1, proteins fused with AID were rapidly degraded within 30–60 min after the addition of IAA or naphthaleneacetic acid (NAA), a synthetic auxin. Subsequently, we found that TIR1 derived from *Oryza sativa* (OsTIR1) had better thermostability than AtTIR1 at 37 °C. Using OsTIR1, a POI fused with AID was depleted within 45 min in chicken DT40 and human cells upon the addition of auxin ($t_{1/2} < 15$ min). Holland et al. also reported the successful use of AID in mammalian cells by combining it with siRNA for the suppression of endogenous target proteins, and demonstrated that POI–AID fusions were mostly depleted in less than 60 min ($t_{1/2} = 10$–20 min) [34]. To directly control endogenous proteins in human cells, we fused endogenous RAD21 (a cohesin subunit) and DHC1 (a dynein subunit) with AID using CRISPR–Cas9-based genome editing [35]. In the case of RAD21 mutant cells, the fusion protein was degraded with a half-life of 17 min after the addition of IAA.

The original AID/IAA17 tag has a molecular weight of 25 kDa and contains a possible dimerization domain. We created a truncated mini-AID (7 kDa) lacking the dimerization domain that demonstrated comparable activity to the original AID/IAA17 [35,36]. Other groups identified alternative versions termed AID* and AID[47], both of which have overlaps with mini-AID [37,38]. All shortened AIDs are recognized by OsTIR1 and work efficiently. However, these variants have not been directly compared.

The AID technology has already been used in many functional studies in various fields. For example, it was recently employed to study chromosome organization after rapid depletion of CTCF, cohesin, and condensin [39–41], indicating that this is a powerful technology for studying direct effects after target depletion. This technology has been applied successfully in controlling POIs in fission yeast, nematode, fruit fly, and zebrafish [42–45]. The AID technology appears to deplete AID-fused POIs more quickly than the other technologies described in this review. This feature may be related to TIR1 overexpression and/or the function of TIR1. Auxin directly binds to the pocket of TIR1, but not to AID, and works as a molecular glue to promote the association of AID with the TIR1–auxin complex [30]. In this case, ubiquitylation and subsequent release of an AID–POI may be quicker than with bivalent ligands, which directly bind both the degron and E3 ubiquitin ligase. Comparison of interaction kinetics between ligand-bound ligase and degron is needed to test this hypothesis.

A downside of the AID technology is that degradation can only be induced at higher IAA or NAA concentrations (100–500 μM), but these do not affect cell growth and the transcriptome [35]. To apply this technology to mice, improvements to enhance auxin sensitivity may be needed. Another problem is that, in cells expressing OsTIR1, the expression level of AID-fused proteins is sometimes reduced, even without auxin. This is possibly because the bovine serum used for culture media contains auxin-like indole compounds possibly derived from tryptophan. We avoided this problem by expressing OsTIR1 from a conditional tetracycline-inducible promoter [35]. However, degradation cannot be achieved until OsTIR1 is expressed following activation of the tetracycline-inducible promoter, and this can take many hours. To overcome these problems and apply this technology to mice, further improvements are needed.

There are other phytohormones that bind to an F-box protein and induce degradation of a specific substrate. One such example is jasmonic acid-isoleucine (JA-ile), which binds the F-box protein coronatine-insensitive 1 (COI1) to degrade JAZ family proteins. Lemischka and co-workers successfully controlled POIs fused with a degron derived from JAZ1 in cells expressing a modified COI1 via the addition of JA-ile analog coronatine [37]. A similar system may be able to be developed using other phytohormones that work similar to auxin and JA-ile.

Destabilizing domain (DD)

Wandless and co-workers developed a so-called destabilizing domain (DD), a constitutive degron that is stabilized in the presence of its ligand (Fig. 2E and Table 1). A POI fused with a DD is degraded in the absence of its ligand. In that study,

$FKBP12^{F36V}$ was mutated to isolate unstable mutants having a single amino-acid substitution such as L106P (DD_{FKBP}) that are stabilized in the presence of a synthetic ligand, Shield-1 [46]. A POI fused with DD_{FKBP} was depleted several hours after the removal of Shield-1 and was re-expressed by adding the ligand back into the culture medium. DD_{FKBP}–POI fusions can be expressed in a dose-dependent manner (100 nM–1 μM). The DD_{FKBP} technology has been applied successfully to mice [47] and used in many functional studies utilizing cell culture [48,49]. The same group subsequently reported analogous DDs based on ecDHFR, estrogen receptor, and UnaG with trimethoprim, tamoxifen, and bilirubin, respectively [50–52].

The DD-based technology is different from the other degradation systems discussed above in two ways. First, DDs work opposite to the other systems because they confer constitutive instability to a POI. Second, the degradation pathway for DDs is not well defined, though the proteasome is clearly involved. Protein quality control systems, which are also involved in Halo-HyT-mediated degradation, are likely involved. Even though DD_{FKBP} has been used in many functional studies, these features may not be ideal for target validation in degrader discovery.

To reverse the relationship between ligand-binding and degradation of DD_{FKBP}, the same group developed a ligand-induced degradation (LID) domain by fusing a synthetic 19-aa degron to the C-terminus of an FKBP12 variant (Table 1) [53]. The 19-peptide degron is hidden in the ligand-binding pocket of FKBP12, which protects the degron from degradation in the absence of its ligand, Shield-1. Shield-1 strongly binds to the binding pocket, which leads to exposure of the degron and induction of POI–LID degradation. Notably, the LID tag works only at the C-terminus of a POI, unlike other genetic degradation systems.

Strategy to develop degradation inducers against a novel target protein

Drug discovery based on targeted protein degradation is still in its infancy. Thus far, most of the heterobifunctional degraders have been designed to target validated proteins known to play an important role in tumor formation. Considering its potential, it would be interesting to identify novel targets for degradation inducer discovery. For this purpose, large-scale genetic screening can be performed using siRNA or CRISPR–Cas9 (Fig. 3). Depending on the setting, multiple candidate genes, such as those that confer a growth advantage to tumor cells, may be identified. Subsequently, it is required to validate the effect on growth by depleting the proteins encoded by the identified candidate genes. For this purpose, the ligand-induced genetic degradation technologies discussed in this review provide an ideal solution (Fig. 3). By tagging endogenous gene with a degron using CRISPR–Cas9 or complementing a degron-fused transgene in a knockout background, it is now possible to generate a conditional

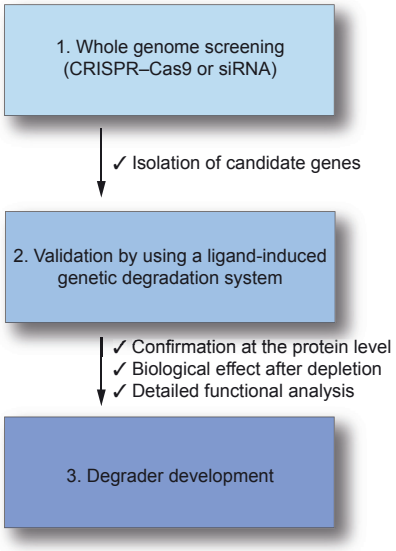

Fig. 3. A strategy for chemical degrader discovery against novel target proteins. Whole genome screening by CRISPR–Cas9 knockout or siRNA knockdown is a first step to discovering candidate target genes. We propose a second step utilizing a ligand-induced genetic degradation system to validate candidate proteins. This is a powerful strategy for assessing the biological impact and allows for various detailed analyses. Finally, the confirmed target proteins are subjected to the rational design of specific degraders. This three-step protein degradation-driven strategy may be proposed as a new time- and resource-efficient approach for degrader drug discovery.

cell line. In fact, the dTAG, AID and DD technologies have already been applied to control endogenous proteins using CRISPR–Cas9 [15,35,48]. Importantly, the POI–degron fusion is degraded by the UPS as in cells treated by a heterobifunctional degrader, and its expression level can be modulated by changing the ligand concentrations. This will provide important information to determine the expected degradation efficacy of a new degrader being developed. Moreover, the established conditional cell lines can be used for detailed functional analyses to understand the immediate consequences of depletion. This will aid in understanding the expected side effects and may lead to new findings in biomedicine. Additionally, adding a degron tag to a POI is advantageous for detection of the fusion protein, in particular to study less known new POIs, because specific antibodies against FKBP12 (dTAG), HaloTag, mini-AID, and DD are commercially available. However, adding a degron tag to a POI might interfere with the function of the POI sometimes irrespective of the size of the tag. It is important to be sure that the POI–degron fusion is functional before depletion. All degron tags except for LID are expected to work at both N- and C-termini. In case a POI–degron fusion was not functional, it would be worth testing to place the degron tag at the opposite terminus of the POI.

As genetic modification is essential for applying a genetic degradation system, genome editing is a crucial technology.

It is important to set-up an efficient pipeline to establish conditional cell lines by combining genome editing and genetic degradation technology. In the future, it may be possible to generate a library of conditional degron cell lines for all human genes. Such a library could be used from the initial screening.

When employing a genetic degradation system for target validation, the question is which system to use. The answer depends on the assay and organism that is being used. A comparison of ligand-dependent genetic degradation systems is shown in Table 1. Researchers have to find the appropriate system for their study because all technologies have advantages and disadvantages. We suggest initially comparing multiple systems. It may be worth employing a system that has been used successfully in multiple functional studies. Such a system must be robust and easier to apply.

The target validation discussed above can be used for drug discovery of conventional inhibitors. However, even if the same protein is targeted, the outcome may be different between inhibitor and degrader. Interestingly, conventional inhibitors can induce target degradation. For example, selective estrogen receptor down-regulators (e.g., fulvestrant) induce degradation of the estrogen receptor and suppress the growth of tamoxifen-resistant breast cancer [54], suggesting that degraders may generally work better than conventional inhibitors. We expect that degraders will be developed to target novel undruggable proteins, and that inducible degron technologies will contribute to future degrader discovery.

Conflict of interest

Y.T. is a founder and shareholder of FIMECS, Inc.

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