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Review

Targeted-Protein Silencing Tools: Overview and Future Perspectives

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Simple Summary: Gene inactivation is a powerful strategy to study the function of specific protein in the context of cellular physiology which can be applied for only non-essential genes, since they destroy the DNA sequence. On the other hand, perturbing the amount of the transcript can lead to incomplete protein depletion and generate potential off-target effects. Instead, targeting at protein level is desirable to overcome these limitations. In the last decade, several approaches have been developed and wisely improved, including compartment delocalization tools and protein degradation systems. This review highlights most recent advances in Targeting Protein Silencing (TPS) and focus on a putative novel tool to specifically degrades endogenous genetically unmodified target protein.

Abstract: Targeted Protein Silencing (TPS) is an elegant approach to investigate protein function and its role in the cellular landscape, overcoming limitations of genetic perturbation strategies. In contrast to CRISPR/Cas9 and RNA interference, these systems act in a reversible manner and reduce off-target effects. Several TPS have been developed and wisely improved, including compartment delocalization tools and protein degradation systems. In this review, we focus on Anchor-Away, deGradFP, auxin inducible degron (AID) and dTAG technologies, and discuss their recent applications and advances. Finally, we propose Nano-Grad, a novel nanobody-based protein degradation tool to specifically proteolyze endogenous tag-free target protein.

Keywords: Targeted Protein Silencing (TPS); Targeted Protein Degradation (TPD); dTAG; FKBP12; von Hippel-Lindau (VHL); degron; deGradFP; Anchor-Away; Nanobody; Nano-Grad

1. Introduction

Disruption of protein homeostasis is a powerful strategy to dissect specific protein function in the context of cellular physiology. Two main approaches are currently used to suppress protein expression: gene knockout by CRISPR/Cas9 and gene knockdown by RNA interference (RNAi) [1].

While gene editing by CRISPR/Cas9 acts at DNA level leading to a complete and irreversible depletion of the protein of interest (POI), RNAi induces reversible transcripts degradation and, in turn, its reduction [2-5]. On the other hand, RNAi can generate off-target effects [6] whereas CRISPR/Cas9 is a very selective genome editor [7].

In the last two decades, RNAi was widely used to achieve protein downregulation at mRNA level when knockout approaches were not compatible with life or a reversible experimental system

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was required. Anyway, the most disadvantage of this approach is that, when RNAi induced, all protein products already translated remain unaffected. Overall, both strategies determine an indirect depletion of the POI and can trigger compensatory mechanisms [8]. Targeting at protein level allows to interfere specifically with protein conformations, including post-translational modifications and splice variants. Besides, such approach would allow the depletion of the POI in specific cellular compartments and modulate its concentration over time.

Therefore, protein degradation techniques arise from the need to control and analyse the function and involvement of gene products in narrow time window of specific phases of various cellular activities including cell division. The rapid effectiveness feasible by protein targeting can be exploited to downregulate POI in a stage-specific way or when time is a relevant factor. In this context, cell division or Drosophila development studies perfectly match with the purpose of the protein interfering. To obtain an accurate and efficient protein silencing, several strategies have been developed. Among these, protein displacement systems and targeted protein degradation (TPD) are emerging strategies aiming to achieve proteolysis and loss of function of POI respectively. Protein displacement systems lead to inhibition of POI by compartment delocalization. Instead, TPD exploits the powerful of TAG, hijacking POI to E3 ubiquitin ligases to determine ubiquitylation and consequentially proteasomal degradation by recruitment of the ubiquitin-proteasome system (UPS). TPD includes several techniques that determine a rapid protein depletion minimizing compensation effects and accumulation of secondary defects [9].

In this review, we focus on widespread of TPS systems and discuss their applications in understanding protein function. We report Anchor-Away (AA), an interesting displacement technology; deGradFP, auxin inducible degron (AID) and dTAG degradation systems and their recent applications and advances. Finally, we propose a nanobody-based degrader (called nano-grader) to specifically degrade endogenous target protein eliminating all the procedure steps for genetic manipulations.

2. Anchor Away (AA)

Protein loss-of-function is a very important method for determine the role of a POI in different cellular and molecular mechanisms. Nuclear-localized proteins are involved in many important cellular processes such as DNA replication, DNA repair and transcription. Unlike all the other approaches described later, AA is not based on a degradation pathway and it mostly used to functionally inhibit nuclear POI. In AA technique, a rapamycin-dependent treatment is used to delocalize the POI from its physiological cellular compartment in order to prevent its functionality. The system is reversible, and following removal with the rapamycin-dependent treatment, the protein can reach its physiological localization to exploit its function. The AA technique was first defined in Saccharomyces cerevisiae [10] and later applied in Drosophila melanogaster [11] and human cell lines [12]. In the latter application, the mammalian mTOR pathway is employed for the association between FKBP12-rapamycin and FRAP, an evolutionary conserved 289-kDa protein. Rapamycin bridges between the POI and the anchor protein, allowing functional inhibition of the target protein [13]. In details, the FKBP12-rapamycin-binding-domain of FRAP (FRB) is fused to a specific POI, while the FKBP12 is fused to the anchor protein.

One of the most prominent application of the AA is to inhibit a nuclear target protein moving it to the cytoplasm. In this case, the anchor must be an abundant, cytoplasmic protein. One good example is Rpl13A, a ribosomal protein that is physiologically imported into the nucleus, assembled to form the ribosome and then permanently exported to the cytoplasm. Thus, in presence of rapamycin, the anchor protein (Rpl13A-FKBP12) interacts with the POI (fused with C-terminal domain of FRAP) forming a stable ternary complex which is exported in the cytoplasm where the POI protein is not able to properly work anymore (Figure 1).

Unfortunately, although AA is a very efficient strategy for non-functional delocalization of nuclear proteins (towards cytoplasm), it is not always true for cytoplasmic proteins on the other way round (towards nucleus). An attempt addressing that was done by Haruki et al., who proposed a

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histone-like anchor for delocalizing cytoplasmic proteins [10]. Changing course certainly represents a focus point in the future perspectives of this attractive technique.

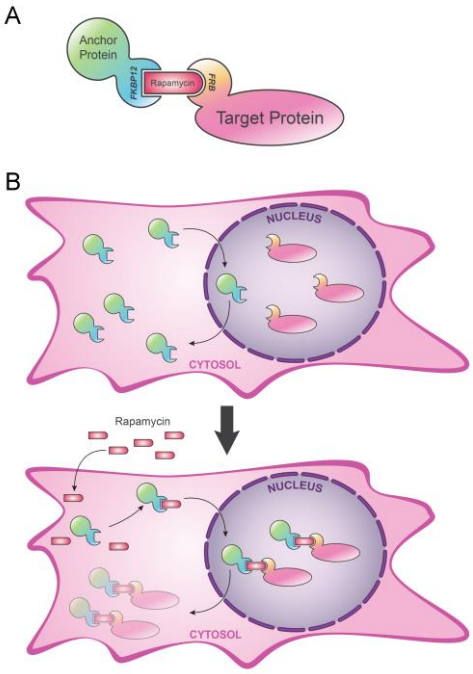

Figure 1. Anchor-Away (AA)

The mechanism of Anchor Away is based on the functional inhibition of the target protein.

A) Target protein is fused with the FKBP12-rapamycin-binding-domain of FRAP (FRB), while the Anchor protein is fused with FKBP12.

B) Added rapamycin acts as bridge between FRB and FKBP12 and POI is functionally inhibited by its own displacement from the physiological localization.

POI: protein of interest. Ub: ubiquitin.

3. deGradFP

DeGradFP technique is based on a E3 ubiquitin ligase chimeric construct (the so called, SCF complex) in which a transgenic adapter is fused to a specific nanobody (a natural single-domain antibody containing only heavy chains from llama or alpaca) for the recognition of GFP-tag, directing the polyubiquitination of the target GFP-protein which is then degraded by the proteasome pathway (Figure 2). The structure of the SCF complex normally comprises a Skp1, Cullin and F-box containing complex: this asset is maintained in the DeGradFP except for the F-box protein subunit, which is substituted by an engineered inducible form (NSlmb) fused to a nanobody (VhhGFP4) against the fluorescent reporter proteins and some derivates (GFP, Venus, YFP and EYFP) [14,15]. The use of fluorescent proteins is a great added value, because fluorescence intensity can be measured to check POI downregulation in time-lapse experiments. Of note, any background noise can be excluded using a Red Fluorescent Protein (RFP) tag signal, which is not recognized by the complex acting as negative control. Experimental evidence shows that the signal starts to decrease at ~30 minutes after induction and only a residual 10% or less can be observed after ~3 hours in most of the cases [14]. Moreover, Caussinus et al. found that the degradation efficiency is equivalent in different cellular compartments; therefore nuclear, cytoplasmatic and transmembrane proteins (H2A.v::GFP, apterous ap::GFP, spaghetti squash sqh::GFP and crumb Crb::GFP) respectively, can be equally polyubiquitinated and hijacked to the proteasome. Anyway, it must be discussed that this system is not applicable in two cases. First, when the POI has such a structure that could internalize the GFP tag preventing the binding of the NSlmb-VhhGFP4 to the target. Second, if the POI is part of a multiprotein complex that could hide GFP tag which is not accessible anymore. It has also been observed that the GFP molecule itself is not recognized by this polyubiquitination complex, unless associated to another protein or peptide (the smallest chain tested is the 3xNLS)

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suggesting a molecule recognition size limit for this system [14-16]. Since several transgenic stocks carrying GFP-tagged proteins for Drosophila and other model organisms already exist, deGradFP can be consider the most versatile system [14,17]. A more recent system based on the adaptor specificity of the E3-ubiquitin ligase have been proposed. In this case, the GFP nanobody VhhGFP4 is attached to SPOP E3 ubiquitin ligase which guide the degradation of nuclear protein more efficiently [18]. The evolutionary conservation of the complex gave the possibility to expand this SPOP-based deGradFP system in different organisms like Drosophila melanogaster [14], Nicotiana tabacum [19], Danio rerio [20] and Parhyale hawaiensis [17] and animal cell lines [16].

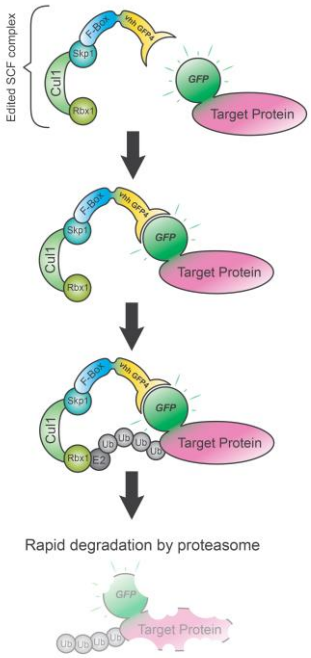

Figure 2. de(G)radation of Fluorescent Protein (deGradFP)

The deGradFP technique rely on the specificity of the SCF complex given by the fused nanobody to a GFP-tag fused protein. The consequent poly-ubiquitination of the target protein results in a proteasome-dependent degradation.
In this system, SCF complex is composed by endogenous components: the adapter elements for the F-box protein (Skp1), cullin scaffold (Cul1) and the RING protein recruiting an E2 ligase (Rbx1). The specificity of the substrate is given by the FBP subunit target recognition (F-box) which is fused to the vhh-GFP4, an anti-GFP nanobody. Upon GFP-tagged protein recognition by F-box/vhh-GFP4, the E2 ubiquitin-protein ligase mediates the poly-ubiquitination of POI which will be immediately degraded by the proteasome.
POI: protein of interest. Ub: ubiquitin.

4. Auxin-inducible degron system (AID)

Auxins such as the IAA (indol-3-acetic acid) are hormones that control many steps of growth and plant development. When their amount increases, it allows the transcription of the auxin-responsive genes through a degradation of the transcriptional repressors AUX/IAA via proteasome.

In this pathway there are three essential components: the E3 ubiquitin ligase enzyme, the target proteins and the phytohormone. As explained above, the E3 ubiquitin ligase is the SCF (Skp1, Cullin 1 and F-box) complex. The F-box subunit is versatile to be modified according to the different applications, in order to mediate the substrate specificity. TIR1 (transport inhibitor response 1) is a particular F-Box protein, which binds to auxins and then leads to the target (AUX/IAA) recognition. Subsequently, an E2 ubiquitin conjugating enzyme is recruited for polyubiquitination and

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degradation of POI. The application of this degradation system in non-plant cells is based on the presence of SCF complex in all eukaryotes, but it is limited because of missing of TIR1 orthologs.

Using genetic manipulation, it was possible to mediate the ectopic expressions of both TIR1 and an AID tag sequence (derived from an AUX/IAA, recognized by TIR1) to tag an endogenous target protein. The exogenous TIR1 is able to form a functional SCF-TIR1 complex that can lead the degradation of the tagged protein by the proteasome pathway in the presence of auxin [19] (Figure 3). This system was successfully used in many organisms including yeast, chicken, hamster and human cells. In yeast, it has been demonstrated that the system works using the IAA17 (also known as AX3) peptide as AID tag and the TIR1 adapter, both derived from Arabidopsis thaliana [21].

Nevertheless, this first-generation system did not work at temperature incubation required for animal cell culture [21], therefore AtTIR1 was replaced with the Oryza sativa ortholog OsTIR1 which is more stable at high temperature. When the auxin is supplied, the activation of the TIR1 protein induces a very rapid POI depletion, with a half-life of 10-20 min [22,23] which can be reverted by removing the auxin [21].

However, the use of the natural auxin IAA is limited in some model organisms [24-26] and all of the secondary effects are abolished by using a synthetic auxin, the NAA (1-naphtalenicetic acid).

The AID system is extensively used to study the function of both non-essential and essential genes in yeast, Drosophila melanogaster and Caenorhabditis elegans, while its application in human cells has been limited because of tricky AID-tagging of endogenous proteins, especially for essential proteins [21]. To overcome this problem, CRISPR/Cas9-based genome editing tool has been exploited to fuse the AID tag to essential genes in human cell line [27]. The introduction of m-AID tag (minimum AID tag, 7 kDa) coupled to CRISPR/Cas9 approach has been successfully used in other studies that involved human essential genes with roles in cell division like APC4 [28] or Cdc7, a serine-threonine kinase involved in many processes including Aurora B activity stimulation [29], a master essential kinase required during mitosis. In some cases, to obtain a faster and more efficient POI degradation, AID system has been coupled to Tet-OFF promoter system, in which the simultaneous IAA+Doxycycline (dox) treatment results in a more rapid and complete POI degradation compared with a depletion mediated only by dox or IAA [30]. This combined system has been used in investigation of human essential proteins like CDK2, a kinase essential for the S-phase progression; Cyclin A, partner of CDK1 and CDK2, involved in the control of the S-phase and mitosis; and TRIP13, a protein that regulates the Spindle Assembly Checkpoint (SAC) [30].

A limitation of the original AID system is the premature degradation of the target also when auxin is not added in the culture medium [31-33]. This auxin-independent degradation is the consequence of the high expression rate of TIR1. The use of a Tet-Promoter in combination with the AID system was also employed to overcome this problem putting the Os-TIR1 gene under a tetracycline-regulated promoter [27]. However, the tet-OsTIR1 expression could be slow and have an influence on the degradation timing.

To prevent basal auxin-independent depletion of the target protein, many efforts have been spent to develop an improved auxin-inducible degron system in which ARFs (auxin transcription factors) are co-expressed together with components of the original AID system. In plant cells, when the auxin level is low, ARFs bind AUX/IAA proteins [34]. Alternatively, when the auxin levels increase, TIR1 interacts with AUX/IAA (which are both leaded to the proteasome) and ARFs proteins are released to regulate transcription of auxin responsive genes. In this improved system in which ARF-AID are co-expressed, native levels of POI are preserved by formation of a stable ARF-AID tag complex which change the conformation of AID-tag preventing its premature association with auxin-unbond-TIR1 and POI degradation [35].

A more recent improvement of the original AID system consisting in the generation of a bicistronic all-in-one plasmid that mediates the expression of TIR1 together with the AID tag fused POI in order to reduce genetic manipulation [36]. However, although this new approach relies on randomly plasmid integration in the host genome, a POI expression comparable to that of the endogenous protein can be achieved. Moreover, the control of OsTIR1 and AID tagged POI

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expression by the same promoter, determinates a proportional expression of them avoiding that overexpressed TIR1 can lead to auxin-independent degradation.

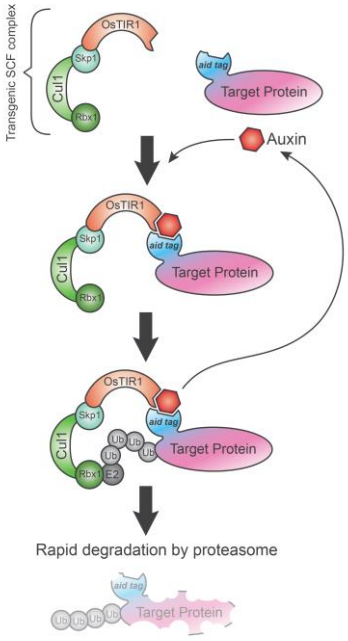

Figure 3. Auxin-inducible Degradation (AID)

The AID system required the ectopic expression of both OsTIR1 gene and aid-tagged POI.

As shown in the figure, the exogenous OsTIR1 gene can form a functional transgenic SCF complex by connecting with the endogenous elements of the complex (Skp1, Cul1 and Rbx1). When auxin is added in the culture medium, it binds OsTIR1 triggering POI recognition and its subsequent polyubiquitination by an E2 ubiquitin-conjugating enzyme. The tagged protein is rapidly leaded to the proteasome for degradation. POI: protein of interest. Ub: ubiquitin.

5. degradation TAG (dTAG) system

The degradation TAG system is a technology for rapidly, reversible and selectively depletion of a POI developed by Nabet, et al. [37]. This approach needs three major components: a FKBP12F36V-fused POI, a small synthetic molecule, defined degrader, and the endogenous E3 ligase complex (Figure 4). Target protein is fused with the 12kDa cytosolic prolyl isomerase engineered variant (FKBP12F36V), by using transgene expression or CRISPR-mediated locus-specific knock-in [38,39]. A heterobifunctional degrader (e.g. dTAG-13) recruits FKBP12F36V-fused POI to cereblon (CRBN), the recognition unit of CRL4-CRBN E3 ubiquitin ligase complex, leading to exclusive POI degradation by proteasome. In brief, authors have developed a series of degrader molecules consisting of AP1867, a synthetic FKBP12F36V selective ligand, and thalidomide binding CRBN, connected to each other by different linkers.

The dTAG system was first employed to evaluate the consequences of acute degradation of ENL and MELK, a transcriptional regulator and a promoting proliferation kinase respectively [40,41]; and later it was successfully tested in cells to rapidly and selectively degrade a panel of FKBP12F36V-fused chimeras like BRD4, HDAC1, EZH2, MYC, PLK1, and KRASG12V. Reported data shows specific variation rates of POI degradation depending on target subcellular compartmentalization. Moreover, Nabet et al. extended dTAG-strategy to in vivo targeted degradation studies using mouse as model. Recent applications of this technology were adapted to rapidly deplete all IE2 protein isoforms, to elucidate the role of these proteins in HCMV late infection [42], and also to degrade solute carrier (SLC) proteins, the largest class of transporters with multi-pass transmembrane domain topology [43]. Based on reported data, dTAG platform is a versatile system triggering selective depletion without off-target effects [44]. This powerful approach would be an ideal validation strategy of therapeutic targets in in vivo applications [37,40].

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More recently, Nabet, et al. [45] have expanded the suite of dTAG molecules developing dTAGV-1, a degrader that engages the von Hippel-Lindau (VHL) E3 ligase complex. This second generation of dTAG overcomes limitations of dTAG-13 in degradation of several protein, exemplified by EWS/FLI, a driver of Ewing sarcoma. dTAGV-1 compared to dTAG-13 show an increased pharmacokinetic and pharmacodynamic profile with a longer half-life, a greater exposure, and an improved duration of degradation. This implemented tool make dTAG strategy a universally applicable and a good candidate to be considered the most efficient and less time- consuming TPD approach.

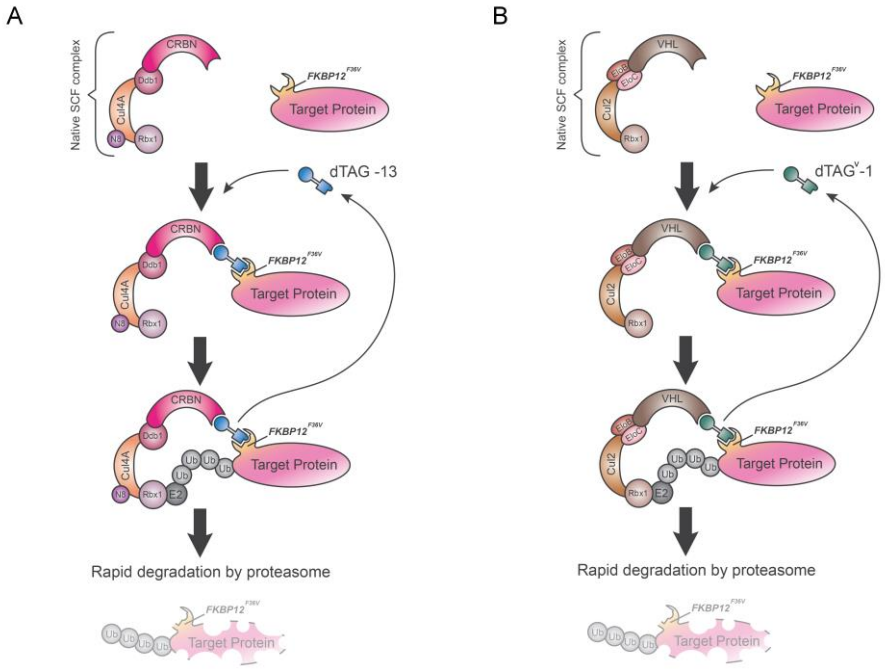

Figure 4. degradation TAG (dTAG)

A) Heterobifunctional dTAG-13 molecules engage FKBP12F36V-fused POI and Cereblon (CRBN), hijacking it towards endogenous proteasome machinery for POI degradation. dTAG-13 contains AP1867 and thalidomide, FKBP12F36V and CRBN selective ligands, respectively. CRL4–CRBN E3 ubiquitin ligase recruited by dTAG-13 is composed of cullin scaffold (CUL4A), adaptor protein (DDB1), substrate receptor (CRBN), the RING protein (Rbx1) recruiting an E2 ligase and N8 ubiquitin-like protein (NEDD8).

B) dTAGV-1 molecule recruits the von Hippel-Lindau (VHL) E3 ligase complex, increasing dTAG system efficiency. VHL complex consists of a cullin scaffold (Cul2), two adaptor proteins (EloB and EloC), a substrate receptor (VHL), and Rbx1. POI: protein of interest. Ub: ubiquitin.

6. Conclusions and Future Perspectives

During the last decade, the field of TPS had a massive growth and spread rapidly in cell and developmental biology. We discussed different approaches to modulate proteins functionality and study their physiological roles. Although Anchor-Away system has advantage to be inducible/reversible (by Rapamycin), it has some disadvantages also. Indeed, it cannot be applied to all proteins but only for the nuclear ones. Thus, more versatile methods have been developed. One possible alternative is based on the use of ubiquitinoylation pathway to induce targeted protein degradation via proteasome. Caussinus et al. engineered an anti-GFP nanobody fused to F-box to channel GFP-tagged protein to the proteasome for degradation. This strategy proved to be highly beneficial for time-lapse microscopy to follow POI degradation in vivo. Despite deGradFP is a more suitable approach for potentially any protein, it is not a reversible system and requires adding a relatively big tag (GFP) to POI risking to compromise its functional folding. These limitations carried out to further improve these strategies and reduce both tag size and genetic modifications. To address these, two further methods were developed and discussed in this review: Auxin Inducible Degron and dTAG systems.

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While the AID is an auxin-dependent degradation system which still requires expression of an adaptor from planta (TIR1), the dTAG does not need of any added transgenic element because it exploits native degradation pathway triggered by a very permeable and small heterobifunctional degrader (e.g. dTAG-13) approximating tagged-POI and proteasome machinery. Even though dTAG represents the latest frontier in TPD, it still requires the POI to be fused to a tag, therefore, cloning and stable cell lines generation procedures cannot be skipped in any way. Hence the need to eliminate protein tagging process and notwithstanding flexibility of the system.

Addressing that, Bery et al. set up an original cell-based screen to identify nanobodies that selectively degrade the RHOB-GTP fraction engineering them with a functionalized F-box domain [46]. The F-box/intrabody-mediated protein degradation represents the first approach to selectively target tag-free POI, active form of small GTPases or other proteins with multiple cellular activities, although the preliminary nanobodies identification process is hardworking and highly time consuming [46]. A further attempt was done with TRIM away and PROteolysis-Targeting Chimeras (PROTACs) systems.

In TRIM away, POI-specific antibody bringing between TRIM21 and POI and marking it for degradation through ubiquitination. This approach is extremely versatile, but not feasible for a large-scale use as the antibody must be internalized by injection or electroporation [47]. Unlike most of the degradation systems, PROTACs do not necessitate genetic modification of target gene as they work against endogenous POIs by using small bivalent molecules which are able to simultaneously bind POI and an E3 ligase component, ultimately leading to POI ubiquitination and degradation. Undoubtedly, identification or development of PROTACs that can freely pass cell membrane and target different E3-ligase and POI requests a great effort, and it is an extremely high time-consuming procedure [48].

In order to overcome the above-mentioned limitations, we are working on the optimization of an innovative strategy exploiting the efficiency of nanobody and dTAG system to approach close together POI and proteasome machinery to stimulate rapid degradation: Nano-Grad (Prozzillo Y, et al., in preparation). In our model (Figure 5), VHL ligand is covalently linked to POI-specific VHH (nanobody) to generate a new type of degrader which we call Nano-Grader (NG) (Figure 5A). Likewise dTAGv-1, the hetero-bifunctional NG is able to approximate VHL subunit to free-tag POI and trigger its rapid degradation via proteasome. A crucial step for the success of this method, is the internalization of the NG into the cells. To allow this passage, we propose to generate cell-permeable nanobodies by site-specific attachment of a cyclic arginine-rich cell-penetrating peptides, as previously done by [Herce, et al. [49]. Theoretically, Nano-Grader strategy may represent a highly versatile system, as nanobodies against every POI could accept these modifications to develop itself into NG and trigger tag-free target protein degradation.

Although this innovative approach indicates several advantages clearly shows many advantages (Table 1); one limit could be the strength of the bond between antigen and nanobody, which can vary quiet substantially. Another aspect that needs to be discusses is that proteasome machinery could potentially incorporate NG for degradation, leading to a progressive accumulation of VHL ligand molecules resulting toxic for the cell, such as the unidirectional interaction with only proteasome can make it inactive in a dominant-negative manner. On the other hand, cell machinery could compensate for this toxic effect by physiologically eliminating the thalidomide. Nonetheless, Nano-Grad approach contains elements of novelty and advancement which are worth to be tested and optimised. In conclusion, Nano-Grad could become the last generation of TPS and open the way to definitely forsake the need to tag POI and get tangle up with cumbersome genetic manipulations.

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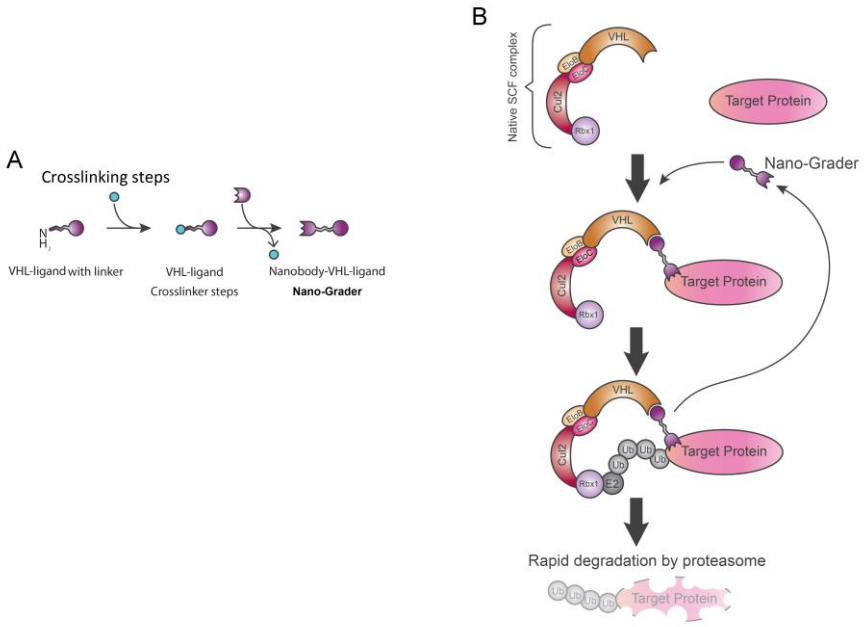

Figure 5. Nano-Grad (NG)

A) Nano-Grad contains VHL selective ligand, an anti-POI specific nanobody and an arginine-rich tails for cellular permeabilization.

B) Heterobifunctional Nano-Grader molecules bring together the von Hippel-Lindau (VHL) E3 ligase complex and POI catalysiNng proteasomal degradation of target protein.

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Table 1. Advantages and Disadvantages of Targeted Protein Silencing systems.

System	Tag size	Degrader (MW g/mol)	Organisms	Advantages	Disadvantages	Reversible/ Inducible	Transgenic elements
Anchor-Away	12 kDa	Rapamycin (914)	- Fly - Yeast - Human cell lines	- Functional inhibition of multiple tagged proteins - No stress response mechanism is activated - *In vivo* applicability - High selectivity	- Usable only for nuclear POIs - Requires engineering fusion between proteins - Several pilot experiments are needed to understand the concentration of Rapamycin - Long-time degradation (the silencing is detectable 6 hours after treatment)	Yes/Yes	2
deGradFP	27 kDa		- Zebrafish - Crustacean - Fly - Plants - Animal cell lines	-Nuclear, cytoplasmatic and trans-membrane targets available - *In vivo* applicability - Possibility to follow the degradation process by fluorescence - Availability of large libraries of GFP::proteins for different model organisms	- Long-time degradation (less the 10% of the EGFP signal after 3h) - Requires genetic engineering (endogenous expression of GFP::POI); - Tag size - Some fusion proteins cannot be detected by the system	No/No	2
AID system	7 kDa	IAA (175) NAA (186)	- Fly - Worm - Yeast - Chicken, Human, and murine cell lines	- Rapid POI degradation - Useful for both nuclear and cytoplasmatic proteins - Two kind of inducers (natural or synthetic auxins) - Couplable with other systems (CRISPR-Cas, Tet promoters) - Preserves native levels of POI	- Usable only in non-plant cells - Limited by presence of only few orthologs of TIR1 - Requires genetic manipulation (TIR1 and Tag fused protein expression)	Yes/Yes	2
dTAG system	12 kDa	dTAG-13 (1049)	- Mouse	- High selectivity	- Different rates of POI degradation depending	Yes / Yes	1

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		dTAG^{V}-1 (1361)	- Human and murine cell lines	- Rapid POI degradation - Small TAG size - *In vivo* applicability - Excellent pharmacokinetic and pharmacodynamic properties (long half-life of degrader and great exposure)	on subcellular compartments - Limited to the cell systems in which CRISPR/Cas9 modifications are feasible - Tested in few organisms		
Nano-Grad	No	N/A	N/A	- Preserves native levels of POI - *In vivo* applicability* - No genetic manipulation is required	- Nano-Grader may be also degraded by proteasome*	Yes/Yes	0

* These represent only putative advantages/disadvantages since they have not yet been experimentally verified.

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