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Design and functional characterization of synthetic E3 ubiquitin ligases for targeted protein depletion:

Selective protein knockout using engineered ubiquibodies

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Abstract

A number of techniques now exist for decreasing the expression of cellular proteins without the need for genomic modification. One such technique involves engineered protein chimeras that combine the ubiquitination activity of E3 ubiquitin ligases with the binding affinity and substrate specificity of designer binding proteins (DBPs). These chimeras, called “ubiquibodies”, are capable of selectively and controllably steering virtually any protein to the ubiquitin proteasome pathway (UPP) for degradation, making ubiquibodies a powerful addition to the protein knockout toolbox. A distinguishing feature of ubiquibodies is their modularity – simply swapping DBPs can generate a new ubiquibody with specificity for a different substrate protein. Moreover, by employing DBPs that recognize particular protein states (*e.g.*, active vs. inactive conformation, mutant vs. wildtype, post-translational modification), it becomes possible to deplete certain protein subpopulations while sparing others. This protocol outlines the steps necessary to design and functionally evaluate ubiquibodies for customizable silencing of cellular proteins.

Keywords

antibody; non-antibody protein scaffolds; protein degradation; protein silencing; ubiquibody; ubiquitination; ubiquitin proteasome pathway (UPP)

INTRODUCTION

Determining the function of a specific cellular protein is commonly achieved by assessing the consequences of its removal. A number of technologies for decreasing the expression of cellular proteins have emerged, enabling target-specific silencing at the DNA, RNA, or protein level. For example, genome editing using CRISPR-Cas9 is a convenient approach for knocking out a gene of interest (Cong et al., 2013; Mali et al., 2013) but is irreversible and may not be feasible for many targets such as those that are essential. Alternative silencing

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Conflict of Interest Statement

None

approaches involving antisense deoxyoligonucleotides (Nasevicius & Ekker, 2000) and RNA interference (Fire et al., 1998), which target mRNA transcripts, are attractive because they do not require genomic modification. However, these methods can result in incomplete knockdown especially for long-lived proteins, cannot distinguish among post-translational protein populations, and are prone to off-target effects (Jackson & Linsley, 2010; Kok et al., 2015). Chemical inhibitors can achieve protein silencing with post-translational precision, but often require lengthy screening to identify lead candidates, which may lack the specificity and/or affinity necessary for efficient silencing. Antibodies and antibody fragments circumvent this issue with their exquisite affinity, specificity, and modularity. However, antibodies and their fragments require disulfide bonds for proper folding and do not always function in the reducing environment within cells. This has led to the development of intracellular antibody fragments (intrabodies) and antibody mimics such as the human fibronectin type III domain (FN3) and designed ankyrin repeat proteins (DARPin)s, all of which can be engineered to bind their antigens inside living cells (Kawe, Forrer, Amstutz, & Pluckthun, 2006; Koide, Abbatiello, Rothgery, & Koide, 2002; Martineau, Jones, & Winter, 1998). Unfortunately, these “inhibition-by-binding” approaches involving chemical inhibitors and antibody/antibody-mimics have two significant drawbacks: (i) the necessity for a 1:1 stoichiometry between binder and target; and (ii) the inability to hit so-called undruggable targets (Lazo & Sharlow, 2016).

Recently, several alternative methods have been developed that harness the power of the ubiquitin proteasome pathway (UPP) to achieve protein-level silencing via an “inhibition-by-degradation” approach. The UPP is the primary mechanism utilized by eukaryotic cells to maintain protein homeostasis by preventing the accumulation of abnormal or toxic proteins that are misfolded or damaged. The canonical ubiquitination cascade involves three enzymes, termed E1, E2, and E3, that tag proteins for degradation through the covalent addition of a poly-ubiquitin chain (Figure 1). Ubiquitin, a 76-amino-acid protein, is first activated by E1, and then subsequently transferred to the carrier E2. Lastly, the E3 ubiquitin ligase conjugates ubiquitin to an exposed lysine residue on the target protein. Importantly, substrate specificity is conferred by the E3 (Kerscher, Felberbaum, & Hochstrasser, 2006), whose intrinsic structural flexibility enables accommodation of substrates with different sizes and structures (Qian et al., 2009). A handful of strategies that exploit the UPP for targeted proteolysis involve substantial modification of the target protein with: (1) destabilizing domains called degrons that are conditionally activated in the presence of drug or light (Bonger, Chen, Liu, & Wandless, 2011); (2) degrons that induce proximity between the target protein and an E3 ubiquitin ligase (Holland, Fachinetti, Han, & Cleveland, 2012); or (3) the green fluorescent protein (GFP) (Causinus, Kanca, & Affolter, 2011).

A number of more practical strategies have been described that harness the UPP to degrade unmodified proteins. For example, protein knockout using the SCF system has been developed by introducing established protein-protein interaction modules to the F-box-containing substrate receptor for specific recruitment of target proteins (Hatakeyama, Watanabe, Fujii, & Nakayama, 2005; Zhang, Zheng, & Zhou, 2003) or using peptide-small-molecule hybrids, known as proteolysis targeting chimeras (PROTACs), that bridge the interaction between the intended target and the F-box (Sakamoto et al., 2001). More recently, Portnoff et al. described a universal strategy involving engineered protein chimeras

comprised of an E3 ubiquitin ligase and a DBP (Portnoff, Stephens, Varner, & DeLisa, 2014). These chimeras, called “ubiquibodies” (uAbs), combine the flexible ubiquitin-tagging capacity of the human E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) with the engineerable affinity and specificity of DBPs, including single-chain Fv (scFv) intrabodies, FN3 and DARPin. The result is a customizable technology for efficiently directing otherwise stable proteins to the UPP for degradation. Importantly, uAbs offer several advantages. First, they are highly modular – simply swapping DBPs can generate a new uAb with specificity for a different substrate protein. Second, by adapting uAbs with DBPs that recognize particular protein states (*e.g.*, active vs. inactive conformation, mutant vs. wildtype, post-translational modification), it becomes possible to deplete certain protein subpopulations while sparing others. Here, we describe protocols for the design and characterization of uAbs. Basic Protocol 1 describes how to create uAbs with desired specificity. Basic Protocols 2 to 5 detail how to characterize the activity of uAbs using a combination of cell-free and mammalian cell-based methods.

STRATEGIC PLANNING

The general workflow for designing and functionally characterizing uAbs with desired specificity is provided in Figure 2. This workflow involves methods for cell-free (**Basic Protocol 2**) and mammalian cell-based (**Basic Protocol 3**) characterization, which can be complementary but the importance of each will depend on individual objectives. The cell-free characterization methods should be used to confirm substrate binding and ubiquitination activity, including the linkage of ubiquitin chains (*e.g.*, Lys48), but do not provide any information regarding the extent of target degradation. For cell-free characterization, it is imperative that the uAb and target protein have distinct detection tags, or can be detected directly by existing affinity reagents. Identification of a suitable target-specific DBP is a critical step in the design of a new uAb molecule. If a DBP is not available for the target of interest, one can be isolated *de novo* using established techniques like yeast surface display, filamentous phage display, etc. However, these techniques are outside the scope of the protocols provided here.

BASIC PROTOCOL 1

Design and Cloning of uAbs with Desired Specificity—The following protocol describes how to clone a uAb with desired specificity by replacing the existing DBP, namely β -galactosidase-specific scFv13-R4, with a new target-specific DBP. All uAbs should be cloned into either a mammalian or bacterial expression vector, depending on the characterization route desired (Figure 2). First, identify a DBP that specifically binds the desired protein target. We have achieved success with a number of different formats including scFv intrabodies, FN3s, and DARPins. Other scaffolds may also work, provided that they are small, fold efficiently in the reducing cytosolic environment, and exhibit high affinity (low μ M to nM) and specificity towards the intended target (Sha, Salzman, Gupta, & Koide, 2017). There have been several reports describing successful fusions between DBPs and truncated E3 ubiquitin ligases (Caussinus et al., 2011; Portnoff et al., 2014; Shin et al., 2015). In our hands, the flexibility and solubility of human CHIP are advantageous properties in the context of uAbs; hence, these protocols will focus on the implementation of

CHIP-based uAbs. The human E3 ligase CHIP (C-terminal U-box ligase domain) is modular in nature, containing an N-terminal tetratricopeptide repeat (TPR) domain, a helical linker domain, and a C-terminal U-box ligase domain (Figure 3a). The TPR domain binds molecular chaperones Hsc70-Hsp70 and Hsp90, resulting in the ubiquitination of a broad range of chaperone-bound client proteins (Cyr, Hohfeld, & Patterson, 2002). The helical linker domain is necessary for protein dimerization and substrate ubiquitination (Nikolay et al., 2004). The U-box domain binds the E2 enzyme, facilitating the transfer of ubiquitin from the E2-ubiquitin complex to the substrate protein (Jiang et al., 2001). The uAbs described here utilize a truncated variant of CHIP, CHIP-TPR, which lacks the substrate-recognition TPR domain (Figure 3a).

We employ a dual FLAG-6×His tag on the C-terminus of our uAbs. The FLAG epitope (DYKDDDDK) aids in solubilization, while the 6×His tag is used for purification. Both can be utilized for immunoblotting detection, although the FLAG-tag has a lysine residue, which can be ubiquitinated and potentially inaccessible to binding by the anti-FLAG antibody.

Materials—Plasmid DNA encoding uAb (pET28a-R4-uAb or pcDNA3-R4-uAb (Figure 3b); both described elsewhere (Portnoff et al., 2014) and available on Addgene #101800 & #101801)

- DBP DNA template
- Primers, with designed restriction overhangs
- DNA polymerase
- dNTPs
- Restriction enzymes
- Agarose (molecular biology quality)
- SybrSafe (Thermo Fisher Scientific)
- DNA gel extraction kit
- DNA ligase and buffer
- Competent *Escherichia coli* cells (chemically or electro-competent)
- Antibiotics (based on expression vector chosen)
- Super Optimal Broth (SOB; see recipe)
- Luria-Bertani Agar (LBA; see recipe)
- Plasmid DNA miniprep kit
- Equipment
- Thermocycler
- Gel imaging system capable of detecting DNA
- Gel electrophoresis system

Power supply

Protocol

1. Design primers to amplify the new target-specific DBP with the necessary restriction overhangs (see Figure 3 for details).
Verify that the DBP sequence does not contain the chosen restriction sites. To create a proper genetic fusion, do not include a STOP codon in your reverse primer. If cloning into pcDNA3 (+), or other mammalian vector, the addition of a Kozak sequence can help proper protein translation.
2. Perform PCR of your DBP according to recommended DNA polymerase conditions.
3. Visualize and verify PCR products by DNA gel electrophoresis. Purify correct PCR products with DNA gel extraction kit.
4. Digest both the backbone plasmid encoding the R4-uAb and the purified DBP PCR product insert with corresponding restriction enzymes according to manufacturer's protocols. Visualize proper digestion for both digested products by DNA gel electrophoresis. Purify both using DNA gel extraction kit.
A properly digested backbone plasmid should produce two clear bands, the smaller corresponds to the ~750-bp DNA encoding the scFv13-R4 DBP and does not need to be purified. The larger is the linearized backbone plasmid and should be purified for use in the ligation reaction.
5. Set up ligation by mixing DNA ligase with purified empty plasmid and purified PCR product inserts, and perform ligation according to manufacturer's recommended DNA ligase conditions.
Typically, ligations should be set up at variable insert:backbone ratios (3:1 and 5:1 are good starting points). A control reaction containing no insert DNA should also be performed to assess backbone self-ligation and background.
6. Transform ligations into suitable competent *E. coli* strain (e.g., BLD21(DE3)). Plate onto LBA plates with appropriate antibiotics. Incubate plates at 37°C overnight.
Typically, self-prepared chemically competent cells can be transformed with ligation reactions to yield colonies; however, if transformation efficiencies are low, electrocompetent cells should be used.
7. Screen colonies for correct insert by colony PCR or restriction digest following purification of plasmid using miniprep kit.
8. Confirm correctly cloned constructs by DNA sequencing.

BASIC PROTOCOL 2

Cell-free Characterization of uAb Activity: Purification of 6×His-tagged uAbs

from E. coli—This protocol is designed to purify uAbs for downstream experiments, including characterization and determination of binding affinity and/or catalytic activity. This same protocol can also be applied to any control protein, such as the unfused DBP or E3 domain, and the target protein itself, so long as they are tagged with a 6×His epitope.

Materials

Bacterial expression strain (*e.g.*, BL21(DE3)) carrying plasmid encoding uAb (see **Basic Protocol 1**)

Luria-Bertani (LB) medium (see recipe)

Isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.1M stock solution

Immobilized metal affinity column (HisPur Ni-NTA Resin, Thermo Scientific 88222)

Gravity column

Phosphate-buffered saline (PBS; see recipe)

Imidazole

Protein Concentrators PES, 10K MWCO, 0.5mL (Pierce 88513)

Equipment

Homogenizer

Refrigerated centrifuge

Spectrophotometer

Protocol steps: Day 1

- 1 Inoculate 10 mL LB + antibiotics with expression strain carrying uAb-encoding plasmid. Grow overnight at 37°C with shaking.

Day 2

- 2 Dilute overnight culture 1:100 into 500 mL of fresh LB medium.
Culture volumes can be adjusted between 100 mL and 4 L depending on the amount of purified uAb required. Most FLAG-6×His-tagged uAbs express extremely well under standard conditions.
- 3 Grow at 37°C with shaking until OD₆₀₀ is between 0.4 and 0.6 (~2–3 hr).
- 4 Induce expression with IPTG (0.1 mM final concentration).
- 5 Incubate culture at 30°C with shaking for 6 hr.
Expression times can be optimized by monitoring protein production between 4 and 24 hr post-induction.
- 6 Harvest cells by centrifugation at 3000 × g for 30 min at 4°C.

- 7 Freeze pellet at -80°C . Pellet can be stored for up to 1 month before purification.

Day 3

- 8 Thaw cell pellet on ice (~30 min)
- 9 Prepare the following buffers:
 - a. Equilibration buffer: PBS with 10 mM imidazole, pH 7.4
 - b. Wash buffer: PBS with 25 mM imidazole, pH 7.4
 - c. Elution buffer: PBS with 250 mM imidazole, pH 7.4
- 10 Resuspend pellet in 20 mL chilled equilibration buffer.
- 11 Lyse cells by preferred mechanical lysis method (*e.g.*, liquid homogenization).

These culture volumes are best suited to lysis by liquid homogenization or sonication, depending on available equipment. If not available, cells can also be lysed in detergent solutions, such as BugBuster (Millipore 70584), or by repeated freeze-thaw cycles.
- 12 Fractionate sample by centrifugation at $12,000 \times g$ at 4°C for 30 min. Collect the supernatant, which contains uAb in soluble form.
- 13 Purify uAb by gravity flow according to the HisPur Ni-NTA Resin protocol.
- 14 Desalt and concentrate purified uAb from equilibration buffer into PBS using protein concentrator following manufacturer's instructions.

This step is important for the removal of imidazole, which can inhibit downstream applications. It is advantageous to perform desalting and concentration in the same step.
- 15 Quantify yields by measuring absorbance at 280 nm (A280) or total protein assay. Purity can be measured by SDS-PAGE followed by staining with Coomassie blue.
- 16 Store purified uAb at 4°C for up to 1 month or at -80°C in 25% glycerol for up to 12 months.

Basic Protocol 3

Characterizing uAb target specificity by ELISA—This protocol is used to characterize the binding activity of the uAb. The assay confirms DBP specificity for its cognate antigen in the context of the E3 fusion. It is important to include the unfused DBP as a positive control, and the unfused CHIP TPR domain as a negative control (Figure 4). This assay is performed in 96-well plates with purified protein, in technical triplicate (*i.e.*, eight dilutions of four analytes can be tested in a single plate).

Materials—Purified uAb, purified target protein, and purified control proteins (see **Basic Protocol 2**)

Phosphate buffered saline (PBS, see recipe)

PBST (PBS + 0.05% Tween 20, v/v)

Blocking buffer (PBST + 3% milk, w/v)

Detection antibody (*i.e.*, anti-6×-His tag-specific antibody conjugated to horseradish peroxidase (HRP), Abcam, ab1187)

ELISA chemiluminescent substrate

Equipment

96-well plate (EIA/RIA, 96 well, flat bottom, clear, high binding, VWR 29442-322)

Multi-channel pipette

Micro-titer plate shaker

Micro-titer plate reader

Protocol: Day 1

- 1 Dilute target protein (antigen) to 4–12 µg/mL in PBS. Add 50 µL/well of diluted antigen to a 96-well plate and incubate at room temperature for 2 hr or 4°C overnight.

Carbonate/bicarbonate coating buffer (pH 9.6) can also be used to dilute the antigen. The high pH of this buffer aids in the electrostatic absorption of protein to the polystyrene plate.

Day 2

- 2 Remove antigen solution by inversion (*i.e.*, pouring the solution into the sink and tapping the plate against paper towels to wick away remaining liquid)
- 3 Wash three times with 100 µL/well of PBST for 5 min with gentle shaking, discarding liquid by inversion between washes.
- 4 Fill each well with blocking buffer (270 µL completely fills the wells). Incubate at room temperature for 1–3 hr with gentle shaking. Remove blocking buffer by inversion.

Blocking can be performed at 4°C overnight.

- 5 Make uAb and control protein dilutions:
 - a. Normalize samples by total protein, as measured by Bradford, Lowry, or equivalent protein quantification assay. Dilute samples 10–50 µM in blocking buffer. Add 100 µL/well of diluted sample to wells in the first row (*i.e.*, for technical triplicate, 100 µL diluted uAb would be added to wells A1, A2, and A3, 100 µL diluted DBP would be added to wells A4, A5, and A6, etc.)

- b. For two-fold dilutions, add 50 μ L blocking buffer to all wells in rows 2–8.
- c. Make 1:2 serial dilutions of the samples down the lanes of the 96-well plate by taking 50 μ L of the diluted samples in the first row and mixing via pipetting with the 50 μ L of blocking buffer in the second row. Repeat for each row in the plate, taking 50 μ L from the previous row and mixing it with the buffer in the subsequent row, discarding the final 50 μ L from the final wells.

For initial testing, it is recommended to start with a higher dilution (50 μ M) and performing larger dilutions (1:4 or 1:10) to experimentally determine the appropriate range.

- 6 Incubate the plate for 1 hr at room temperature with gentle shaking. Remove the dilutions from the wells by inversion.
- 7 Wash four times with 100 μ L PBST for 5 min with gentle shaking, discarding liquid between washes.
- 8 Dilute detection antibody in blocking buffer according to manufacturer's recommendation (5 mL is sufficient for one 96-well plate.) Add 50 μ L/well of antibody dilution and incubate for 1 hr at room temperature with gentle shaking. Remove liquid by inversion.

HRP-conjugated primary antibodies specific for FLAG or other epitope tags can be used. Similarly, using an unconjugated primary antibody, in conjunction with an HRP-conjugated secondary, can be used to amplify signal. Antibody dilution may need optimization to minimize background signal. Note that if the detection antibody recognizes the target antigen, the signal will be obscured.

- 9 Wash six times with 200 μ L PBST for 5 min with gentle shaking, discarding liquid between washes.
- 10 Quantify binding with ELISA chemiluminescent substrate as described by the manufacturer.
- 11 Measure absorbance in each well at appropriate wavelength for your substrate with micro-titer plate reader.

Basic Protocol 4

Characterization of uAb activity by cell-free ubiquitination assays—Cell-free ubiquitination assays are used to determine the activity of uAbs by monitoring the addition of ubiquitin to the cognate target in the presence of upstream UPP components. There are two primary ways to setup a cell-free reaction: time course and component necessity. A time course assay monitors the sequential addition of ubiquitin to the target over a period of time (e.g., 0–2 hr); a component necessity assay confirms that target ubiquitination is the result of the canonical UPP cascade. Results of both types of assay are evaluated by Western blot analysis (Figure 5).

Materials—ATP

E1 (Ube1; R&D Systems E-305)

E2 (UbcH5α; R&D Systems E2-616)

Ubiquitin (R&D Systems U-100H)

Purified uAb, target, and control proteins (see **Basic Protocol 2**)

Ubiquitination reaction buffer, 10× (see recipe)

Laemmli buffer, 2× (see recipe)

SDS-PAGE running buffer, 1× (see recipe)

SDS-PAGE gel (4–20%)

Polyvinylidene difluoride (PVDF) membrane

Powdered milk

Tris-buffered saline with Tween 20 (TBST; see recipe)

Anti-6×His tag antibody, HRP conjugate (abcam ab1187)

Anti-ubiquitin antibody, mouse (MilliporeSigma 05-944)

Antibody specific for target protein

Goat anti-mouse antibody, HRP conjugate (Santa Cruz sc-2005)

HRP conjugated secondary antibody for detection of anti-target antibody

Chemiluminescent Substrate

Equipment

Power supply

Gel electrophoresis system

Gel transfer system

Gel imaging system capable of chemiluminescence detection

Protocol**Time Course Assay**

- 1a** Calculate reaction volume based on number of time points desired (10 μL/time point).

Time points at 0, 15, 30, 60, 120 min are recommended for initial tests, but can be adjusted depending the kinetics of uAb-mediated ubiquitination.

- 2a** In a microcentrifuge tube, combine all reagents to the final concentration shown below and adjust to final volume with ddH₂O. Incubate at 37°C.

Reagent	Concentration
Buffer	1×
ATP	4mM
Ubiquitin	50μM
E1	0.1 μM
E2	2 μM
uAb	3 μM
Target	3 μM

The order of addition of components matters. Competing auto-ubiquitination reactions can begin before the addition of the target. Therefore, add ubiquitin and ATP last. Keep samples on ice until all components are added.

- 3a** At each time point, remove 10 μL of reaction mixture and halt reactions with 10 μL of 2× Laemmli buffer. Boil samples for 2 min at 100°C.
- 4a** Analyze results by SDS-PAGE followed by Western blot analysis using appropriate primary and secondary antibody pairs (*e.g.*, anti-ubiquitin followed by anti-mouse HRP conjugate for visualizing ubiquitinated species; anti-6×His antibody HRP conjugate for visualizing uAb; antibody specific for target protein followed by appropriate secondary for visualizing the target) (Figure 5a).

*Western blot analysis with antibodies that recognize different types of polyubiquitin linkages (*e.g.*, anti-K48-linked polyubiquitin antibody) can be used to provide additional information about the mode(s) of target ubiquitination. Reaction products can also be analyzed by mass spectrometry to identify sites of ubiquitination, but is beyond the scope of this protocol.*

Component Necessity Assay

- 1b** Combine all ubiquitination reagents in microcentrifuge tube as Step 2a above; however, omit a single UPP component (*e.g.*, E1 enzyme) from the reaction (Table 1). Adjust to final volumes with ddH₂O, and incubate at 37°C.
- 2b** To systematically evaluate the omission of other UPP components, additional reactions can be identically set up as in Step 1b.
- 3b** Halt reactions by combining with equal volumes 2× Laemmli buffer and boiling for 2 min at 100°C.

The total reaction time should be chosen based on the results of the time course analysis outlined above. It is recommended to choose a reaction time where clearly detectable levels of target ubiquitination are obtained when all UPP components are present.

4b Analyze results as above (Figure 5b).

BASIC PROTOCOL 5

Characterizing uAb-mediated protein degradation in mammalian cells—This protocol describes methods for evaluating the ability of uAb to degrade target protein in mammalian cells. This approach involves ectopic expression of the uAb (and target if necessary) and evaluates target degradation by Western blot analysis of cell lysates (Figure 6). Any mammalian cell line (*e.g.*, HEK293T, HeLa, etc.) that can be transfected should be suitable, but the protocol here describes optimized conditions for HEK293T cells.

Materials—HEK293T cell line (ATCC CRL-3216)

Complete DMEM with antibiotics (see recipe)

Trypsin-EDTA (0.05%)

JetPRIME DNA and siRNA transfection reagent, Polyplus-transfection (VWR 89129)

Mammalian expression plasmid encoding uAb

Mammalian expression plasmid encoding target

Empty mammalian expression plasmid

Nonidet P-40 lysis buffer (NP-40, see recipe)

Detergent-compatible total protein assay

Laemmli sample buffer, 2× (see recipe)

Equipment

6-well plate for cell culture

Refrigerated microcentrifuge

Protocol

Cell transfection and harvest: Day 1

- 1** Plate HEK293T cells in 6-well plates (1 well/sample), such that the next day they are 60–80% confluent.

Important controls include: target only, uAb only, non-specific uAb, and cell only samples.

Day 2

- 2 Transfect each well with 2 μ g of total plasmid DNA using a 2:1 ratio of jetPRIME:DNA (v/w), according to manufacturer's instructions. Replace media 4–6 hr post-transfection. Suggested starting conditions are 1.0 μ g for uAb plasmid and 0.1 μ g for target plasmid.

It may be helpful to perform an experiment with a gradient of uAb plasmid DNA for a given target concentration. A higher concentration of uAb plasmid DNA does not always equate to better degradation.

Day 3

- 3 24 hr post-transfection, wash cells with 1 mL of PBS per well. Add 200 μ L 0.05% trypsin-EDTA per well and incubate at 37°C for ~10 min or until cells have detached. Quench trypsin with 1 mL of complete DMEM per well and transfer samples to a microcentrifuge tube.
- 4 Centrifuge at $500 \times g$ for 5 min to pellet cells. Decant media. Freeze pellets at -20°C ; pellets can be stored for 1–2 weeks or processed immediately for Western blot analysis.

Time-course experiments between 1 and 4 days can also be performed to determine the optimal window for maximal target degradation.

Western blotting

- 5 Lyse cell pellets by resuspending in 200 μ L NP-40. Mix end-over-end at 4°C for 20 min. Centrifuge lysed cells for 20 min at $18,000 \times g$ at 4°C and collect the supernatant as the soluble fraction.

NP-40 is appropriate for whole cell fractions of HEK293T, COS-7, BHK21, HeLa, and MCF-7 cells. Consider cell line and cellular localization of target when choosing a lysis buffer. Smaller volumes of NP-40 can be used if more concentrated lysates are needed.

- 6 (Optional) If soluble ubiquibody expression is low, the ubiquibody could be partitioning to the insoluble fraction. To collect the insoluble fraction, wash the pellets from Step 1 with 50mM Tris-HCl and 1 mM EDTA, pH 8 and pellet at $18,000 \times g$ for 5 min. Solubilize the pellet by adding an equal volume of 2% SDS in PBS and boiling for 10 min. Centrifuge the boiled samples for 10 min at $18,000 \times g$ at room temperature to remove the remaining cell debris and collect the supernatant as the insoluble fraction.
- 7 Quantify total protein content of soluble (and insoluble fractions) so that samples can be normalized for Western blot analysis. Any detergent compatible total protein assay is sufficient.
- 8 Boil samples in 2 $\times$ Laemmli buffer for 15 min.

- 9 Load SDS-PAGE gel with sample volumes such that each well contains 10 µg of total protein. Perform electrophoresis and Western blotting according to standard procedures.

It is important to immunoblot against the target, uAb, and a housekeeping protein (e.g., GAPDH, tubulin, etc). Optimize total protein loading amounts depending on the expression level of the target protein.

REAGENTS AND SOLUTIONS

Use deionized, distilled water (ddH₂O) in all recipes and protocol steps

Phosphate-buffered saline (PBS)

8 g NaCl

0.2 g KCl

1.44 g Na₂HPO₄

0.24 g KH₂HPO₄

To prepare 1 L, dissolve above reagents in water. Adjust pH to 7.4 using HCl or NaOH.

Filter sterilize for use with mammalian cells. PBS can be stored at room temperature for >1 year.

Luria-Bertani (LB) medium

10 g NaCl

10 g tryptone

5 g yeast extract

To prepare 1L, dissolve above reagents in water. Adjust pH to 7.0 using 5N NaOH.

Autoclave to sterilize. LB can be stored ~1 month at room temperature.

LB with agar (LBA)

Prepare LB as described above.

Add 15g/L Bacto agar

Autoclave to sterilize. Allow LBA to cool to ~50°C before adding antibiotics and mix well before pouring into a petri dish to cool/solidify.

Super Optimal Broth (SOB)

0.5 g NaCl

20 g tryptone

5 g yeast extract

To prepare 1L, dissolve above reagents in water. Adjust pH to 7.0 with 5N NaOH.

Autoclave to sterilize. SOB can be stored ~1 month at room temperature.

Tris-buffered saline with Tween 20 (TBST)

137 mM NaCl

2.7 mM KCl

19 mM Tris Base

Dissolve above reagents in water to desired volume. Add 1 mL Tween 20 per liter.

Nonidet P-40 lysis buffer (NP-40)

50 mM Tris-Cl (pH 7.4)

150 mM NaCl

1% Nonidet P-40

Dissolve above reagents in water. Store at 4°C for ~1 year.

Laemmli sample buffer (2×)

4% (w/v) SDS

20% glycerol

120 mM Tris-Cl (pH 6.8)

0.02% (w/v) bromophenol blue

Dissolve above reagents in water to desired volume. Store buffer at room temperature.

SDS-PAGE buffer (1×)

3 g Tris base

14.4 g glycine

1.0 g sodium dodecyl sulfate (SDS)

Dissolve above reagents in 1L of water. Store buffer at room temperature.

Ubiquitination reaction buffer (1×)

20 mM MOPs

100 mM KCl

1 mM DTT

5 mM MgCl₂

Dissolve above reagents in water to desired volume. Adjust to pH 7.2. Store at 4°C for ~1 year.

Complete DMEM with antibiotic

Dulbecco's Modification of Eagles Medium (DMEM) with 4.5 g/L glucose and L-glutamine, without sodium pyruvate (VWR, 45000-312)

Fetal bovine serum (FBS, FetalClone I, Fetal Bovine Serum Alternative, VWR 16777-232) Antibiotic/Antimycotic (ThermoFisher15240062)

In sterile environment, add FBS to 10% v/v and antibiotic to 1% v/v. Store at 4°C and warm to 37°C before use.

COMMENTARY

Background Information

Explanation of the physiological function of a cellular protein often requires assessing the consequences of its removal. By operating at the post-translational level, protein knockout techniques have the potential to dissect complicated protein functions without modifying the protein of interest and at a potentially higher resolution than approaches functioning at the level of DNA or RNA. One such method for post-translational protein interference involves the use of stand-alone DBPs, which achieve interference according to an “inhibition-by-binding” mechanism. However, because there are no natural elimination pathways for the complexes formed between a DBP and its cognate target, the intracellular level of a DBP must exceed the expression level of its target, which can be challenging due to the inefficiency of most existing gene/protein delivery methods. This strategy can be further limited by the reversible nature of target binding, which invariably leads to “escape” of the target and thus only partial inhibition. Even when high-affinity binders are available, target inactivation is not guaranteed because not all DBP binding events result in neutralization.

To address these shortcomings, we and others have developed “inhibition-by-degradation” strategies that link a DBP with the cell’s natural degradation machinery – the UPP – such that the steady-state levels of the DBP’s intended target are systematically and reversibly depleted. Indeed, several methods have been reported for steering cellular proteins to the UPP for degradation. Most of these have been based on a multi-protein complex called Skp1-Cul1-F-box (SCF), which functions as an E3 ligase in the UPP pathway. The F-box proteins serve as modular adaptors that (i) connect to the core SCF complex by binding to Skp1 and (ii) recruit substrates through a protein-protein interaction domain, such as the WD40 domain or a leucine rich repeat. Targeted protein depletion using the SCF system has been accomplished by engineering fusions between the F-box domain and a DBP against a target of interest (Caussinus et al., 2011; Zhang et al., 2003). A similar technique has been developed that employs a chimeric fusion between a DBP and the von Hippel-Lindau (VHL) protein, which serves as a bridge between the target substrate and a different multi-protein E3 complex known as a Cullin (CUL) RING (really interesting new gene) E3 ligase (CRL) (Fulcher, Hutchinson, Macartney, Turnbull, & Sapkota, 2017; Fulcher et al., 2016). While these approaches have proven successful to varying extents, a potential limitation is their dependence on the endogenous SCF or CRL core machinery to carry out ubiquitination. If the core ligase machinery becomes overwhelmed upon high level expression of engineered F-box or VHL chimeras, then the degradation of both the intended target as well as native substrates could become suppressed (Zhou, 2005). A related concern is the complexity of

SCF and CRL complex formation, which requires the presence of numerous protein components in a very precise molecular ratio. Finally, reconstitution of the multi-protein SCF or CRL complexes makes cell-free experiments such as described above extremely difficult. To overcome the potential issues that may be encountered with such multi-protein E3 ligases, we have developed a viable alternative called uAbs, which leverage “stand-alone” E3s such as human CHIP that bind their substrates and transfer ubiquitin to these substrates without the need for additional accessory factors (Portnoff et al., 2014). By exploiting the highly modular architecture of human CHIP, an E3 ubiquitin ligase with exquisite structural flexibility that accommodates substrates with different sizes and structures, we have found engineered uAbs are a generalizable platform for directing otherwise stable proteins to the UPP for degradation.

Critical Parameters

DBP—Although a particular DBP may be validated as a binder to the target of interest, it may not function as desired in the context of a uAb fusion. When possible, test several target-specific DBP clones for uAb activity.

Cell-free component quality—For all components, purchased or purified, minimize the number of freeze-thaw cycles by storing reagents as small aliquots. ATP, in particular, is susceptible to hydrolysis with improper storage conditions.

Mammalian transfection conditions—JetPrime works well as a transfection agent for HEK293T cells, but alternative cell lines might require alternative reagents (Yamano, Dai, & Moursi, 2010). Furthermore, each uAb:target pair is unique and will require an optimization of several parameters for optimal degradation, including amount of DNA transfected, ratio of transfection agent:DNA, ratio of uAb:target DNA, and post-transfection incubation time. If unfamiliar with the cell line and transfection reagent being used, the ratio of transfection agent:DNA is easily optimized by performing mock transfections with a plasmid encoding a fluorescent protein like GFP or mCherry. Determine optimal transfection conditions by analyzing samples by Western blotting or fluorescence microscopy. Preliminary degradation experiments can be executed with a fixed quantity of target DNA, varying the uAb DNA in samples to obtain a 1:1 to 25:1 uAb:target DNA gradient. Once desirable uAb:target DNA ratios have been identified, single uAb:target DNA ratios can be evaluated between 1 to 4 days to determine optimal incubation time.

Troubleshooting

Table 2 describes some commonly encountered issues with expressing and characterizing uAbs in cell-free and in mammalian cell-based experiments, along with potential causes and solutions.

Anticipated Results

Basic Protocol 1 should produce a genetic fusion of the chosen DBP as an N-terminal fusion to CHIP TPR. In **Basic Protocol 2**, it is expected that the newly cloned uAb will be expressed highly in *E. coli* and purify to >95% purity. When tested for binding activity in **Basic Protocol 3**, the newly cloned uAb should bind coated antigen as strongly as the DBP

itself (Figure 4). The negative control, CHIP TPR, should not bind. In **Basic Protocol 4**, the anti-ubiquitin immunoblot should reveal prominent laddering or “smearing” for uAb, but not for the unfused DBP alone (Figure 5). This “smearing” can indicate auto-ubiquitination, which can be verified by the anti-6×His immunoblot. Target ubiquitination should be verified with a target-specific antibody. This “smearing” is indicative of polyubiquitination and can be further interrogated by linkage-specific anti-ubiquitin antibodies or mass spectrometry. Sometimes ubiquitination is not as extensive, and only a single higher molecular weight band is seen above the native target band. In **Basic Protocol 5**, immunoblots should reveal consistent GAPDH bands, indicating equivalent loading among samples (Figure 6). The uAbs and DBPs should be visible upon probing with the anti-6×His antibody, confirming their expression. Lastly, when cognate uAb and target are expressed, the target band should be significantly lighter or undetectable, indicating degradation.

Time Considerations

The amount of time to complete **Basic Protocol 1** will depend greatly on the ability to identify/obtain DNA encoding the required DBP. Once obtained, the design steps should take 1 day, while cloning steps could take 4–7 days. This does not include time to receive designed primers or order necessary plasmids.

Basic Protocol 2 can be completed in 3 days, with days 1–2 devoted to bacterial cell growth and day 3 to lysis, purification, and storage steps. **Basic Protocol 3** requires 2 days, with the first only requiring dilution of antigen onto the 96-well plate. **Basic Protocol 4** can be completed in 1–2 days, largely dependent on Western blot expertise.

Basic Protocol 5 can be performed in 3–4 days once mammalian cells are at a proper confluence for passage. If required, cell lysates can be frozen on day 2 for 1–2 weeks before lysing and analyzing by Western blot.

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Figure 1. Mechanism of UPP-mediated targeted proteolysis by engineered ubiquibodies
 Schematic showing the intersection of the native UPP pathway with laboratory engineered uAbs. The creation of uAbs involves genetic fusion of a truncated E3 (E3*) to a designer binding protein (DBP), which remodels the specificity of E3* for a non-native target protein (T). In the engineered uAb pathway, the DBP-E3* chimera catalyzes the specific attachment of ubiquitin to the target, after which the polyubiquitin-tagged target becomes degraded by the 26S proteasome.

Figure 2. General workflow for development of uAbs with desired specificity

Figure 3. Design of genetic fusions for plasmid-based uAb expression

(a) Linear representation of CHIP, CHIP TPR and scFv13-R4-based uAb (R4-uAb) that is specific for *E. coli* β -galactosidase. Numbers refer to amino acid positions from N terminus (N) to C terminus (C). The proteins are aligned vertically with the coiled-coil and U-box domains of CHIP. CHIP TPR is a truncated version of CHIP lacking the TPR domain. R4-uAb was designed with an additional Gly-Ser (GS) linker connecting the scFv13-R4 intrabody to CHIP TPR. (b) Plasmid map for pcDNA3-R4-uAb, which encodes the R4-uAb in the mammalian expression vector, pcDNA3.1. A detailed description of how this plasmid was created can be found elsewhere (Portnoff et al., 2014).

Figure 4. Characterizing uAb target specificity

Representative ELISA to measure uAb activity towards the substrate. Negative controls include the CHIP TPR and non-specific uAb constructs, which should exhibit no binding.

Figure 5. Characterizing uAb ubiquitination activity

(a) Representative Western blot of samples derived from time course ubiquitination assay reveals high-molecular weight substrate species that become more abundant with time. **(b)** Representative Western blots of samples derived from component necessity assay shows that substrate ubiquitination is dependent on every component of the ubiquitination pathway including target-specific uAb.

Figure 6. Characterizing uAb-mediated target degradation in mammalian cells

Representative Western blots of soluble lysates prepared from transfected mammalian cells display uAb-specific target degradation. Target-specific blot (top) reveals faint target bands in the presence of target-specific uAb, but not the CHIP TPR and non-specific uAb negative controls. α -FLAG blot (middle) confirms expression of uAb and negative controls, while α -GAPDH blot (bottom) confirms equal loading of samples.

Table 1

Set-up for component-based cell-free ubiquitination assay

Reagent	Reaction									
ATP	-	+	+	+	+	+	+	+	+	+
Ubiquitin	+	-	+	+	+	+	+	+	+	+
E1	+	+	-	+	+	+	+	+	+	+
E2	+	+	+	-	+	+	+	+	+	+
uAb	+	+	+	+	-	+	+	DBP	CHIP	TPR

Table 2

Troubleshooting Commonly Encountered Issues

Problem	Possible Cause	Solution
Poor expression of uAb in bacteria	Incorrect cloning	Confirm plasmid sequence
	Need longer induction	Induce cells for a longer amount of time
No binding in ELISA	Target not coated	Optimize target coating and detect with target-specific antibody to confirm
	Not enough uAb incubated	Start with higher protein quantities
	DBP doesn't bind target	Perform ELISA with DBP only control
	Wrong antibody dilution	Optimize antibody conditions for maximal signal
No auto-ubiquitination in cell-free assay	Left out component/component inactive	Try fresh aliquots of components.
	uAb inactive	Try fresh purification
		Test alternative protein linkers between DBP and CHIP TPR
No target ubiquitination in cell-free assay	DBP doesn't bind target	Confirm specificity by ELISA
	Left out component/component inactive	Try fresh aliquots of components
Poor expression of uAb in mammalian cells	Incorrect cloning	Confirm plasmid sequence
	Low solubility	Add tag to enhance solubility, like FLAG tag
	Not enough plasmid	Transfect more plasmid
	Insufficient lysis	Optimize lysis conditions
No degradation of target in mammalian cells	Poor expression of uAb	Optimize as described above
	No binding of uAb to target	Optimize as described above
	Too much target	Transfect less target plasmid