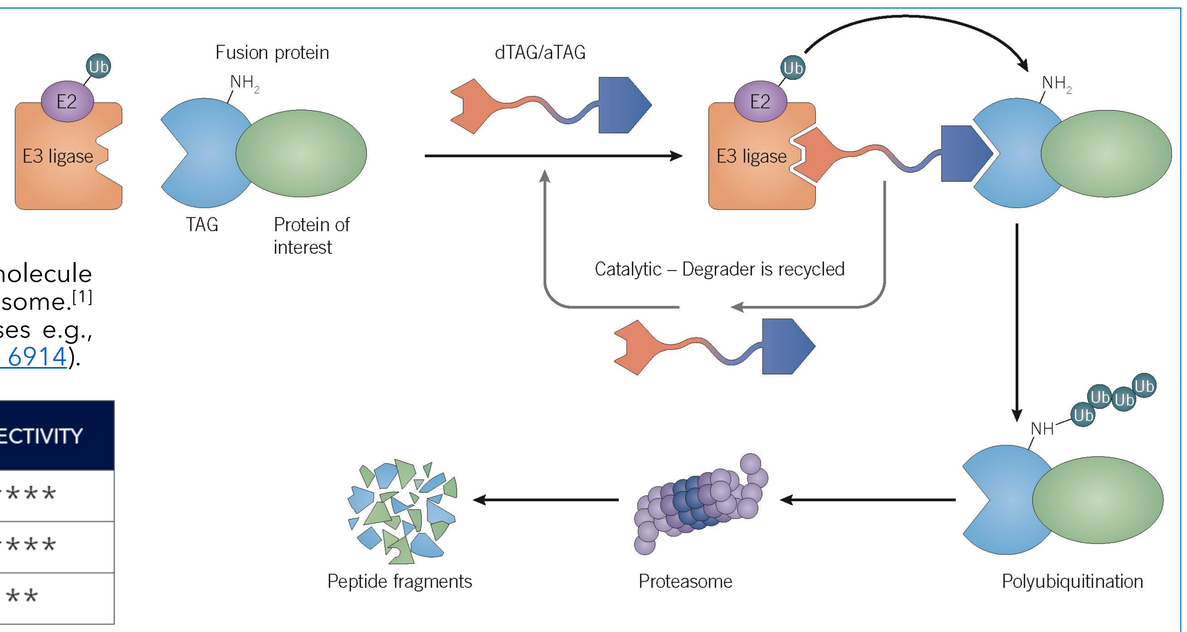
Validating Targets for TPD using dTAG – a comprehensive workflow solution

Pouya Amrollahi, Marnie Wallin, Nithya Jesuraj, Jeff Cooper, Joel Cresser-Brown, Chris Heger, Charles Haitjema, Hannah Maple

INTRODUCTION

TAG degradation technology offers a generalizable strategy to degrade, in principle, any intracellular protein of interest (POI). Its key benefit is that it does not rely on the pre-existence of a ligand or PROTAC® for the POI. The broad applicability that it offers makes this a useful strategy for exploration and validation of targets, particularly in the context of new Degrader development programs in the field of targeted protein degradation (TPD).



The POI is expressed as a fusion protein with a dTAG domain (identity: FKBP12^{F36V}), the dTAG degrader molecule recruits the fusion protein for ubiquitination by an E3 ligase, which targets it for destruction by the proteasome.[1] Several dTAG Degraders and matched pair negative controls are available, that recruit different E3 ligases e.g., CRBN[1]: dTAG-13 (Tocris Cat. No. 6605) and dTAG-7 (Tocris Cat. No. 6912) or VHL[2]: dTAG^{V}-1 (Tocris Cat. No. 6914).

	DOSE TUNEABILITY	EFFICACY	REVERSIBILITY	KINETICS	SELECTIVITY
TAG Degradation Platform (dTAG/aTAG)	***	****	****	***	****
Gene knockout e.g. CRISPR/Cas9	*	****	*	*	****
Gene knockdown e.g. RNAi	*	***	*	*	**

Key advantages of the dTAG platform include the ability to tune the extent of protein knockdown by varying the Degrader dose, and the more rapid onset of action (kinetics) for studying 'fast biology'. In addition, dTAG Degraders can be washed out of cell culture media, reversing their effect.

METHODS

TAG Cell-line Generation

Cells were transfected with Cas9 Nuclease ribonucleoprotein (RNP) complexes targeting the gene of interest (GOI) and single stranded DNA template containing dTAG (FKBP12^{F36V}) and the edits of interest surrounded by flanking arms matching the genomic region of interest. After confirmation of the knock-in event at the population level with junction PCR, cells were cloned and expanded for characterization. Clones containing dTAG -GOI fusion were verified for a homozygous KI via PCR of the edited region and Sanger Sequencing (Figure 1A)

dTAG Degrader Treatment

Cells were cultured in a 6-well plate with RPMI-1640 Media (R&D Systems Cat. No. M30150) supplemented with 10% Premium grade FBS (R&D Systems Cat. No. S11150) and 1% Penicillin-Streptomysin (R&D Systems Cat. No. B21210), reaching 50% confluency after 3 days. 24 hours after stimulation to induce target protein expression, dTAG Degraders dTAG-13 and dTAG^{V}-1 were added at 5 nM, 50 nM and 500 nM, and negative controls dTAG-13-NEG (Tocris Cat. No. 6916) and dTAG^{V}-1-NEG (Tocris Cat. No. 6915) at 500 nM to generate 8 samples per KI. Wild-type samples with the same stimulation were also grown as a control. Cells were harvested after an additional 24 hours of growth. One well of a 6-well plate was used for each sample. Cells were harvested from the plates, washed once with PBS, then flash frozen in liquid nitrogen. Cell pellets were held at -80 °C until time of lysis. Cells were lysed using Lysis Buffer 16 (R&D Systems Cat. No. 895935) with 1 x protease inhibitor (PPI). Cold lysis was performed as follows. 100 µL ice cold Lysis Buffer 16 with 1 x PPI was added to the cell pellets. Cells were vortexed for 10 seconds, then incubated on ice for 15 minutes. Tubes were vortexed again and incubated for an additional 15 minutes on ice.

Detecting Degradation with Simple Western™

The final experimental conditions for Simple Western analysis are shown in Table 1. All experiments were performed according to manufacturer's instructions on Peggy Sue (Figure 1C), a high-throughput Simple Western instrument that can analyse up to 96 samples in one automated run. The samples were diluted with 0.1X sample buffer to the concentration listed in Table 1 prior to analysis.

Target	Antibody	Vendor	Antibody conc.	Lysate conc. (mg/mL)
1	1	Novus	1:33	0.25
FKBP12.6	MAB4174	R&D Systems	1:10	0.8

Table 1. Experimental conditions for Simple Western analysis

RESULTS & DISCUSSION

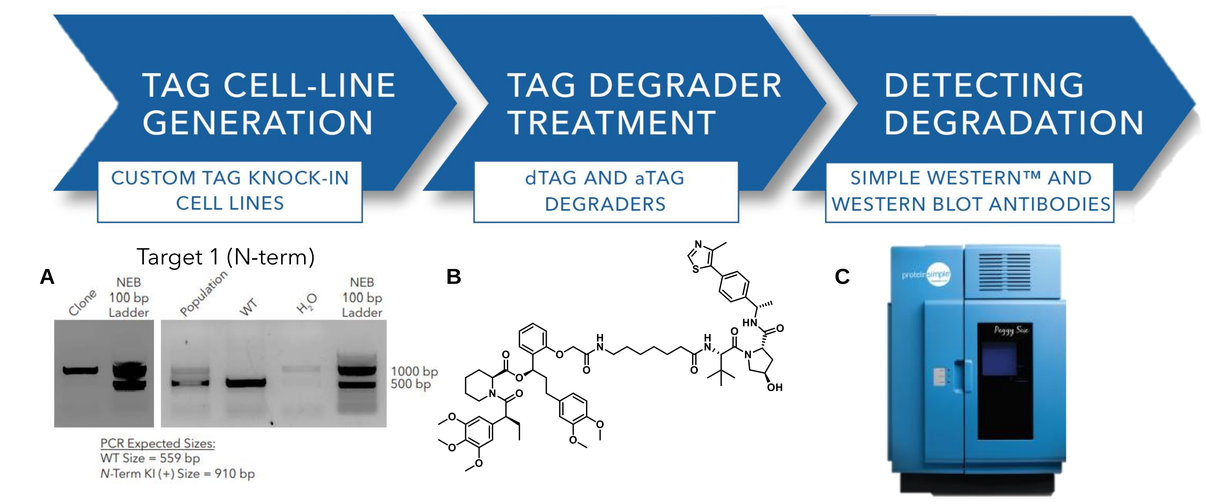


Figure 1. (A) PCR of TAG knock-in cell line, (B) VHL recruiting dTAG-^{V}1 degrader, (C) Peggy Sue automated western blotting instrument

Differences in susceptibility of target degradation by CRBN and VHL recruiting dTAG Degraders

A cell line expressing an N-terminal knock-in of the FKBP12^{F36V} domain with Target 1 was generated and analysed by PCR (Figure 1A, left panel), according to the methods described. Treatment with the CRBN-recruiting dTAG Degrader, dTAG-13, and VHL-recruiting dTAG Degrader, dTAG^{V}-1, to the cell line resulted in dose-dependent degradation of Target 1, with maximum observed degradation at the highest dose tested (500nM, Figure 1A, 1B). Treatment with the corresponding negative controls (NEG) did not result in significant knock-down of the target.T he quantified data (Figure 1A, 1B, right panels) flags a difference in sensitivity for Target 1 between the two dTAG Degraders tested, whereby Target 1 appears to be more sensitive to knock-down with dTAG^{V}-1.

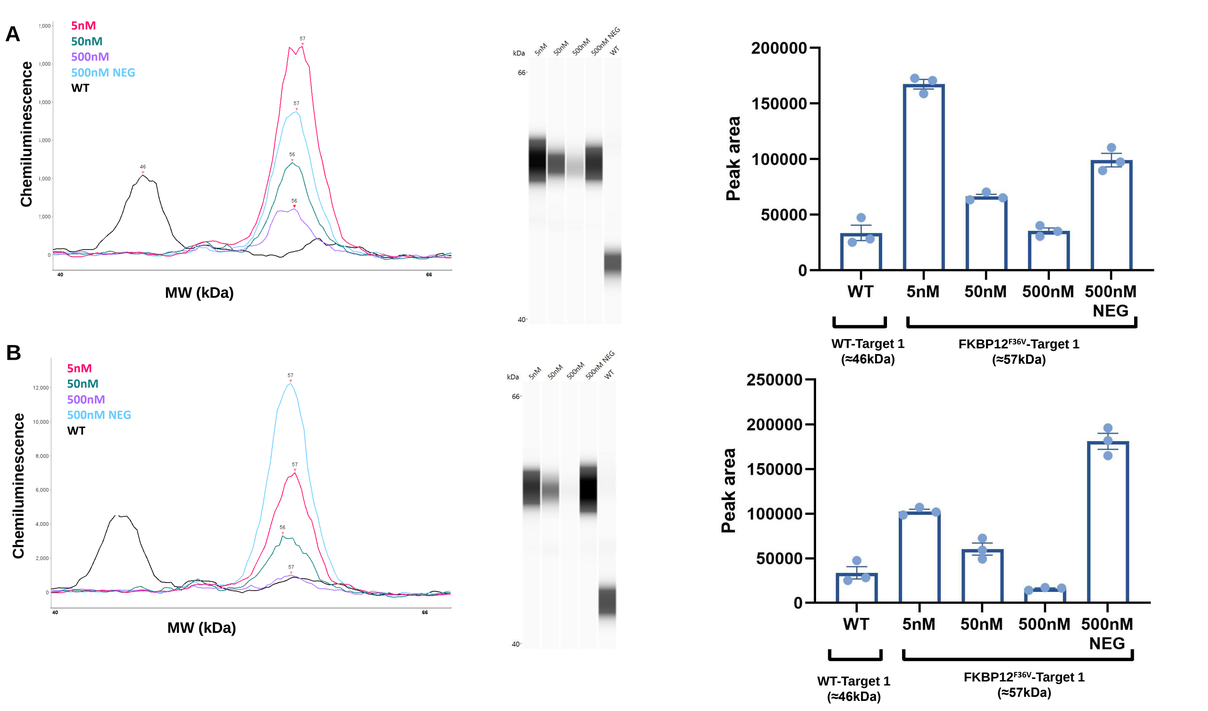


Figure 2. Simple Western data from a dTAG·Target 1 KI cell line following treatment with either dTAG-13 (A) or dTAG^{V}-1 (B) and detection with an anti-Target 1 antibody. The following matched pair negative control degraders were also used as controls: dTAG-13-NEG (A) and dTAG^{V}-1-NEG (B). Degrader treatments are color-coded as indicated by the key top left. Left panel: electropherogram data, Middle panel: lane-view data, Right panel: Graphs showing quantitative data collected by integrating the peaks in the electropherograms (n=3)

Detecting degradation with a 'dTAG' antibody

Antibody validation is a pre-requisite for successful western blotting. In the examples shown previously, the antibody used has reactivity for the target protein, necessitating a different antibody and separate validation for each target protein. Data in Figure 3 was generated using an anti-FKBP12 antibody (MAB4174, R&D Systems). The electropherogram data (Figure 3, left panel) demonstrates that this antibody has reactivity both for WT FKBP12 and the single point mutant used for the dTAG-domain, FKBP12^{F36V}. This dual reactivity can be useful since the WT FKBP12 peak serves as an internal control. The quantified data (Figure 3, right panel) aligns with the data generated using an anti-Target antibody, demonstrating that either antibody can be used in this case to detect degradation.

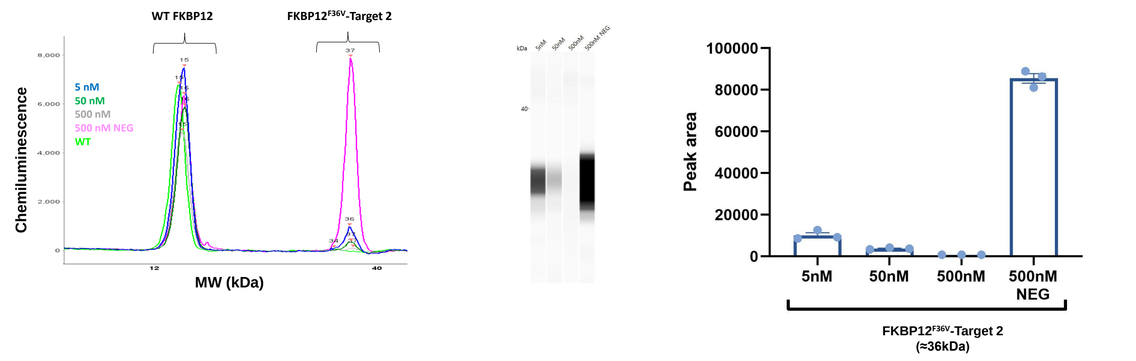


Figure 3. Simple Western data from a dTAG·Target 2 KI cell line following treatment with dTAG^{V}-1 and detection with an anti-FKBP12 antibody. dTAG^{V}-1-NEG was used as a negative control. Degrader treatments are color-coded as indicated by the key top left. Left panel: electropherogram data showing both the peak corresponding to WT FKBP12 and the peak corresponding to the FKBP12^{F36V}·Target 2 fusion protein. Middle panel: lane-view data showing the FKBP12^{F36V}·Target 2 band only, Right panel: Quantitative data collected by integrating the peaks in the electropherogram (n=3)

CONCLUSION

We present data to highlight a full dTAG workflow solution, from custom KI cell lines for a protein of interest, to different dTAG Degraders available for treatment and subsequent characterization using an automated western blotting platform, Simple Western. We also show preliminary data to demonstrate that in some cases it is possible to detect dTAG degradation using an antibody that recognizes the TAG domain (FKBP12^{F36V}), rather than that individual proteins of interest. Initial work should be performed, however, to ensure that the anti-FKBP12 antibody detects the "dTAG" domain when fused with a given POI, to ensure that the anti-FKBP12 targeted epitope has not been blocked during the generation of the dTAG·POI fusion. Finally, we highlight best practise for the use of the dTAG degradation platform, including trying both N- and C-term KI, testing both CRBN- and VHL-recruiting dTAG Degraders to select the most effective compound for a given target, and the use of negative control dTAG Degraders.

REFERENCES

1. Nabet B, Roberts JM, Buckley DL, Paulk J, Dastjerdi S, Yang A, Leggett AL, Erb MA, Lawlor MA, Souza A, et al.: The dTAG system for immediate and target-specific protein degradation. *Nat Chem Biol* 2018, 14:431–441.
2. Nabet B, Ferguson FM, Seong BKA, Kuljanin M, Leggett AL, Mohardt ML, Robichaud A, Conway AS, Buckley DL, Mancias JD, et al.: Rapid and direct control of target protein levels with VHL-recruiting dTAG molecules. *Nat Commun* 2020, 11:4687.

