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Application Note

Visualizing PROTAC® Interactions with Endogenous Proteins Using Duolink® Proximity Ligation Assays

Introduction

Proteolysis-Targeting Chimera (PROTAC®) degraders are a novel class of small molecules that degrade a specific protein by linking it to an E3 ligase, forming a ternary complex, leading to its ubiquitination and labeling for disposal. First described by Sakamoto et al. in 2001, the molecules have gained interest as a potential therapeutic modality with over 40 therapeutic interventions currently in the pipeline (Root Analysis Report, 2020), often targeting oncological or inflammatory diseases.[1,2] Each molecule initiates a series of protein-protein interactions (PPI) - from ternary complex to eventual degradation in the proteasome - which are useful to visualize for confirmation of these activities (**Figure 1**). In this application note, we demonstrate the utility of the Duolink® Proximity Ligation Assay (PLA) for achieving this aim.

Target Degradation System

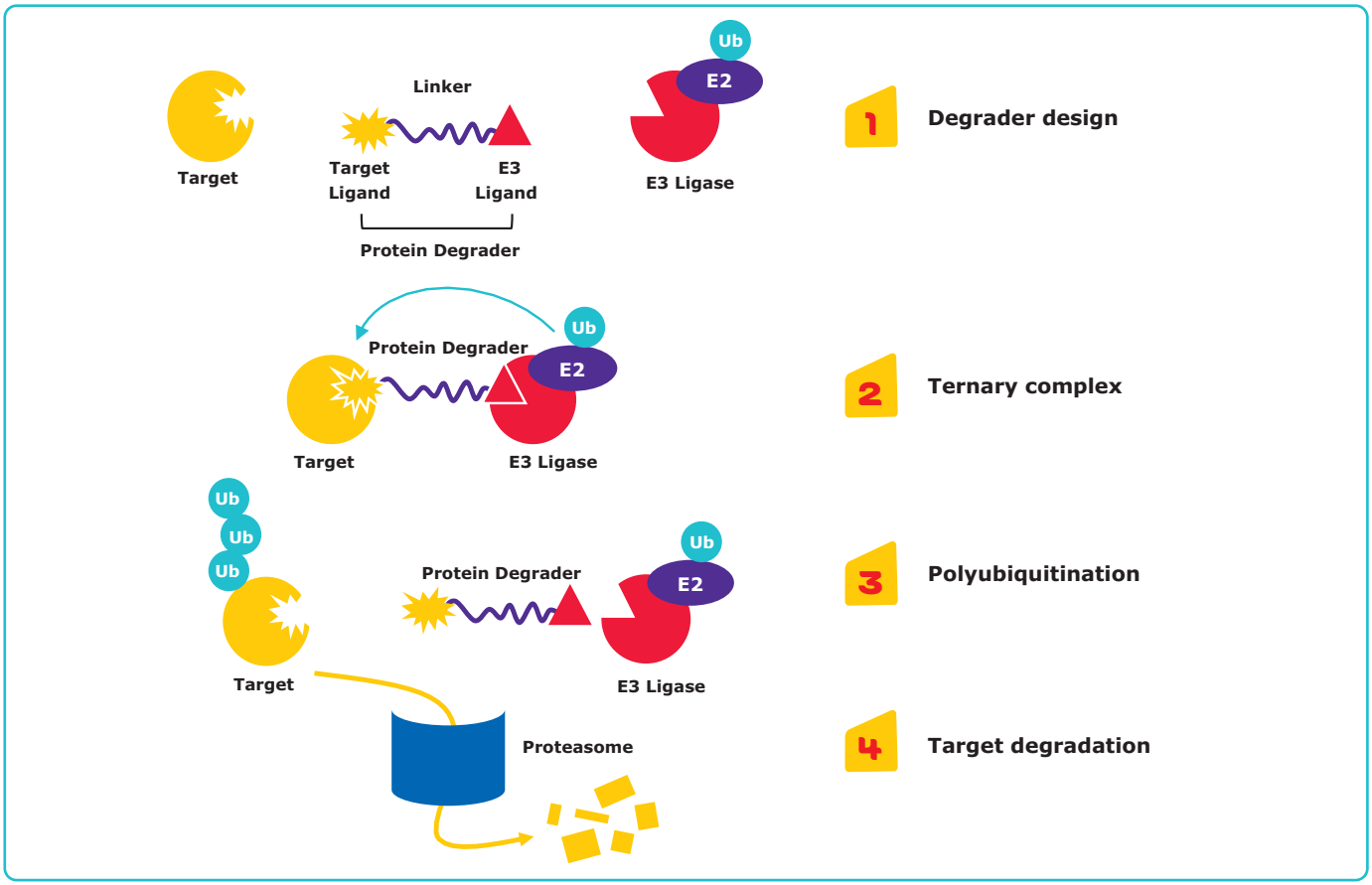

Figure 1. The PROTAC® targeted protein degradation system.

Duolink® PLA is a tool specifically designed for the study of PPIs. It combines the specificity of antibodies with the sensitivity afforded by rolling circle amplification to detect endogenous proteins in fixed cells and tissues. A pair of oligonucleotide-labeled antibodies (PLA probes) generates an amplified signal only when the probes are in close proximity (<40 nm). There are two different assay formats involving either directly labeled antibodies or using labeled secondaries with the latter demonstrated in **Figure 2**.

Duolink® PLA System

Two antibodies raised in separate species bind two proteins of interest or two epitopes of a single protein.

Secondary antibodies with PLA probes (PLUS and MINUS) are added.

Two circle-forming DNA oligonucleotides and the ligation enzyme are added.

Polymerase amplifies the DNA circle. Fluorescently labeled, complementary oligonucleotide probes bind to the amplified DNA.

Each signal is composed of ~1,000 bound fluorescent probes that appear as a distinct dot and can be easily visualized under a microscope.

Figure 2. The steps in the Duolink® Proximity Ligation Assay system.

Methods

Prior to PROTAC® treatment SKOV3 or HCC827 cells were plated and grown on imaging-optimized 96-well plates (ibidi® GmbH black 96-well µ-plate). Once the cells reached ~70% confluency, they were treated with 5 µM MG132 in fresh media for 10 minutes at 37 °C. Cells were then stimulated with 0.1 µM dBet1 or 0.025 – 10 µM PROTAC®3 degrader for 1 hour at 37 °C. Treated cells were washed with Phosphate Buffered Saline, fixed with glyoxal or methanol, and permeabilized with Triton™ NP-40 reagent.

Duolink® assays were carried out using a 96-well assay format with images captured on an IN Cell Analyzer 2200 (Cytiva™ technologies). The materials used are outlined in the table at the end of this application note.

See the full Duolink® PLA fluorescence protocol that was followed at **SigmaAldrich.com/duolink-fluor-protocol**.

Performing Antibody Titrations

One critical feature of designing a Duolink® Proximity Ligation Assay is the identification of appropriate antibody pairs and concentrations. This is especially important when trying to see PROTAC® degrader-driven protein-protein interactions, as there is likely some basal level of POI (protein of interest) : E3 ligase interaction and POI : ubiquitination. A titration of both primary antibodies should be performed to minimize the baseline level of signal, such that few spots are seen in the absence of PROTAC® treatment.

More information on how to optimize your protocol can be found at **SigmaAldrich.com/duolink-tips.**

Results

Visualizing PROTAC® PPI Targeting Brd4 Ubiquitination and Degradation

A key step in the targeted protein degradation discovery workflow is verifying that your molecules are engaging your E3 ligase and protein of interest. This drives complex formation, leading to ubiquitination and eventual degradation. Duolink® PLA allows researchers to screen and verify the protein degradation pathway at native expression levels without the use of specialized equipment.

One established PROTAC® system uses dBet1 to target Brd4 to the cereblon (Crbn) E3 ligase.[3] Following a 60-minute treatment with dBet1 in the presence of MG132 inhibitor to block protein degradation, we were able to monitor Brd4 : Crbn, Brd4 : Ubiquitin, and Brd4 : Proteasome interactions in SKOV3 cells via PLA with the Duolink® orange detection kit (**Figure 3**) following the Duolink® PLA protocol (**SigmaAldrich.com/duolink-fluor-protocol**). The technical control of single antibodies showed no Duolink® signal, indicating that the signal is specific for the targeted protein-protein interactions.

Duolink® Monitoring of Degradation in Action

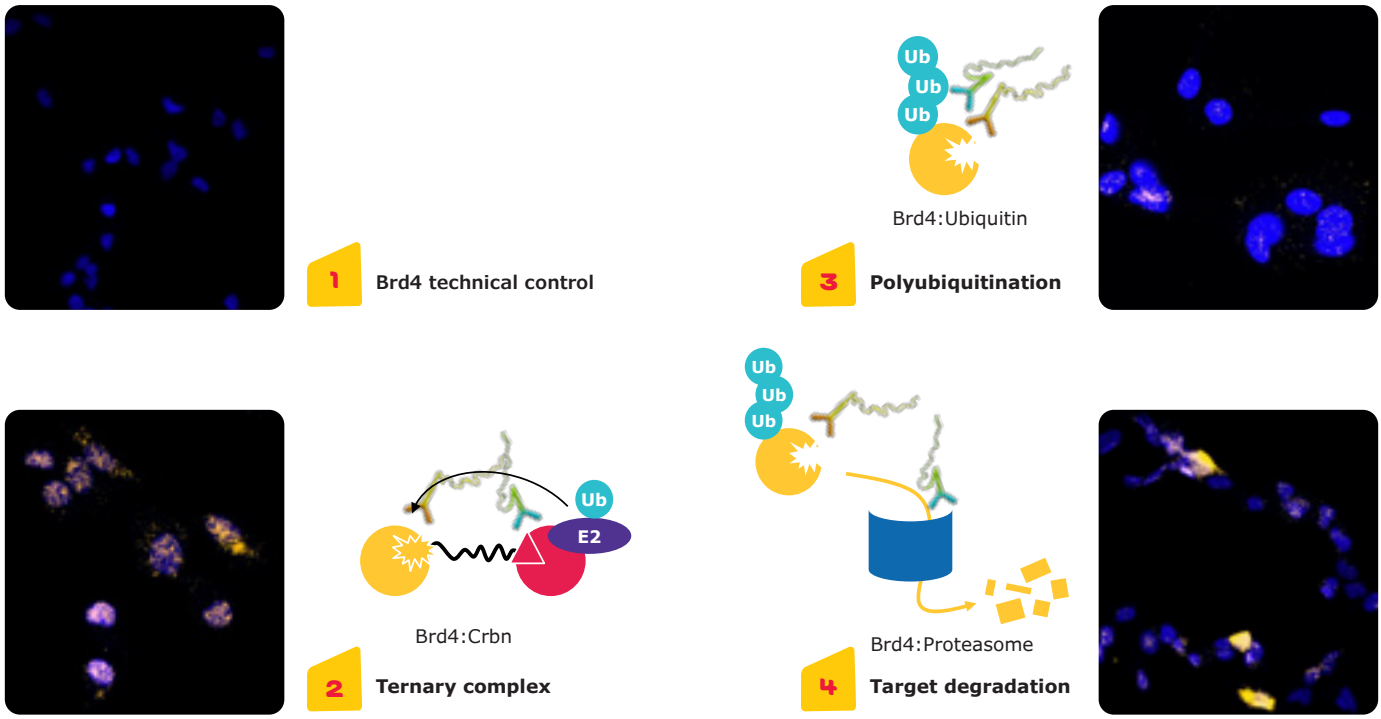

Figure 3. The dBet1 PROTAC® interaction targeting Brd4 ubiquitination and degradation as monitored by the Duolink® Proximity Ligation Assay system. This figure shows dBet1-stimulated SKOV3 cells highlighting the visualization of specific interactions of each step in the degrader cycle: **(1)** Brd4 technical control, **(2)** the ternary complex, **(3)** polyubiquitination, and **(4)** target degradation. Duolink® PLA signal is represented by orange spots and nuclei are stained blue with DAPI.

Measuring EGFR : VHL Complex Formation

Similarly, we investigated EGFR : VHL interactions, this time charting a dose response illustrating increasing EGFR : VHL complex formation with increasing PROTAC®3 concentrations post -1-hour incubation. HCC827 cells harboring EGFR exon 19 deletion were used. The PROTAC®3 degrader utilizes a VHL ligand to drive EGFR : VHL complex formation as described in research by Burslem et al. in 2018.[4] After treatment the cells were fixed and permeabilized as above. Mouse anti-EGFR and goat anti-VHL were used to target the EGFR : PROTAC®3 pair, then a Duolink® *In Situ* Orange kit with anti-goat PLUS and anti-mouse MINUS PLA probes (**Figure 4**) was used for detection. As PROTAC®3 concentration increased, an increase in the EGFR : VHL interaction was evidenced, as seen both visually and when counting spots.

Monitoring PROTAC® Degrader Driven Ubiquitination of EGFR

Following ternary complex formation, the POI must be ubiquitinated so it can be trafficked to the proteasome. Using an anti-K48 ubiquitin antibody we were able to see the PROTAC®3 degrader-driven ubiquitination of EGFR after 1 hour, with saturation of ubiquitination coming at a similar PROTAC®3 concentration as the E3 ligase signal saturation (as shown in **Figure 4**).

Monitoring the Degradation Pathway

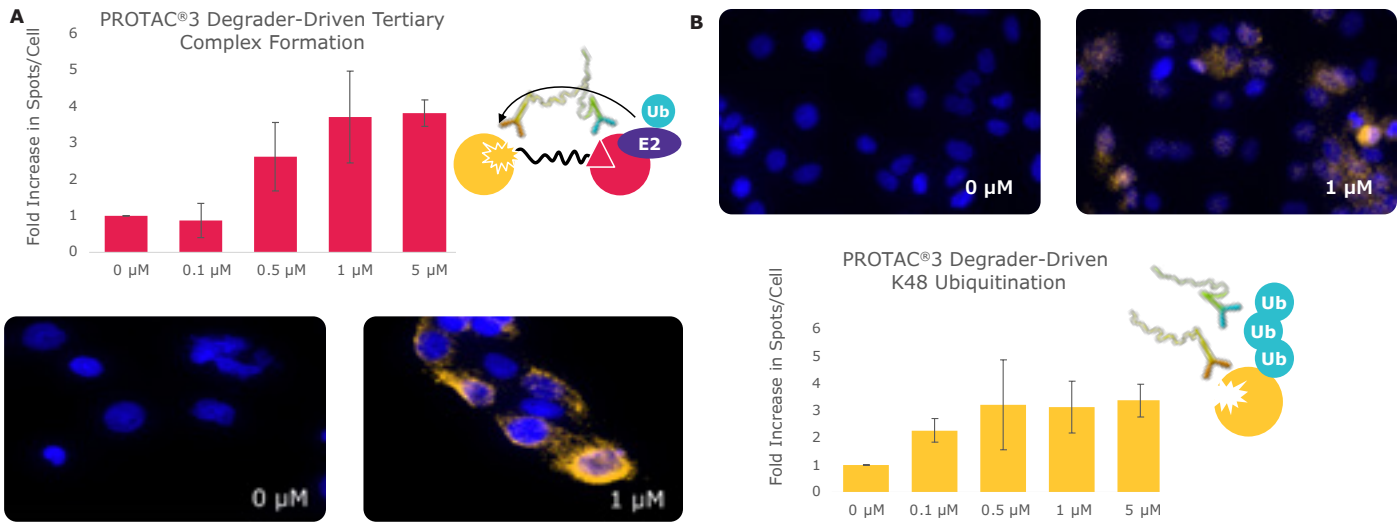

Figure 4. The PROTAC®3 dose-response charts as monitored by the Duolink® PLA-probed EGFR : VHL complex formation. PROTAC®3, a gefitinib-based VHL degrader, in EGFR mutant harboring cell line HCC827 was used to monitor (**A**) EGFR : VHL complex formation and (**B**) K48 ubiquitination. A signal was observed in a concentration range that matches published results for degradation.[4]

Summary

The Duolink® PLA system enables evaluation and visualization for multiple stages of PROTAC® degradation *in situ*, from ternary complex formation to eventual recruitment to the proteasome. As a tool developed for the evaluation of protein-protein interactions, the Duolink® assay enables visual and quantitative confirmation of PROTAC® activity against endogenous levels of the target protein.

Materials

Cat. No.	Description
HPA015055	Anti-Brd4 Antibody Produced in Rabbit
SAB2702287	Monoclonal Anti-Ubiquitin Antibody Produced in Mouse
Product Number Pending	Anti-Ubiquitin K48-Specific Antibody
SAB2501530	Anti-PSMA4 Antibody Produced in Goat
DUO92007	Duolink® *In Situ* Detection Reagents Orange
DUO92003	Duolink® *In Situ* PLA Probe Anti-Goat PLUS
DUO92004	Duolink® *In Situ* PLA Probe Anti-Mouse MINUS
DUO92002	Duolink® *In Situ* PLA Probe Anti-Rabbit PLUS
SAB2501099	Anti-VHL Antibody Produced in Goat
E3138	Monoclonal Anti-EGFR Antibody Produced in Mouse
SML2687	dBet1
HY-123921 (MedChemExpress Cat. No.)	Gefitinib-Based PROTAC®3 Degrader
MAB9574 (Bio-Techne Corporation Cat. No.)	Anti-Cereblon Human CRBN Antibody

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*Merck

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