Targeted degradation of endogenously tagged proteins for phenotypic studies using HaloPROTAC3 and HaloTag® technologies

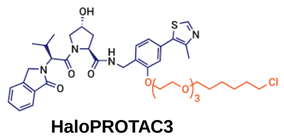
Elizabeth A. Caine[1], Sarah Mahan[1], Kristin Riching[1], Cesear Corona[2], Nancy Murphy[1], Chris Eggers[1], Chris Heid[1], Danette L. Daniels[1], and Marjeta Urh[1]

[1]Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711; [2]Promega Biosciences LLC, 277 Granada Dr, San Luis Obispo, CA 93401

1. Introduction

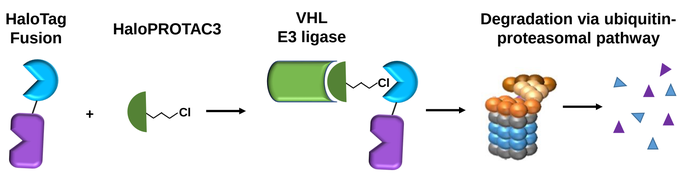
To understand a protein's function inside the cell, studies are often done to remove it using CRISPR-mediated knockout or RNAi knockdown methods. However, these approaches have challenges that include obtaining efficient loss of the targeted protein, or cell death if the protein is found to be essential for cell growth. To overcome these hurdles, we have employed a highly precise and temporally controlled target protein degradation strategy utilizing HaloPROTAC3, a HaloTag® proteolysis-targeting chimera small molecule which specifically degrades HaloTag fusion proteins in live cells. HaloPROTAC3, developed by Prof. Craig Crews at Yale University, recruits HaloTag® fusion proteins to VHL E3 ligase complexes, resulting in ubiquitination and degradation of the HaloTag® fusion via the ubiquitin-proteasomal pathway. Endogenous HaloTag® fusion proteins are developed via CRISPR/Cas9 gene editing into either the N- or C-terminal loci of any target protein. Additionally, we've appended the 11 amino acid HiBiT to the HaloTag® fusion protein, allowing for highly quantitative kinetic monitoring of degradation in live cells with the use of luminescence instead of antibodies. Shown here is the rapid and sustained degradation (80-90% loss) of key therapeutic HiBiT-HaloTag® fusion proteins, BRD4 and β-catenin, after treatment with HaloPROTAC3 in HEK293 LgBiT stable cells. For BRD4, 95% of the protein fusion was lost after 48 hours of treatment with HaloPROTAC3. For β-catenin, phenotypic studies were done to show a muted response to Wnt3a stimulation and TCF/LEF promoter gene activation after degradation of the HiBiT-HaloTag® fusion protein. Together, we demonstrate how these technologies can be used to elicit robust degradation of target proteins, with control over the protein level as well as the time frame for protein degradation. Also, we provide new opportunities for phenotypic studies in order to investigate the function of essential proteins without the need of protein specific PROTACs.

2. HaloPROTAC3 and applications



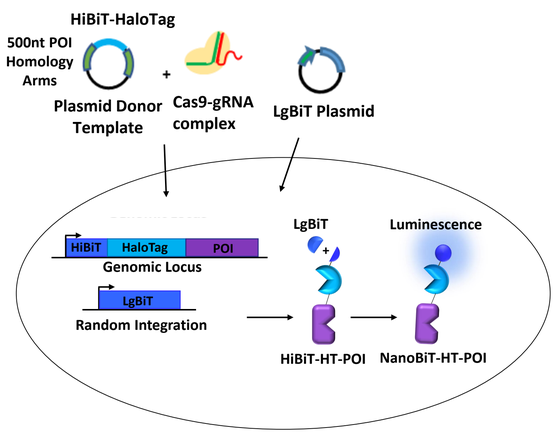
HaloPROTAC3

ACS Chem Biol, **2015**, *10*, 1831



- Phenotypic analysis on endogenous proteins
- Help define targets important for PROTAC development
- *In vivo* (mouse) degradation
- Degradation of proteins without needing to develop PROTAC
- Degrade proteins that are essential for cell growth/survival
- Control over protein level and time frame for protein degradation
- Pair with HiBiT luminescence technology
  - Detection of degradation without the need or antibodies
  - Perform kinetic degradation experiments in live cells

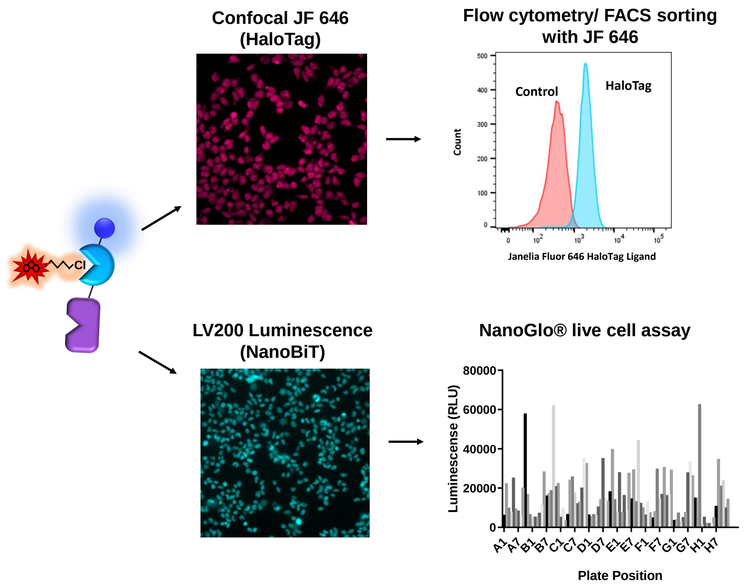
3. HaloTag and/or HiBiT CRISPR insertion



- HaloTag (HT) and/or HiBiT is inserted into genomic locus using donor vector containing 500 nucleotide homology arms to protein of interest (POI)
- Cas9/gRNA complex directs cutting of genomic locus
- LgBiT stable cell lines can be created with use of LgBiT plasmid
- Complementation of LgBiT with HiBiT, generates a functional enzyme with a bright, luminescent signal (NanoBiT)

4. Selection and validation of endogenous tagged protein of interest

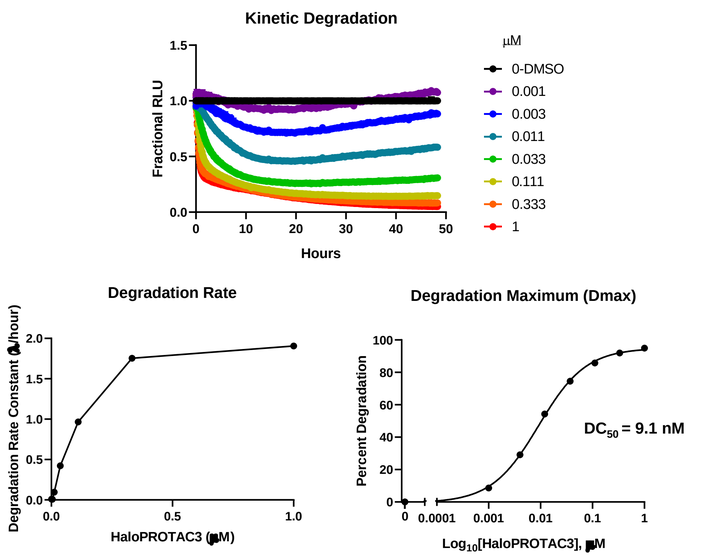
HiBiT-HaloTag-BRD4 HEK293 LgBiT stable cells



- Janelia Fluor® HaloTag® ligands (JF 646) can be used for live cell imaging and FACS sorting to pick out cells with HaloTag insertion through CRISPR
- Combining HaloTag with HiBiT/ LgBiT allows for imaging and live cell assays to identify positive clones after FACS sorting

5. Kinetic degradation with HaloPROTAC3

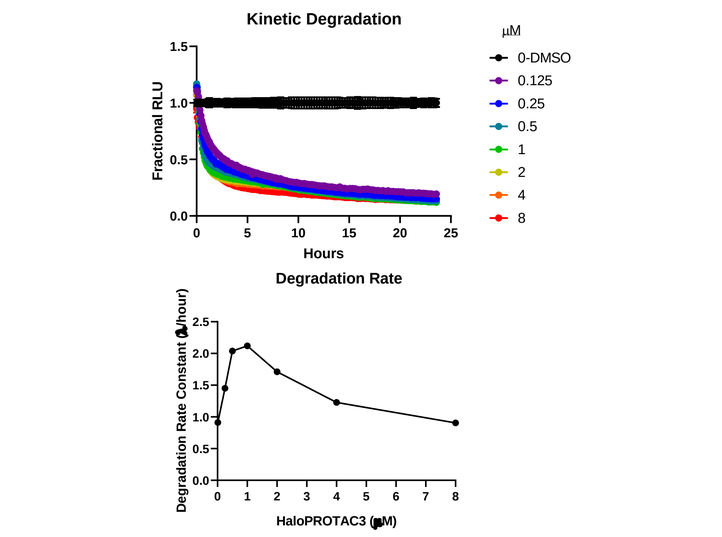
HiBiT-HaloTag-BRD4 HEK293 LgBiT stable cells



- Rapid degradation rate with HaloPROTAC3 with endogenous tagged proteins
- Concentrations between 100 nM-1 µM HaloPROTAC3 appear effective with 95% degradation of HiBiT-HaloTag-BRD4 after 48 hours
- Potency, rate, and degradation maximum can all be calculated from kinetic live cells assays with HaloPROTAC3

6. Toxicity with HaloPROTAC3 concentrations over 1 µM

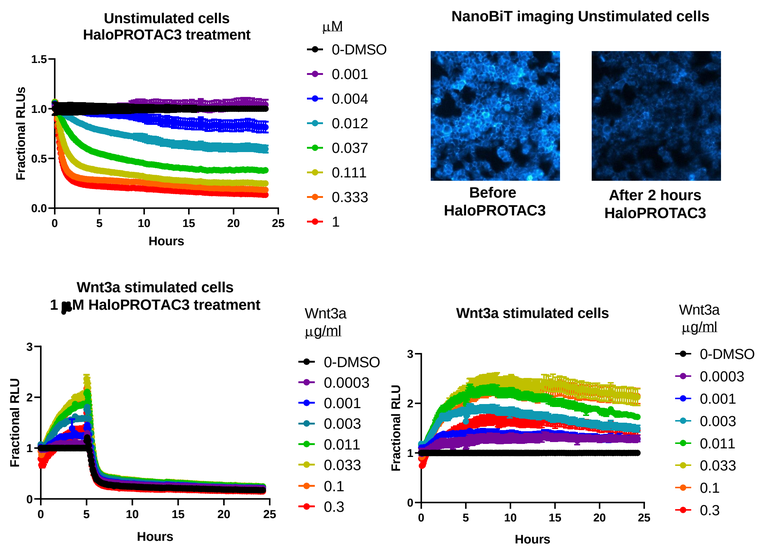
HiBiT-HaloTag-BRD4 HEK293 LgBiT stable cells



- Hook effect observed in degradation rate of HaloPROTAC3
- Decreased rate with concentrations of HaloPROTAC3 above 1 µM
- Similar degradation maximums with HaloPROTAC3 concentrations above 1 µM

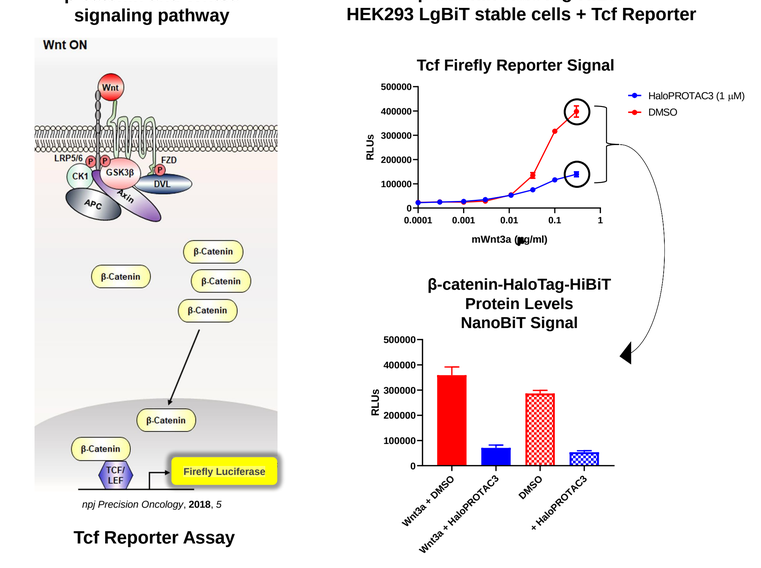
7. Kinetic degradation of β-catenin before and after Wnt3a stimulation

β-catenin-HaloTag-HiBiT HEK293 LgBiT stable cells



- Efficient degradation of HaloTag-HiBiT endogenously tagged β-catenin with HaloPROTAC3 before and after Wnt3a stimulation
- Observe loss of β-catenin-HT-HiBiT after HaloPROTAC3 treatment with LV200 imaging
- β-catenin-HT-HiBiT levels increased after Wnt3a stimulation in live cells

8. Monitoring Wnt3a signaling in the presence of β-catenin degradation



- HaloTag-HiBiT endogenously tagged β-catenin responds as expected to Wnt3a stimulation using dual reporter system with Tcf firefly reporter
- Degradation of β-catenin is observed in presence of Wnt3a and muted response to stimulation
- HaloPROTAC3 is an excellent compound for the study of degradation phenotype and function

9. Advantages and Challenges of HaloPROTAC3

Advantages over other Knock out Technologies

- Control of protein loss (extent and time frame)
- Can study loss of essential proteins that are lethal if knocked out at genomic level
- Protein recovery possible/ reversibility
- Mimic for PROTAC phenotypes
- Study degradation without having a binding ligand

Challenges

- Homozygous CRISPR insertion of HaloTag ideal for phenotype studies
- Localization of target protein to be degraded
- Working in relevant cell type
- Detection of degradation if luminescence is not used
- Limited situations where transient expression is applicable

Corresponding author: elizabeth.caine@promega.com