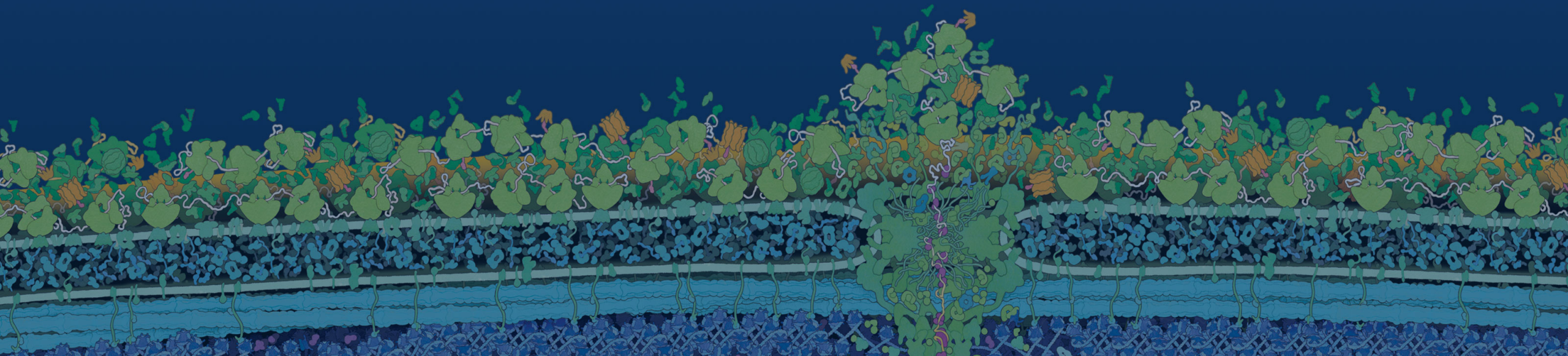
Promega

Targeting Proteins for Degradation: Characterizing PROTAC Kinetics and Mode of Action using Live-Cell Assays

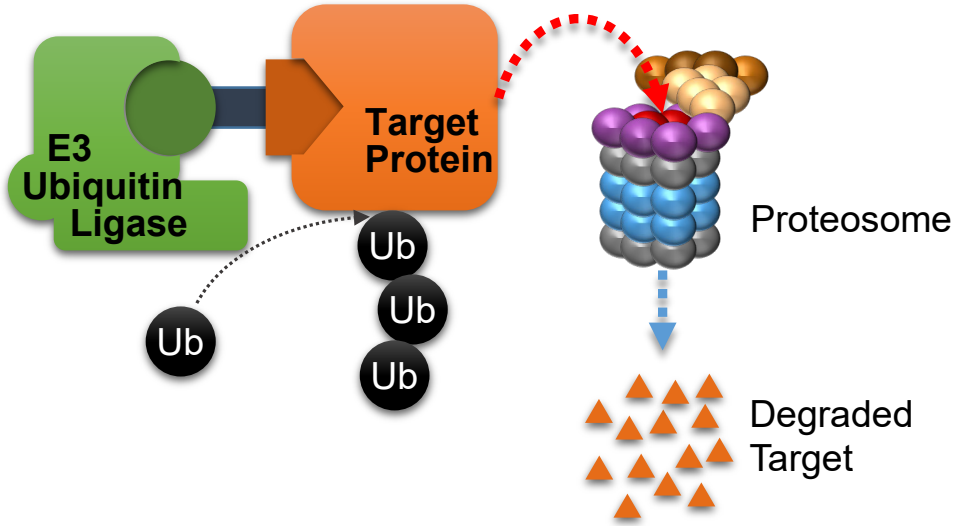
Kristin M. Riching, Ph.D



PROTACs: An Attractive Drug Discovery Strategy

PROTACs, or Proteolysis Targeting Chimeras, induce selective degradation of target proteins by simultaneously binding to target proteins and E3 ubiquitin ligases.

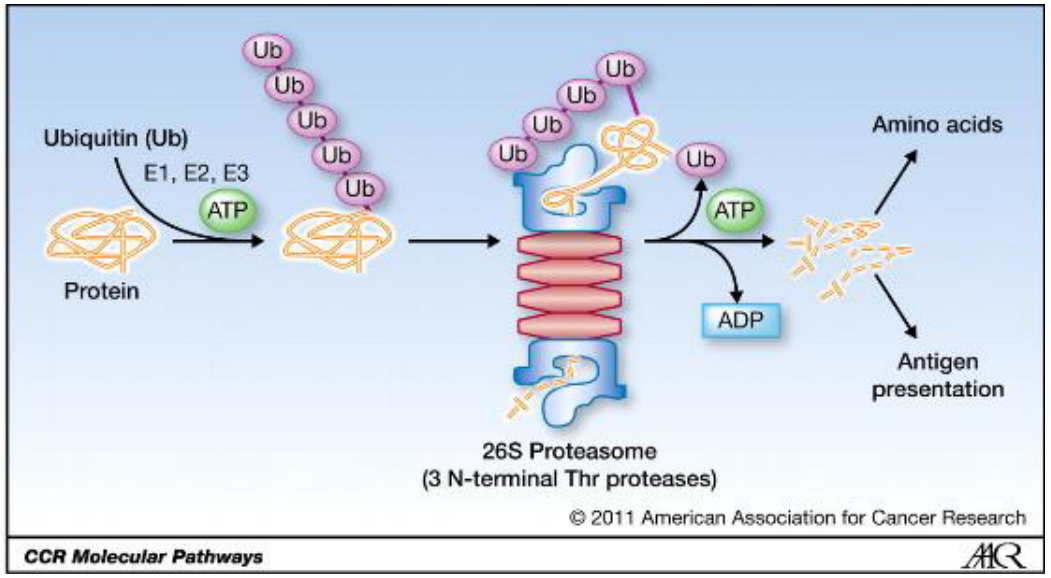
Basic PROTAC Structure



Why PROTACs over classical inhibitors?

- Improved potency due to catalytic mode of action (MOA).
- Prolonged pharmacodynamics due to need for protein resynthesis.
- Potential for targeting “undruggables” with ligands that can bind anywhere on a protein surface.

Understanding the Dynamics and Mechanisms of Protein Degradation



Challenges in understanding degradation and PROTAC efficacy:

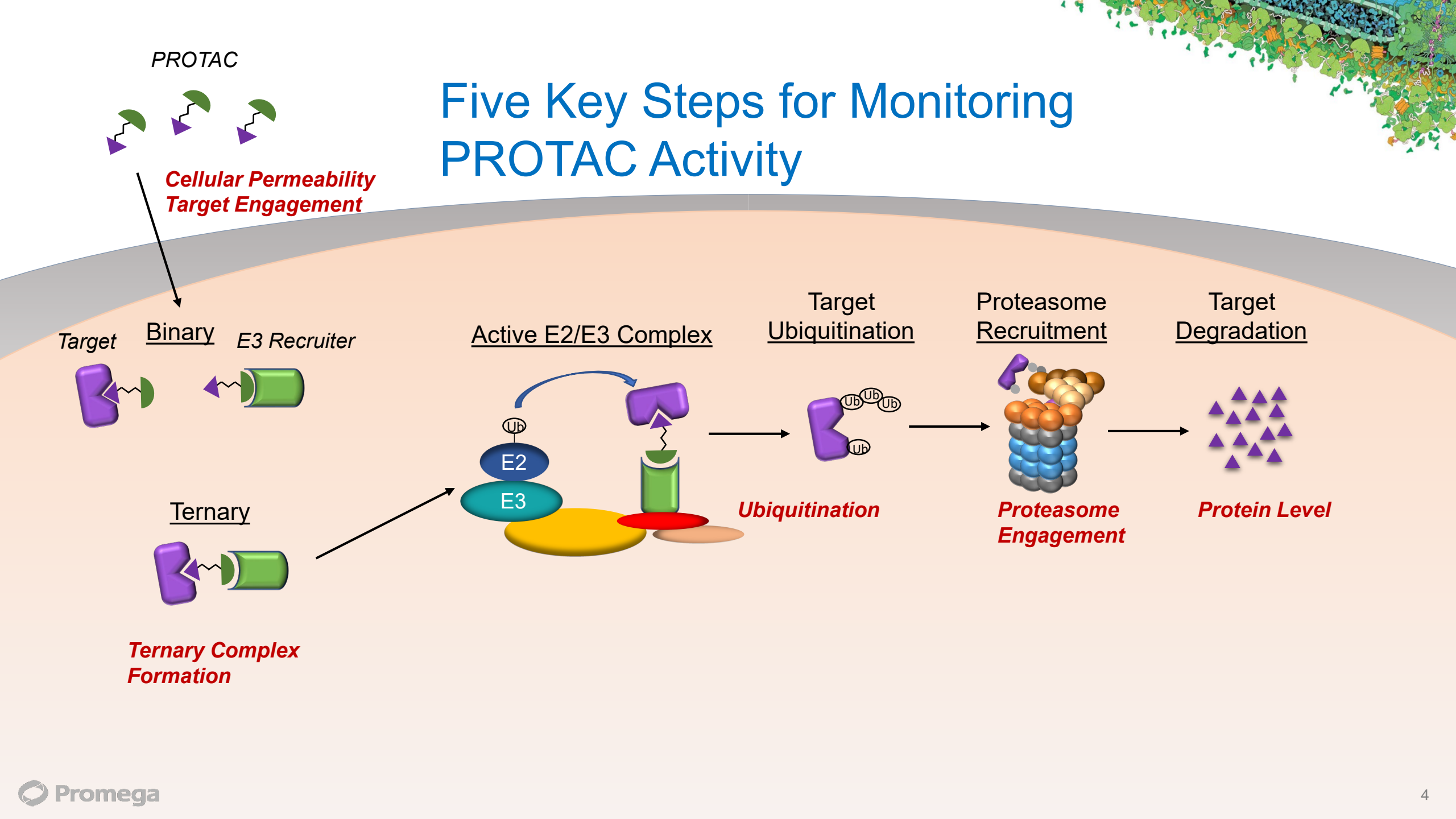
- Protein degradation is a dynamic and complex process.
- How do we assess whether a PROTAC degrades its target?
- If no degradation is observed, what happened?
- How do we know what steps in the pathway to optimize or improve?
- Is degradation efficacy correlated to specific mechanisms?

Solutions

- Develop technologies to understand the key steps for efficient and targeted degradation.
- Use assays to advance research and help development of degradation/PROTAC compounds.

Clin. Cancer Res. 18(1), 15–20. ©2011 AACR

Five Key Steps for Monitoring PROTAC Activity

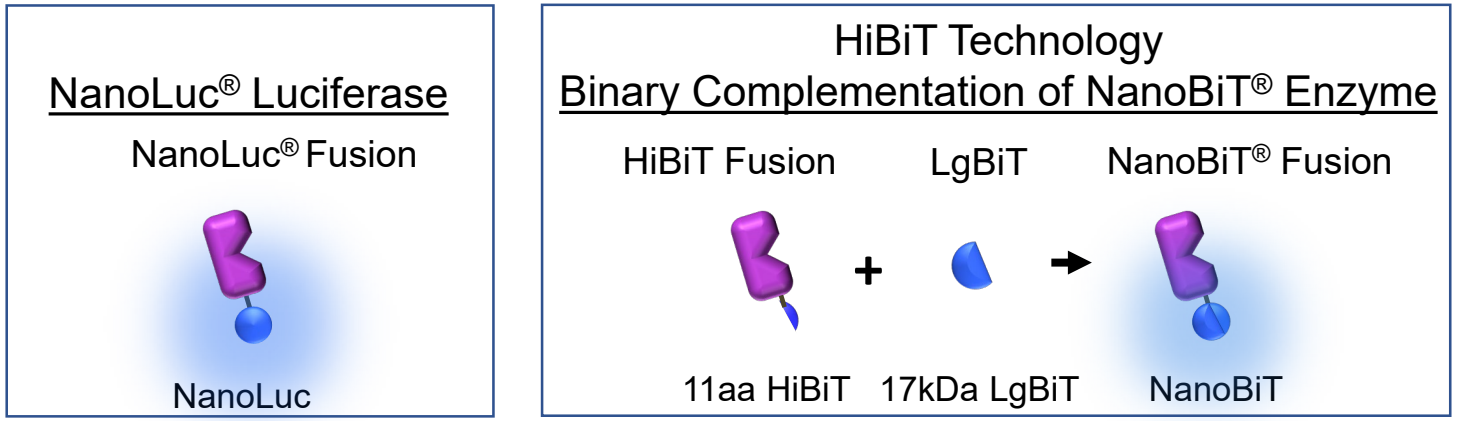


Technologies for Monitoring Events within the Degradation Pathway

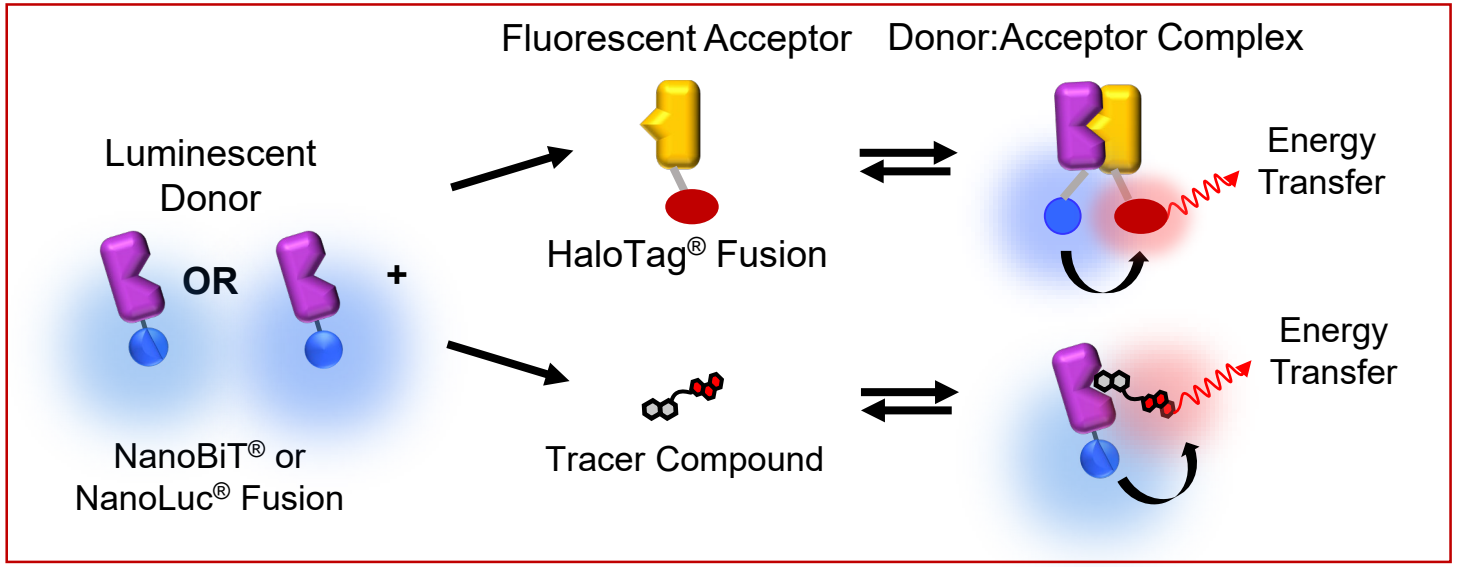
Protein Levels:
Degradation and recovery

Mechanism:
PROTAC binding and protein:protein interactions

Luminescence



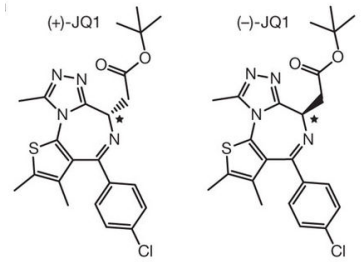
NanoBRET: Bioluminescence Resonance Energy Transfer



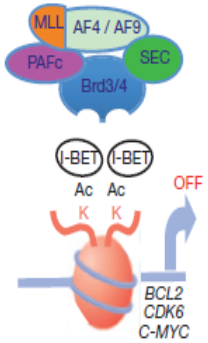
BET Family Bromodomain Inhibitors and PROTACs

Significant therapeutic promise for variety of leukemia, lymphomas and other hematologic cancers

First generation: Protein interaction inhibitors



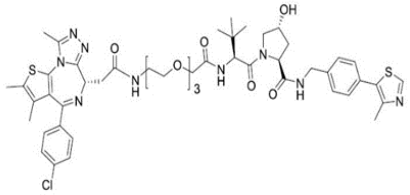
Nature 2010 December 23, 1067–73.



Nature 2011 478, 529–33.

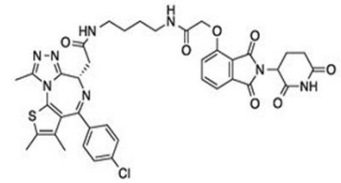
Next generation: Heterobifunctional degraders/PROTACs

MZ1



ACS Chem. Biol., **2015**, *10*, 1770.

dBET1

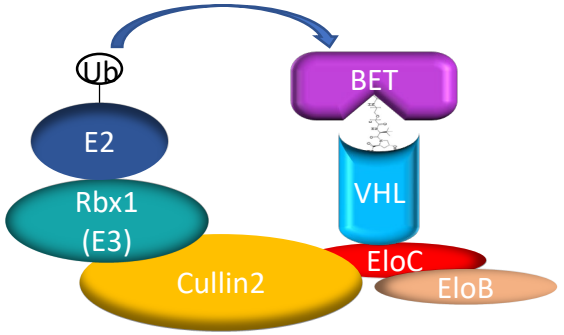


Science, **2015**, *348*, 1376.

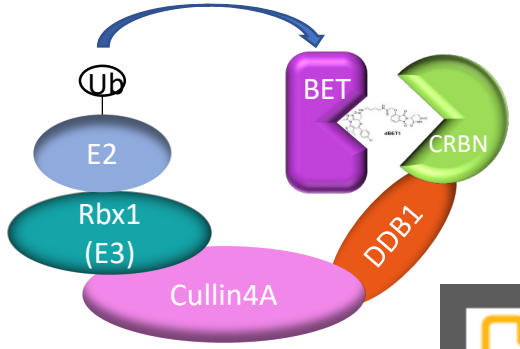
Goals:

- Can we understand the key steps for efficient and targeted degradation?
- Does this insight help development of therapeutic degradation compounds?

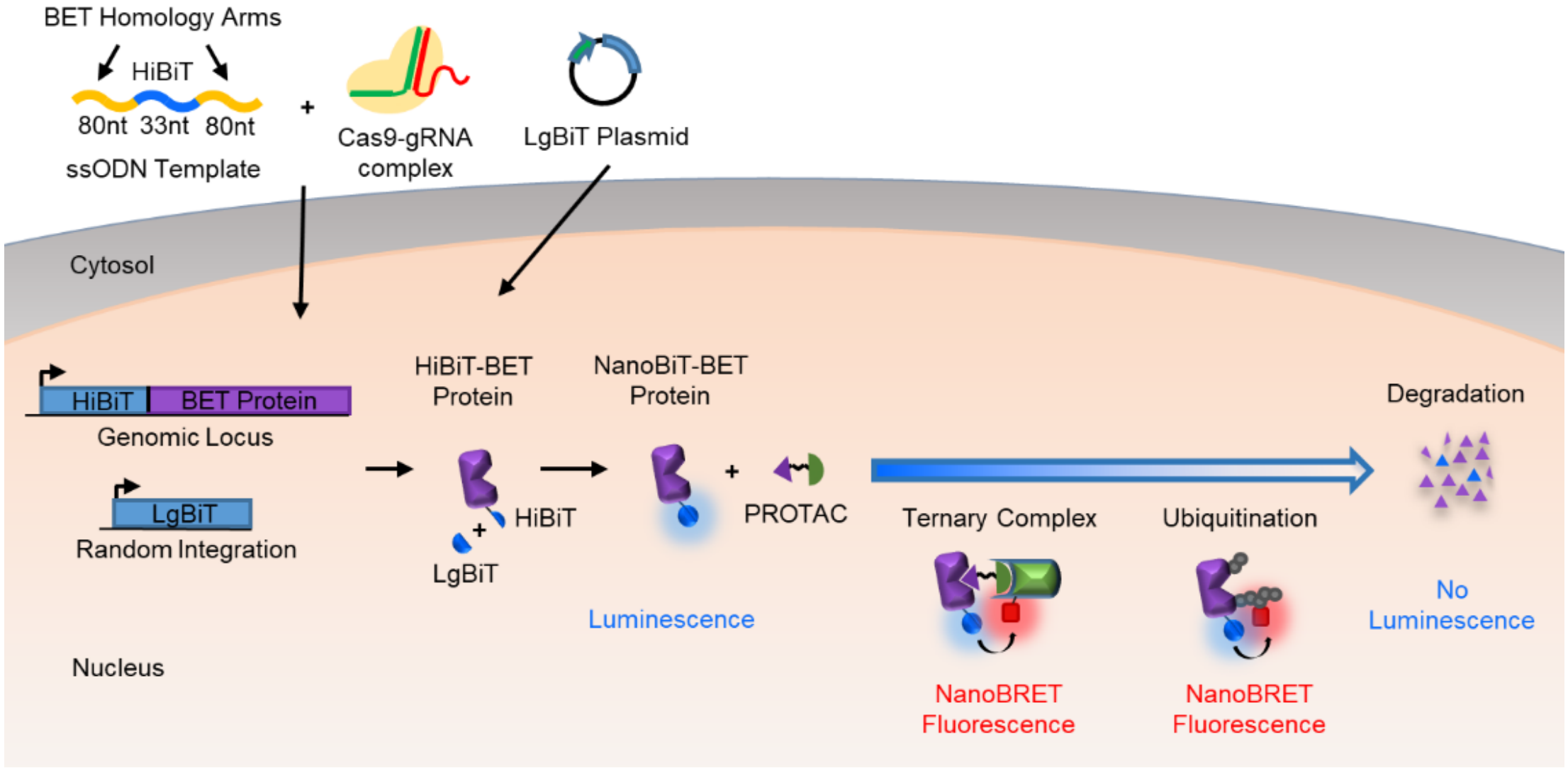
VHL System



CRBN System

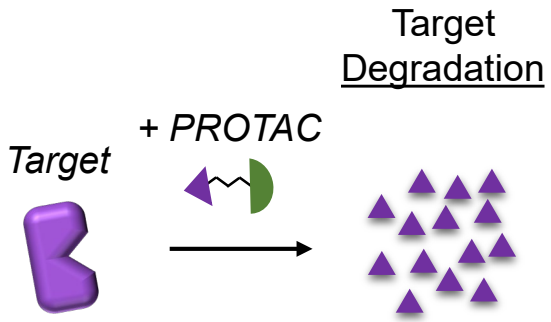


Approach for Studying the BET Family Members and Degradation

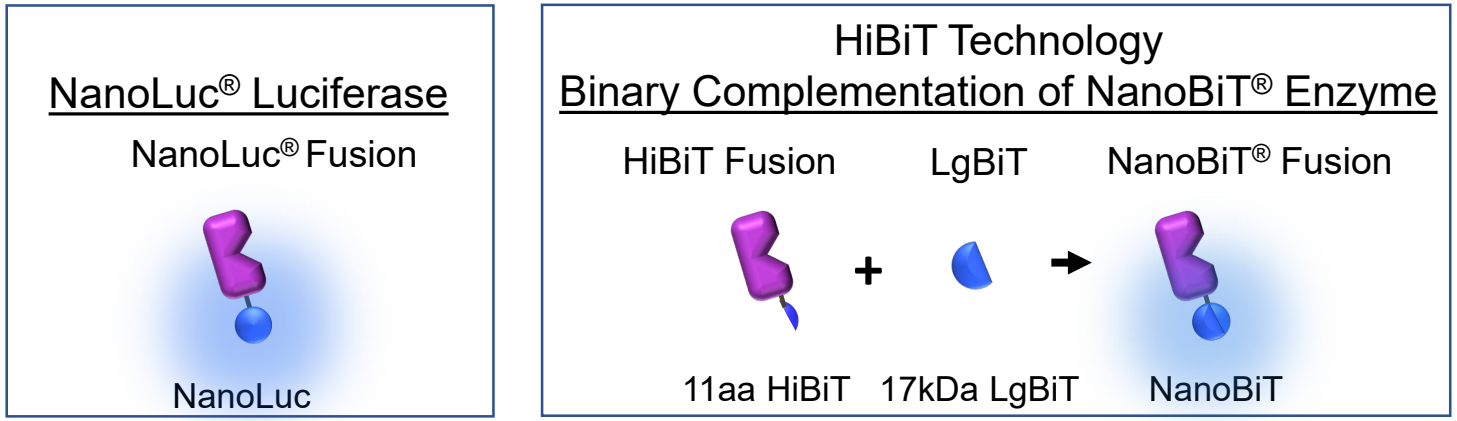


Assay #1: Monitoring Target Protein Degradation

Degradation Assay



NanoLuc® luciferases for protein level detection



Target NanoLuc® and HiBiT fusion proteins can be generated using:

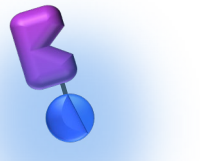
- Transiently or stably expressing fusion vectors.
- CRISPR endogenous tagging–RECOMMENDED for all quantitative calculations (rate, DC_{50}, and Dmax).

Degradation assays can be performed:

- As lytic or in live cells.
- As long-term kinetic analysis (24–48 hours) with Endurazine™ or Vivazine™ extended substrate.

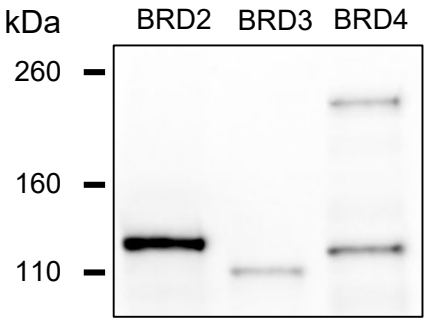
HiBiT CRISPR of BET Family Members and Initial Testing

HiBiT CRISPR
BRD2, 3 or 4



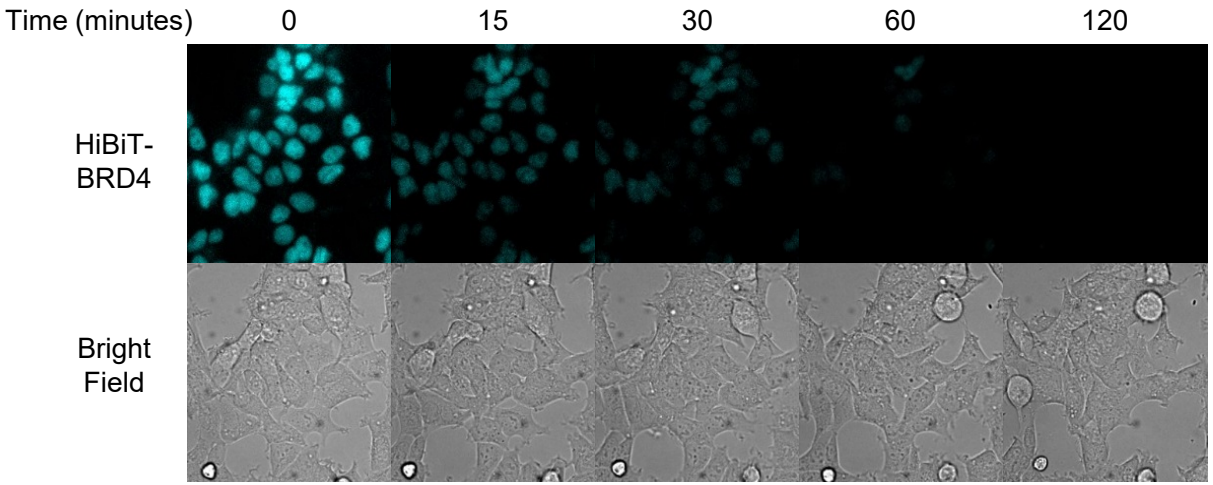
Endogenously tagged protein

Luminescent Blot

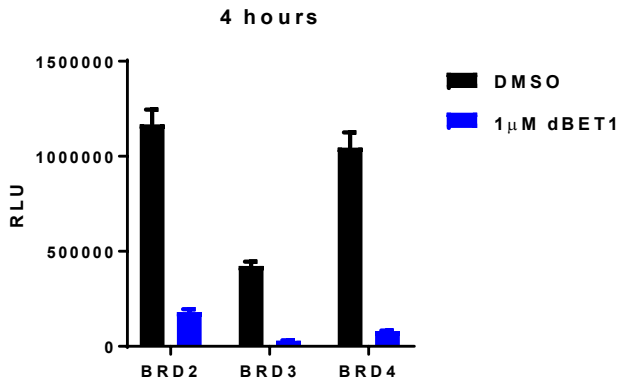


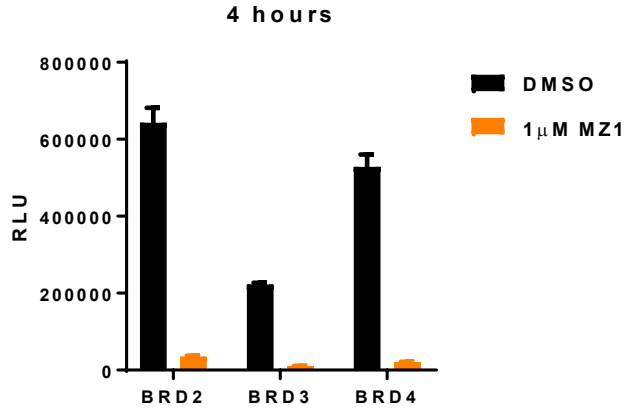
Endogenously tagged BET family members

Luminescent Imaging of HiBiT-BRD4 + MZ1 PROTAC



Endpoint Luminescent (RLU) Analysis with dBET1 or MZ1

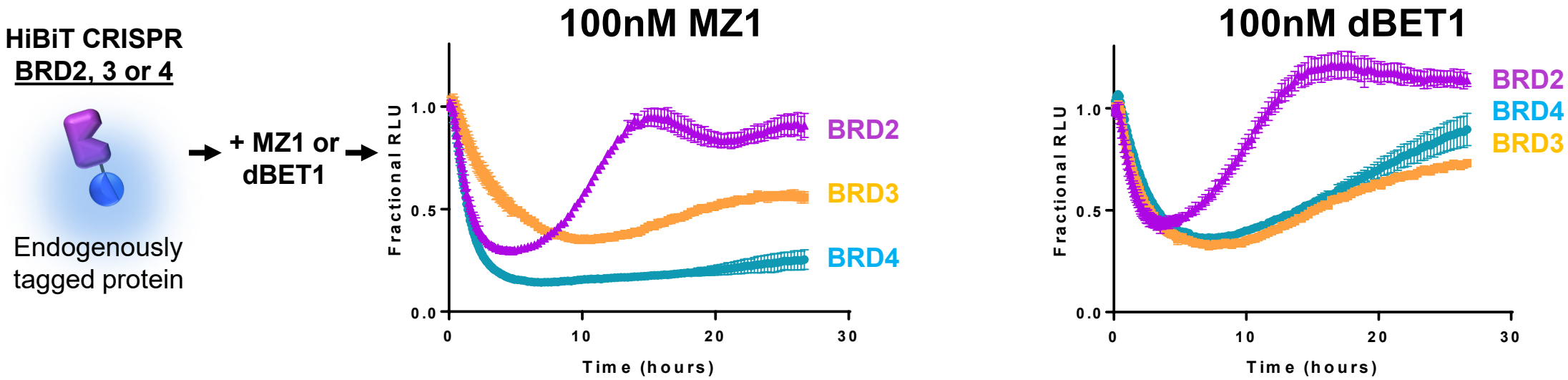




MZ1
ACS Chem. Biol., **2015**, *10*, 1770.

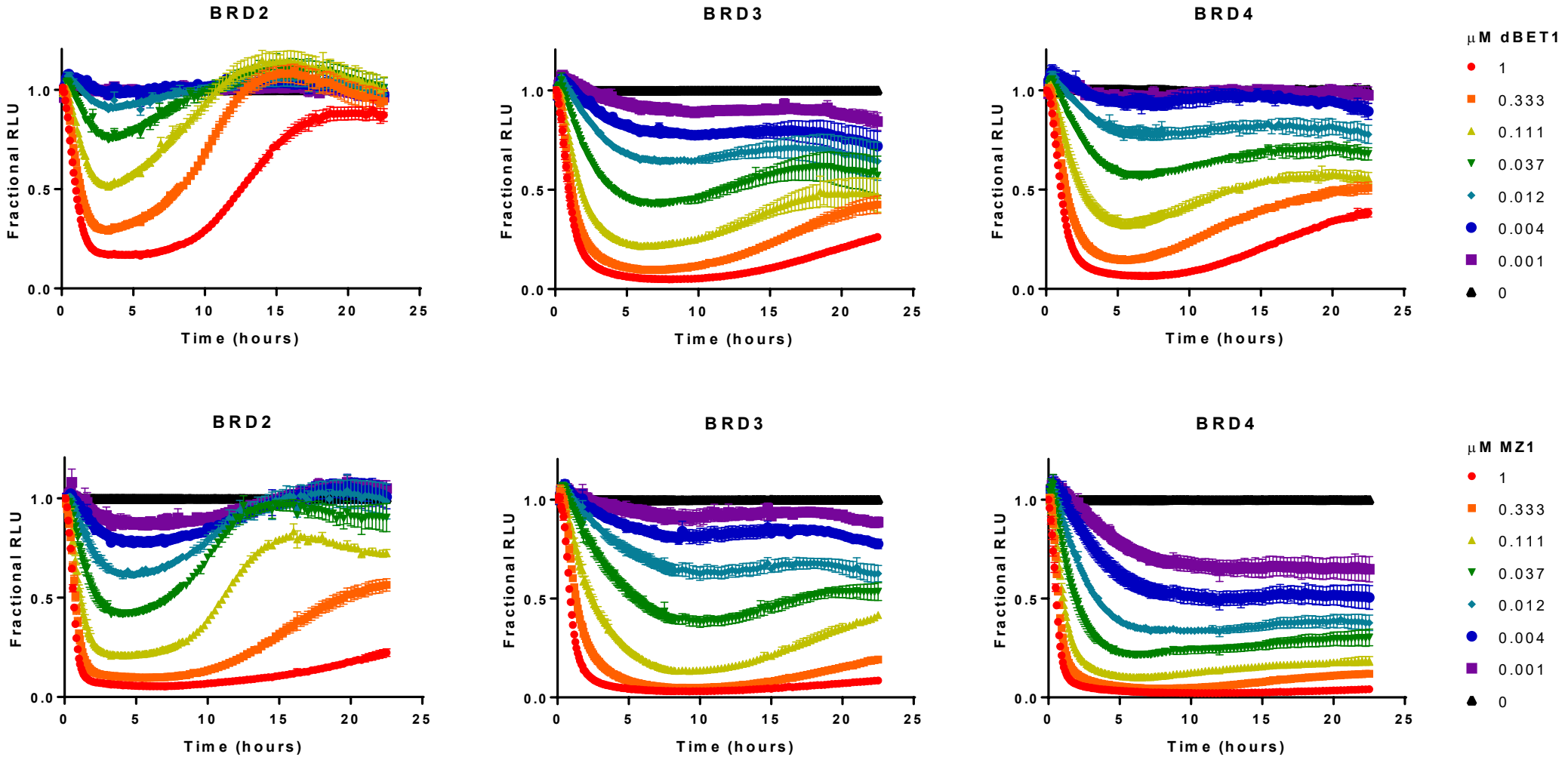
dBET1
Science, **2015**, *348*, 1376.

Live Cell Kinetic Degradation Analysis at Fixed Concentration for 24 Hours



- Each family member revealed both unique degradation and recovery response to MZ1 and dBET1.
- Demonstrated how degradation measurements at fixed points in time can differ drastically.
- Used the extended furimazine substrate, Endurazine, and read continuously on a GloMax® Discover.

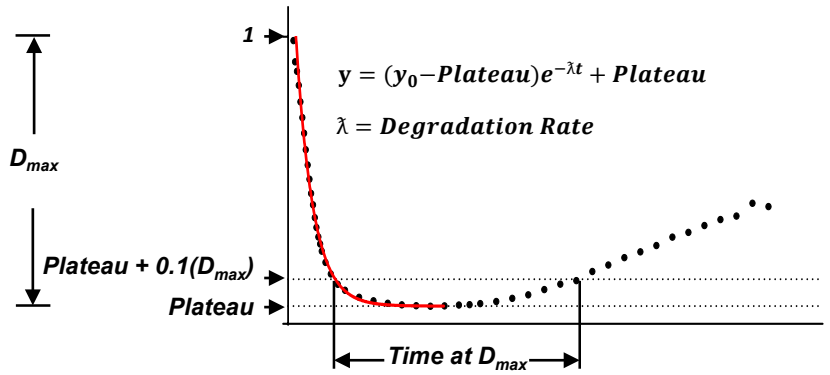
Live Cell Degradation Profiles Across Concentration Series

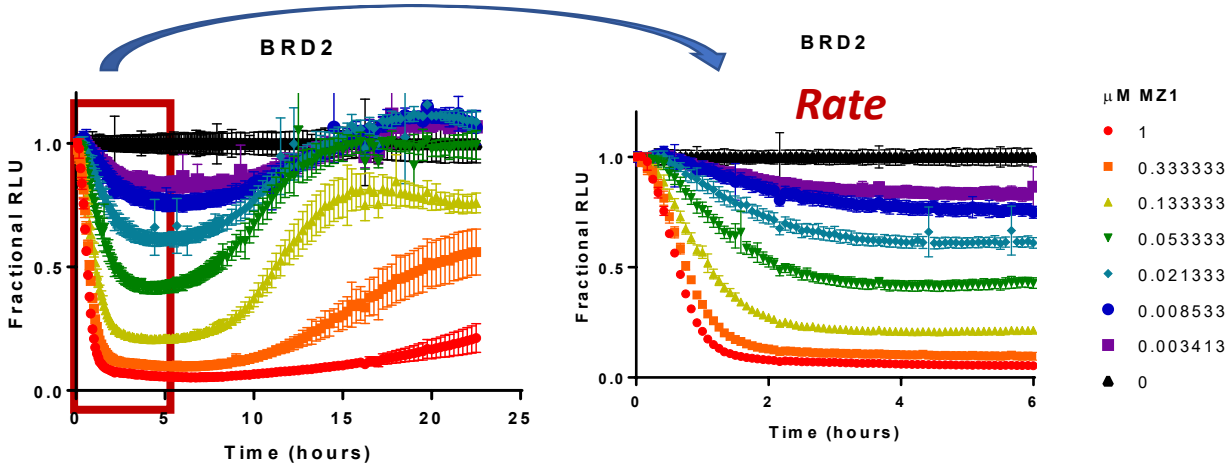


Target and concentration-specific degradation and recovery response to MZ1 and dBET1

Using Profiles to Calculate PROTAC Degradation Parameters

Degradation Profile



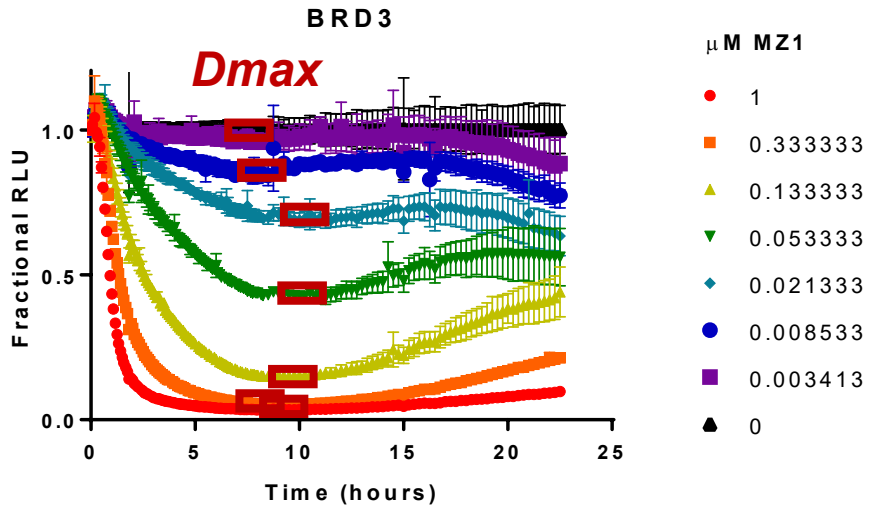


Quantitative Parameters

Rate of degradation

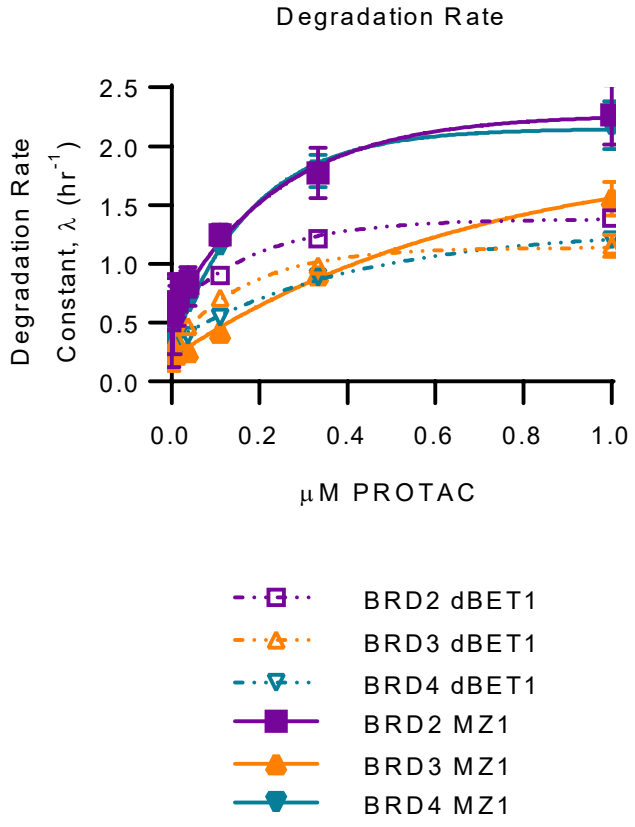
Dmax/DC_{50}

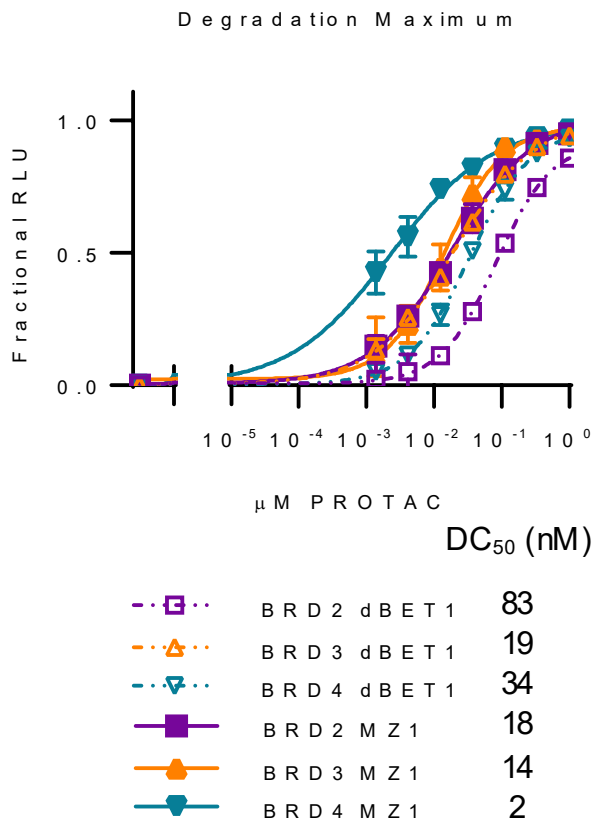
Time at Dmax

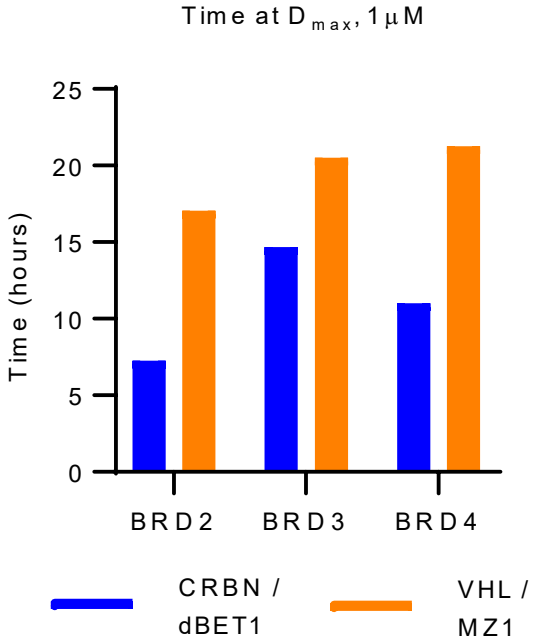


Can calculate multiple parameters in a single degradation profile.

Graphical Representations of Calculated Profile Degradation Parameters

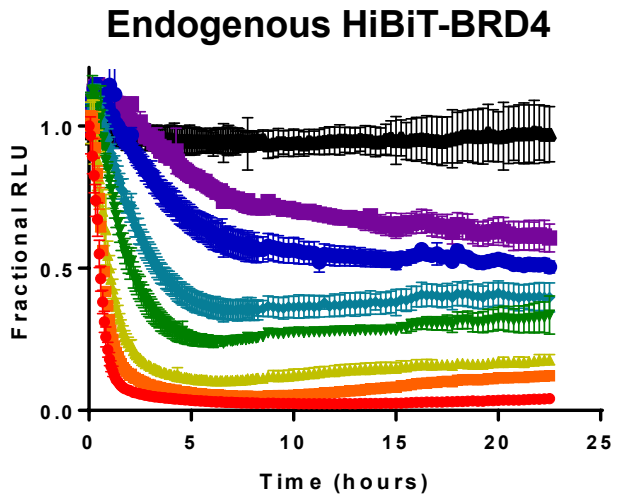


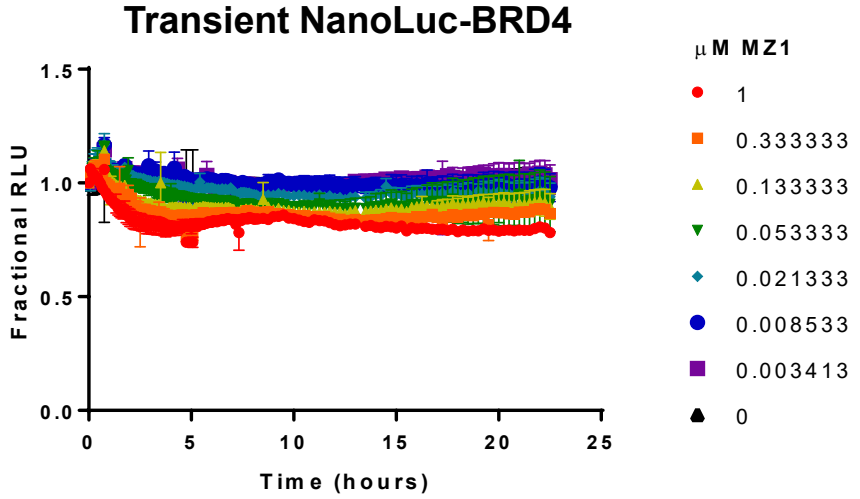


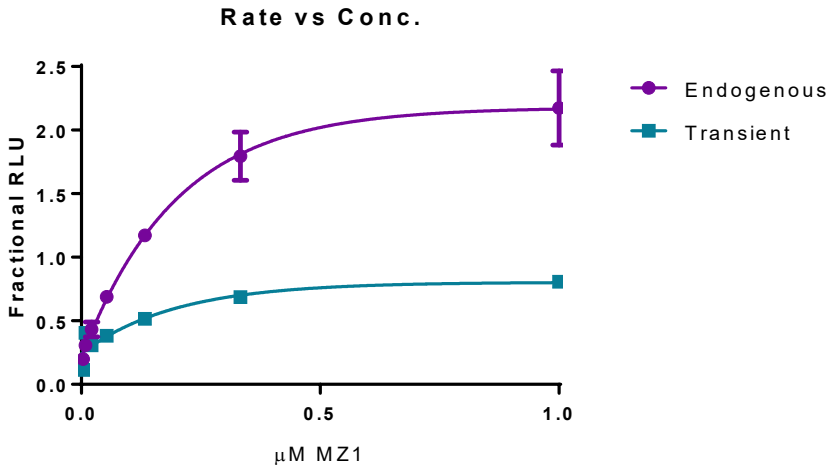


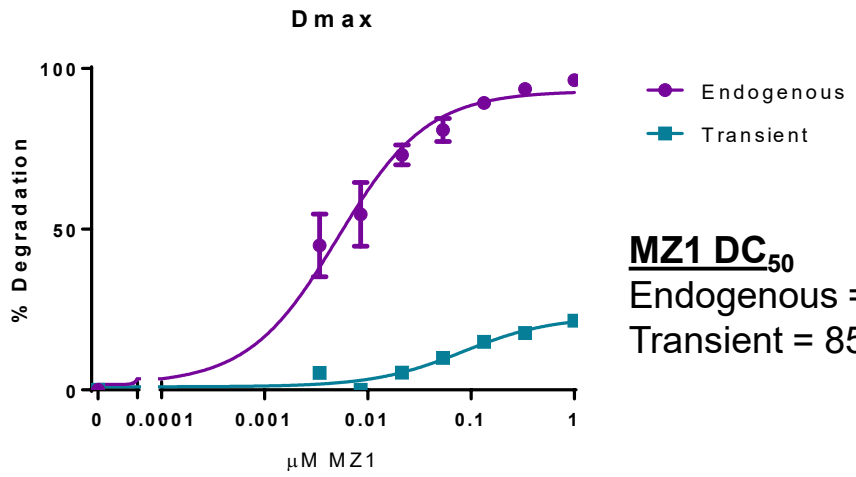
Can rank the order of PROTAC and family member responses across various quantitative parameters.

Comparing Degradation from Endogenous and Ectopic Expression









MZ1 DC_{50}
Endogenous = 8nM
Transient = 85nM

Ectopic expression differs from endogenous by:

- Degradation rate
- Extent
- Protein recovery

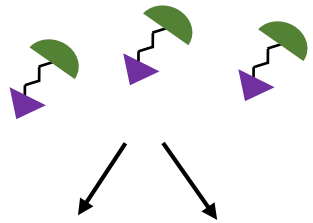
Ectopic expression recommendations:

- Express at low levels.
- Use only qualitatively (yes or no for degradation).
- Stable cell line if possible for more uniform expression.

Assay #2: Monitoring PROTAC Permeability and Target Engagement

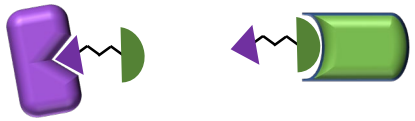
Target Engagement

PROTAC

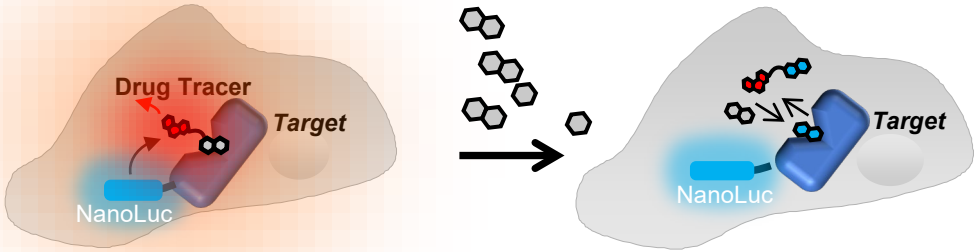


Binary Complexes

Target *E3 Recruiter*



NanoBRET® Target engagement



Intracellular binding Intracellular affinity

- Bioluminescence Resonance Energy Transfer to monitor protein:small molecule interactions
- Highly specific, biophysical measurement
- Monitor binding affinity, residence time and rank order compounds

NanoBRET® target engagement assays can be performed using:

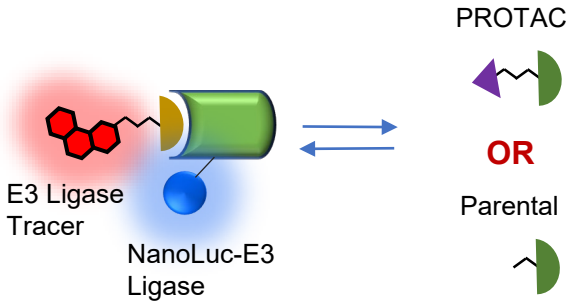
Transient, stable or CRISPR endogenously tagged NanoLuc® or HiBiT fusions

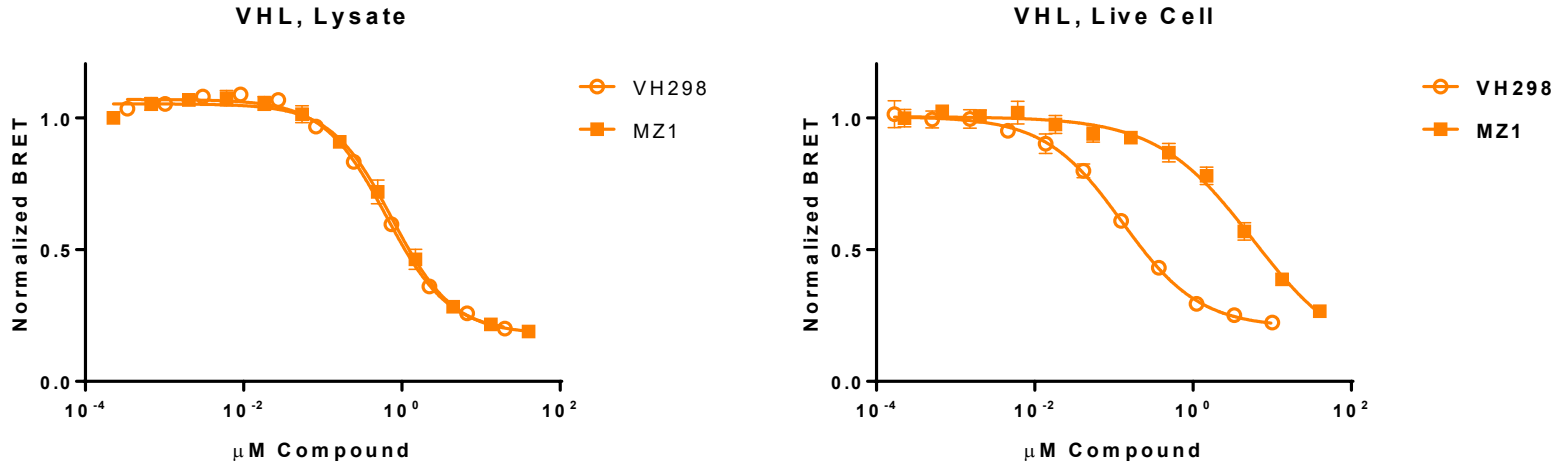
Target specific or E3 ligase fluorescent tracers

Compatible with lytic or live-cell formats to assess cellular permeability.

Comparing Lytic versus Live Cell to Assess Permeability

NanoBRET® TE E3 Competitive Displacement



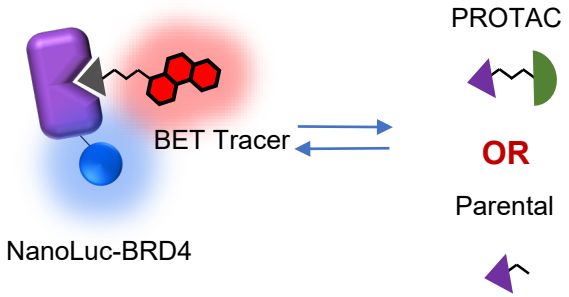


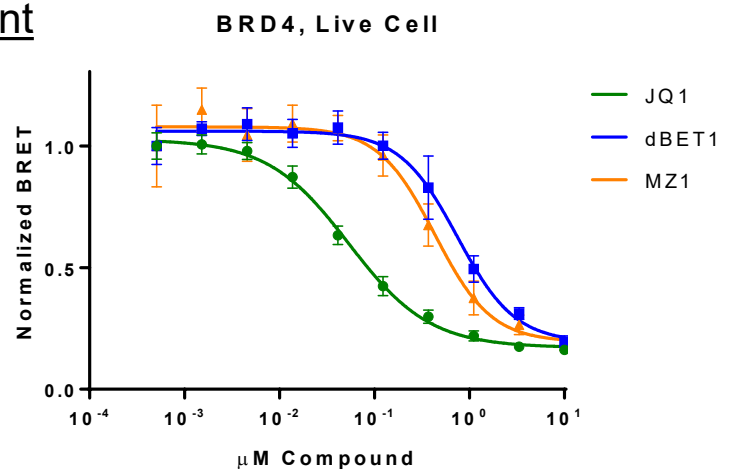
Compound	Target	IC_{50} (nM, Lysate)	IC_{50} (nM, Live Cell)
VH298	VHL	633	127
MZ1	VHL	755	5600

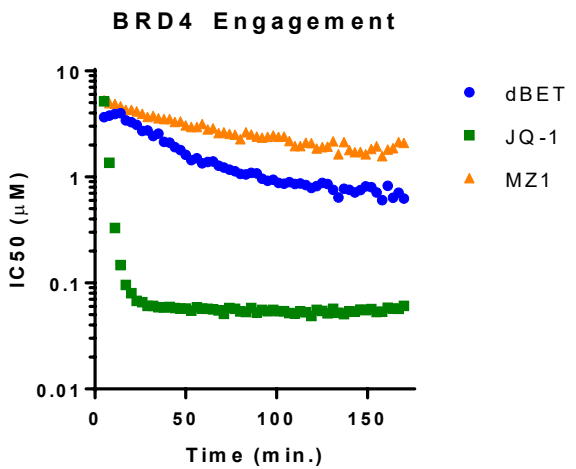
- No difference in VHL binding affinities observed in lytic format between parental (VH298) and MZ1 PROTAC.
- NanoBRET® target engagement in live cells shows binding affinity shift, indicating reduced permeability of MZ1.

Measuring Intracellular Binding to Target BRD4 Protein

NanoBRET® TE Target Competitive Displacement



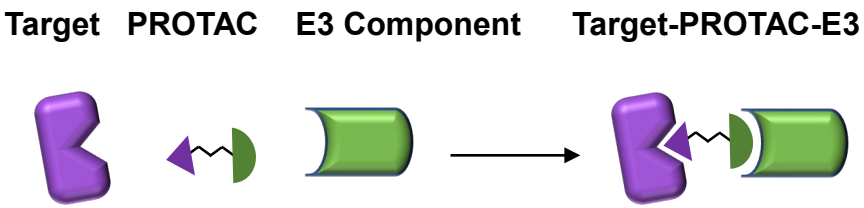




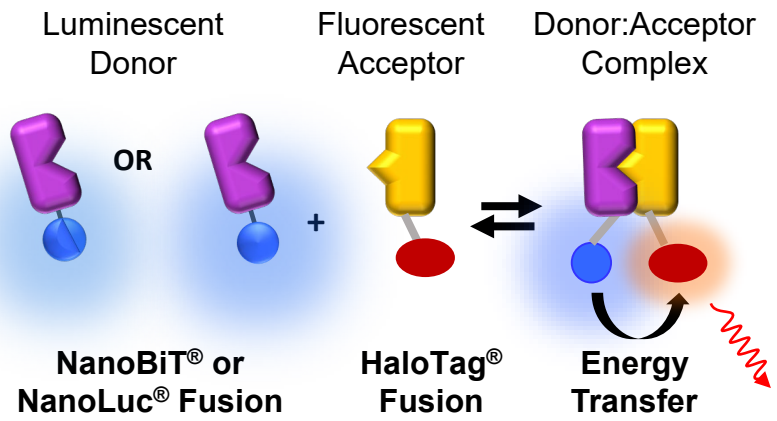
Compound	Target	IC_{50} (nM, Live Cell)
MZ1	BRD4	432
dBET1	BRD4	747
JQ1	BRD4	54

- Significant shift in intracellular binding affinities for BRD4 by MZ1 and dBET1 as compared to parental JQ1.
- Kinetic binding analysis showed slow intracellular engagement, suggesting reduced permeability of PROTACs.

Assay #3: Monitoring E3 Ternary Complex Formation



NanoBRET® protein:protein interactions



NanoBRET® protein:protein interaction assays can be performed using:

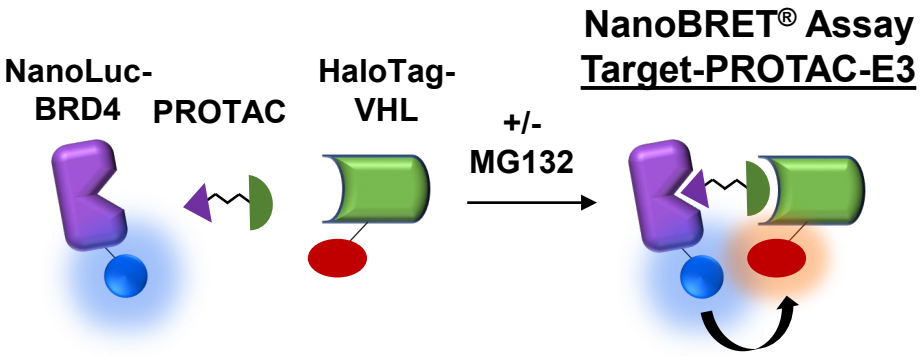
Transient, stable or CRISPR endogenously tagged NanoLuc® or HiBiT fusions as energy donor.

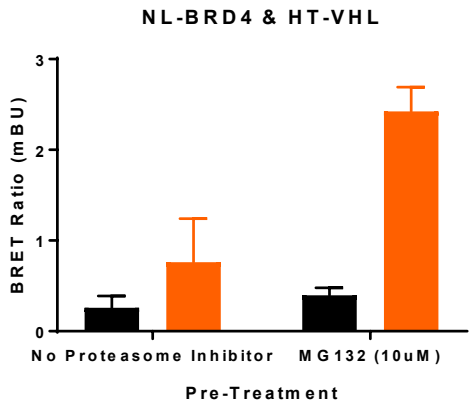
Transient or stable HaloTag® E3 ligases or components as energy acceptors.

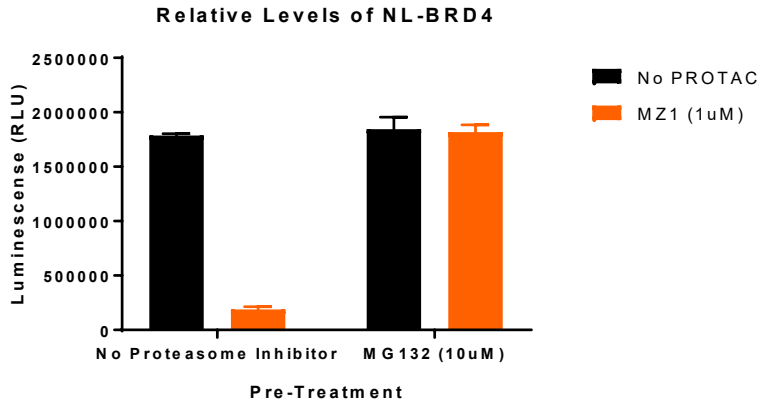
Compatible with lytic or live-cell formats.

Long-term kinetic analysis also possible using stabilized Vivazine™ substrate.

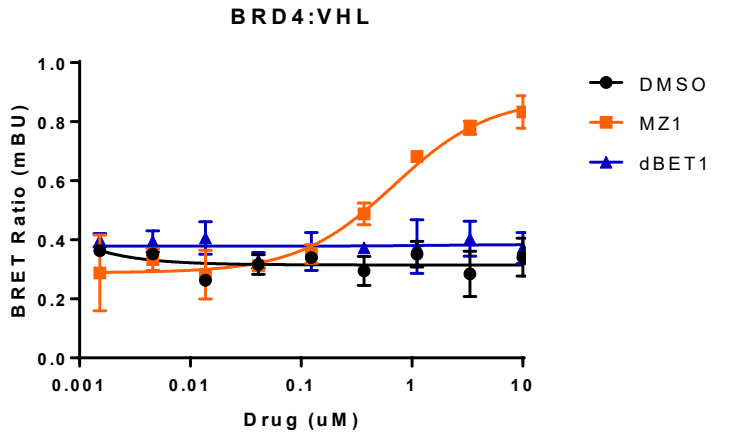
Ternary Complex Detection and Controls





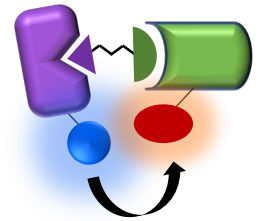


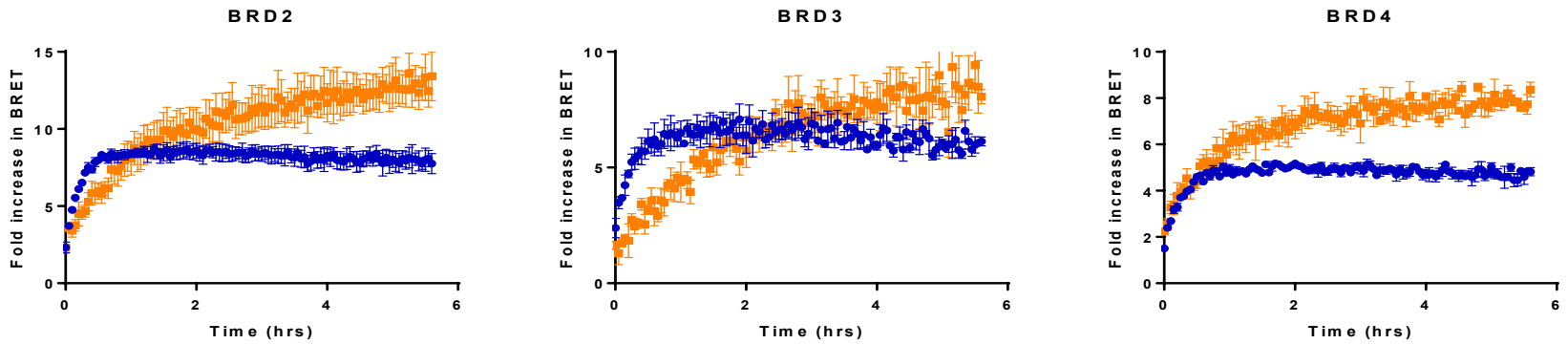
- Monitor ternary complex formation and degradation simultaneously.
- Using MG132 can increase signal window.
- Using irrelevant E3 ligase components demonstrate signal specificity.



Kinetic Monitoring of Ternary Complexes

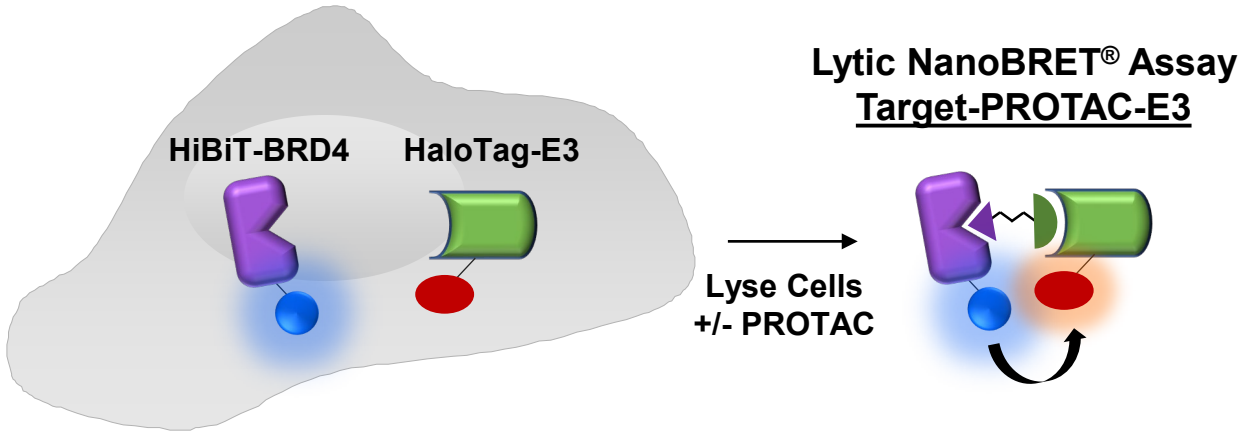
NanoBRET® Assay
HiBiT BET-PROTAC-E3

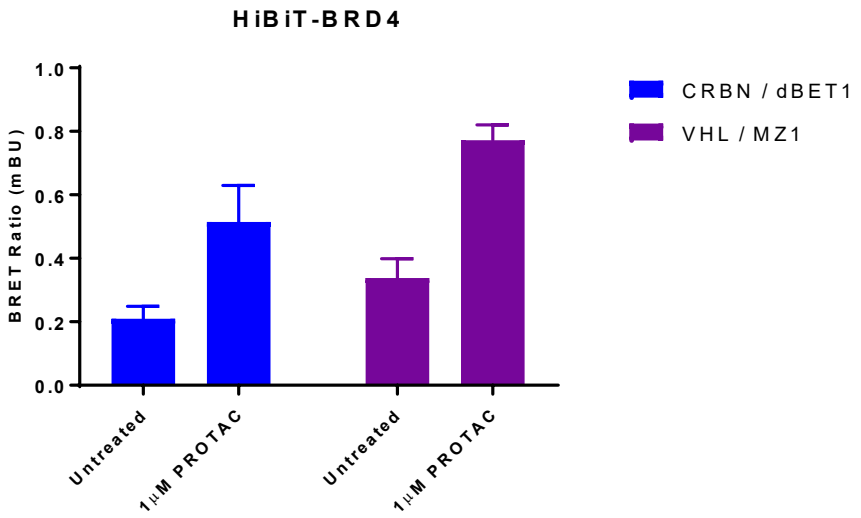




- Kinetic patterns are different for each family member and each PROTAC.
- Analysis in the presence of MG132 enables study of cellular stability of ternary complex.
- Studies greater than 1 hour should use stabilized Vivazine™ substrate.

Monitoring Ternary Complexes in Lysates



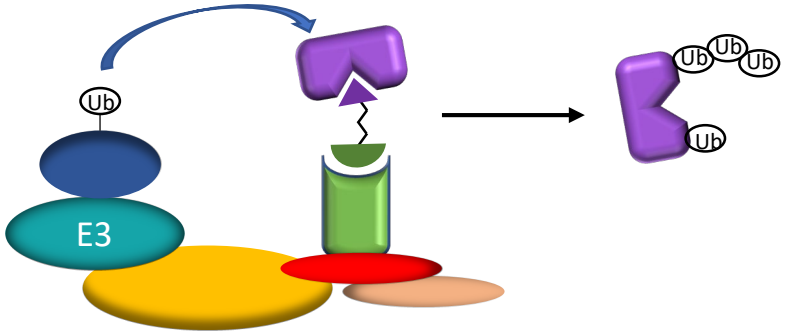


- PROTACs are added after cell lysis with digitonin.
- Can screen for compounds that induce ternary complexes without eliminating hits due to low cell permeability.

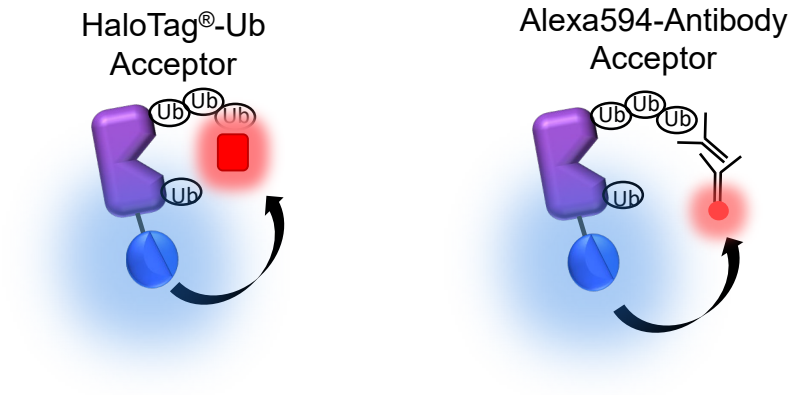
Assay #4: Monitoring Ubiquitination

Active E3 Complex

Target Ubiquitination



Two NanoBRET® Ubiquitination Assays



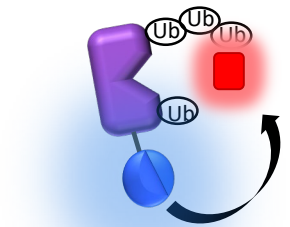
NanoBRET® protein:protein and protein:antibody ubiquitination assays can use the following:

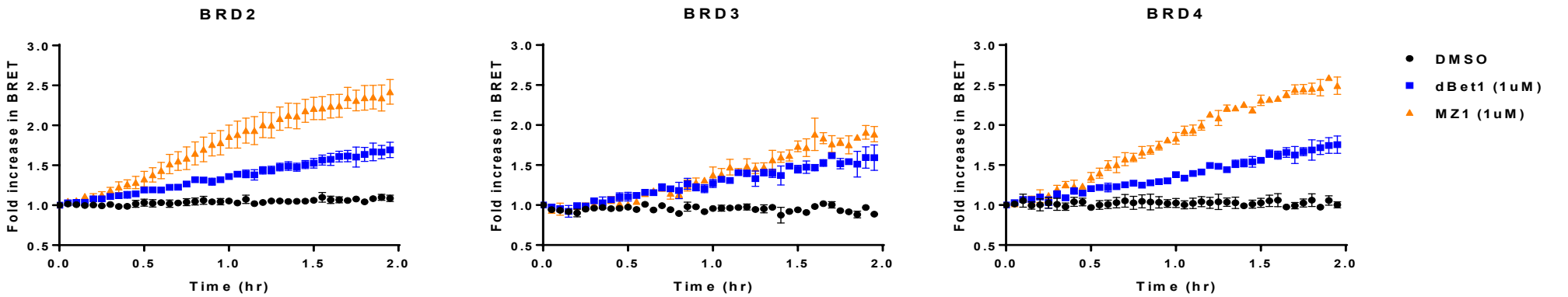
- Transient, stable or CRISPR endogenously tagged NanoLuc® or HiBiT fusions as energy donors.
- Transient HaloTag®-Ubiquitin as energy acceptor (live-cell assay).
- Primary or secondary fluorescently labelled antibody as energy acceptor (lytic assay).

Long-term kinetic analysis possible using stabilized Vivazine™ substrate.

Kinetic Monitoring of BET Family Ubiquitination

NanoBRET® Assay
HiBiT BET-HaloTag®-Ub

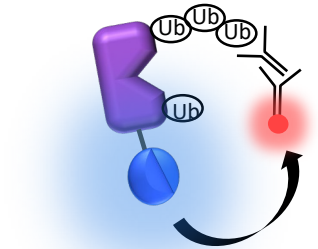


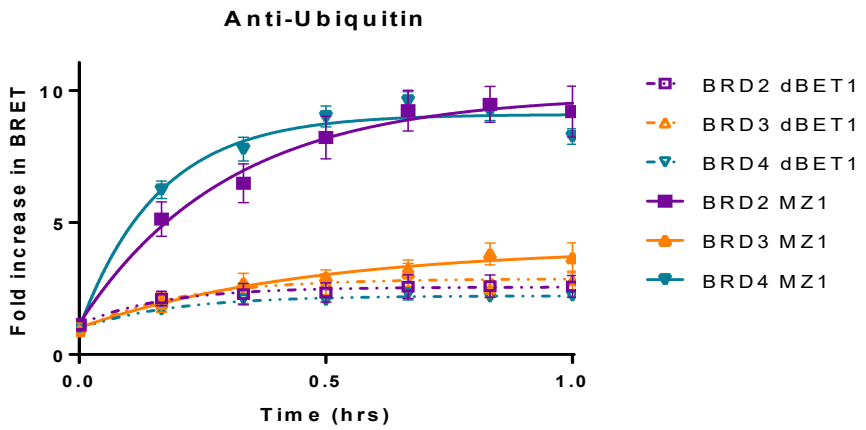


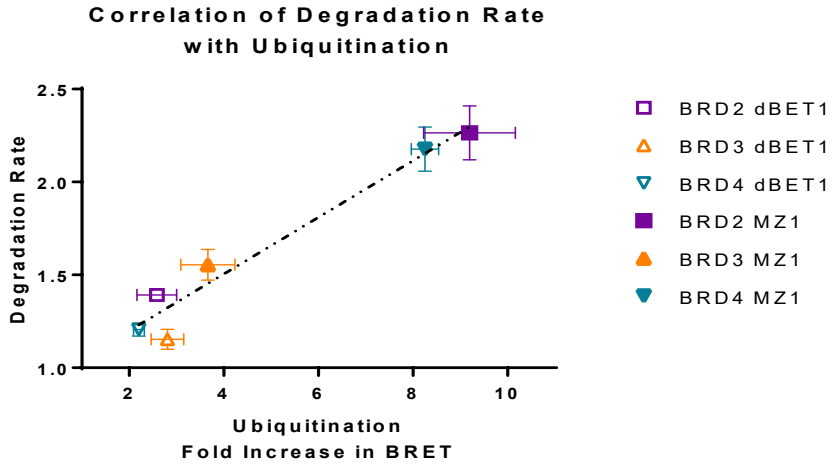
- Live-cell kinetic patterns are different for each family member and each PROTAC.
- The different E3 ternary complexes recruited by dBET1 and MZ1 show differential ubiquitination on respective targets.
- Studies greater than 1 hour should use stabilized Vivazine™ substrate.

Endogenous Ubiquitination Patterns and Correlation with Degradation Rate

NanoBRET® Assay
HiBiT BET-Polyclonal Ub

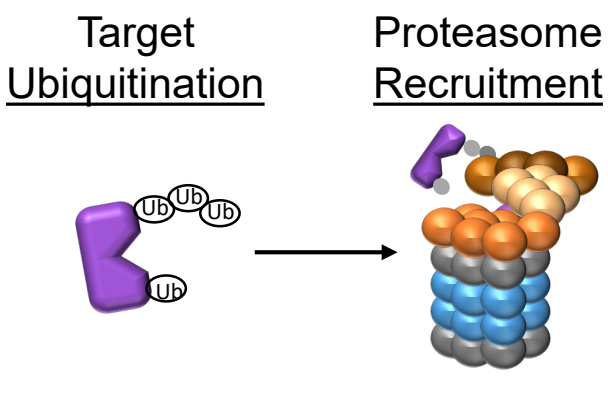


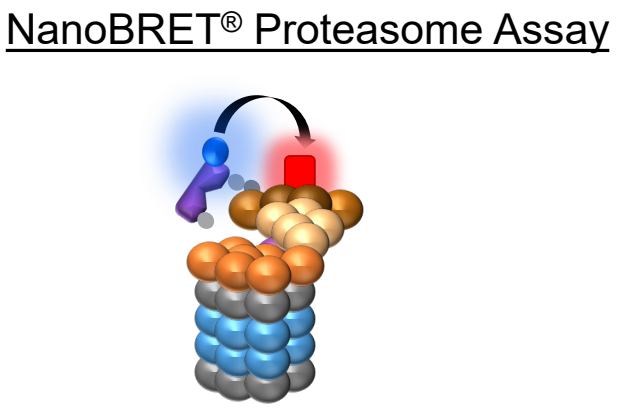




- Relative intensity differences and patterns similar to HaloTag®-Ub NanoBRET® Assay.
- Anti-Ubiquitin measurements are done using lytic, endpoint measurements.
- Level of ubiquitination shows linear relationship, high correlation with calculated degradation rate.

Assay #5: Monitoring Translocation to Proteasome





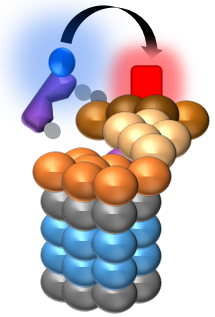
NanoBRET® protein:protein proteasomal assays can use the following:

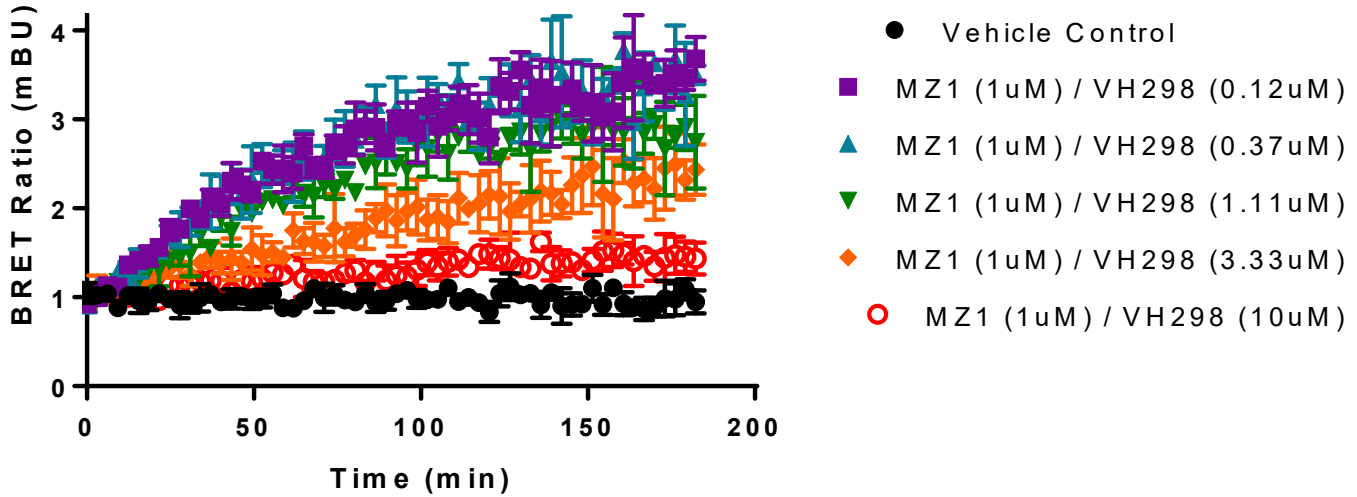
- Transient, stable or CRISPR endogenously tagged NanoLuc® or HiBiT fusions as energy donors.
- Transient HaloTag® -PSMD3 proteasomal subunit as the energy acceptor.

Long-term kinetic analysis possible using stabilized Vivazine™ substrate.

Kinetic Trafficking of BRD4 to the Proteasome

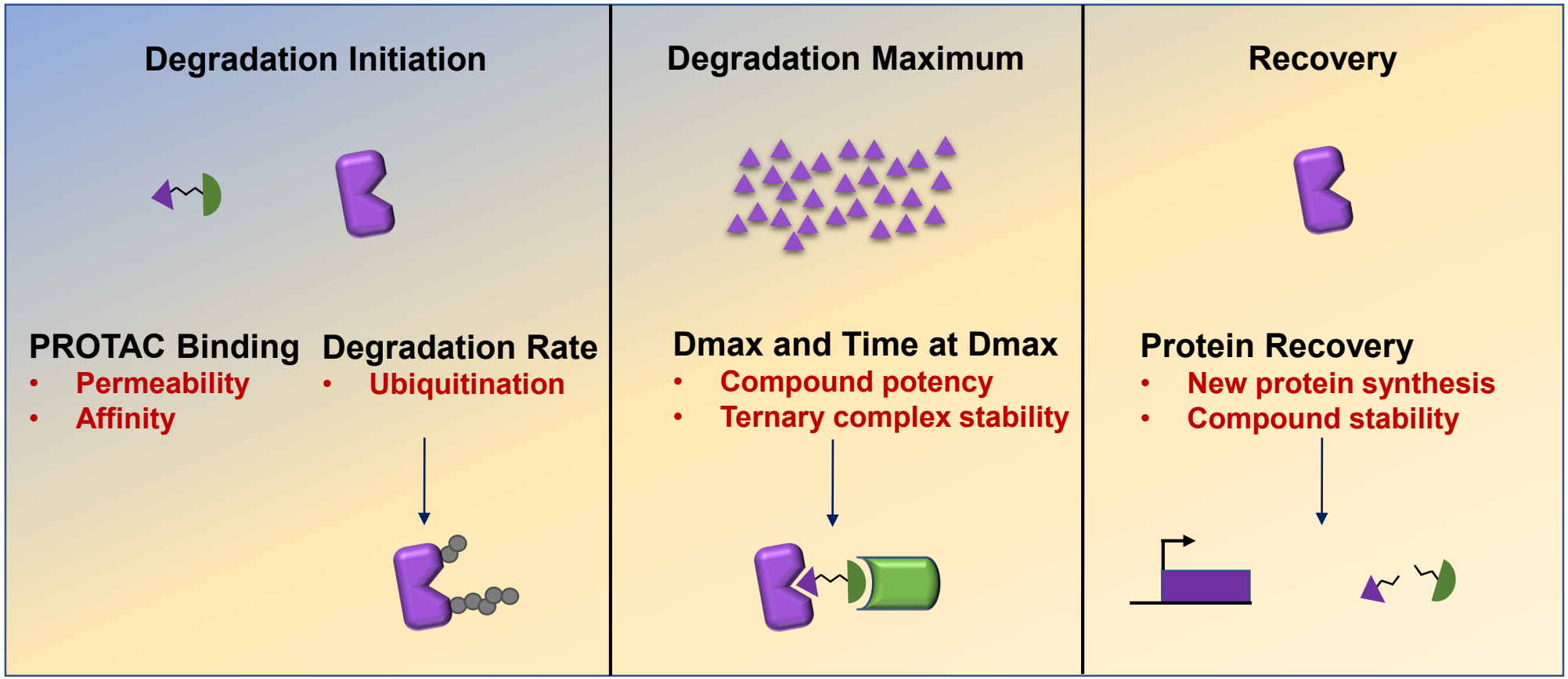
NanoLuc-BRD4:HaloTag-PSMD3





- Detect increase in trafficking of BRD4 to proteasome in the presence of PROTAC.
- Parental compounds can be used as controls.
- We do not recommend using proteasome inhibitors for the assay.
- Can simultaneously monitor loss of target (NanoLuc-BRD4) in proteasome assay.

Mechanistic Contributions to Degradation Phases



Summary

Differentiating cellular technologies are available to study key processes in PROTAC-mediated degradation.

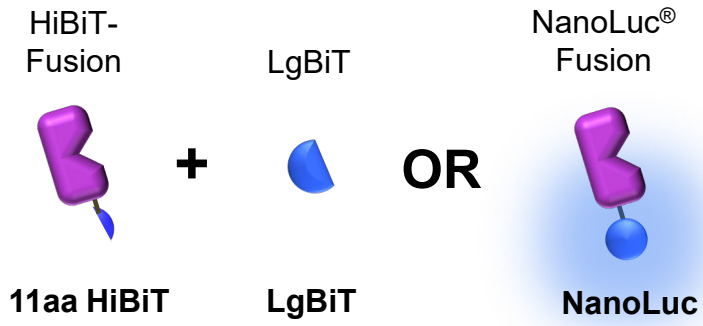
HiBiT and NanoLuc® technology

- Measure kinetics of degradation in live cells.
- Can be used with CRISPR to study endogenous proteins.
- Able to quantitate key degradation parameters.

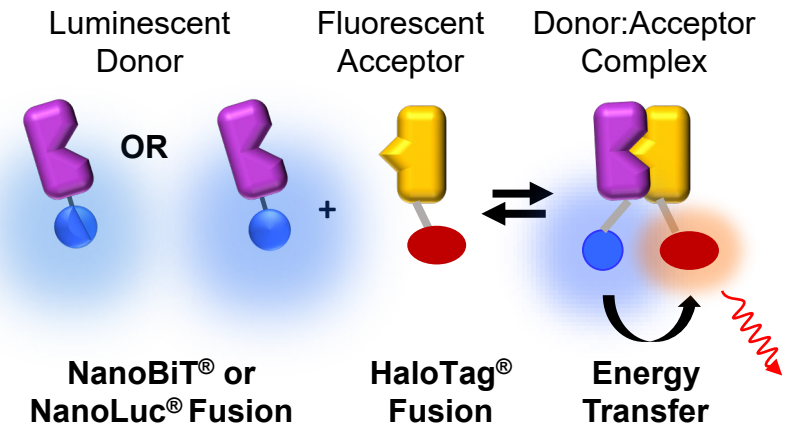
NanoBRET® technology

- Monitor dynamic pathway interactions and signaling mechanisms in live cells.
- Can assess PROTAC cellular permeability.
- Follow induced interactions with E3 ligase components, Ubiquitin and subsequent trafficking to proteasome.

HiBiT or NanoLuc® technology



NanoBRET®
Bioluminescence Resonance Energy Transfer



Promega

Thank You!

Questions?