Detection of Endogenous Protein Turnover Induced by Targeted Degraders

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Abstract

An assay platform that robustly and sensitively quantifies the kinetics of endogenous protein turnover is crucial for discovery of disease-relevant therapeutic agents. This need is particularly relevant for a new class of therapeutics known as protein degraders, such as PROTACs that target specific disease-relevant proteins for degradation by the cellular ubiquitin-proteasome system. Using CRISPR technology and the well-established Enzyme Fragment Complementation system, we introduced a small β-galactosidase fragment into the BRD4 and c-Myc loci in physiologically relevant disease cell models. The homogeneous format and high sensitivity of the EFC assay allows for direct and rapid quantitation of drug-induced changes in endogenous BRD4 and c-Myc protein levels. We tested a panel of PROTACs targeting BRD4 in this system and observed differential kinetics for BRD4 and c-Myc degradation with individual PROTACs that were consistent with previous reports using cell proliferation assays. This suggests that discovery of new molecular entities that modulate the endogenous levels of these proteins is feasible using this assay format. We are currently expanding this cell-based platform, SPRINTer™ protein turnover biosensor assays, to additional protein targets and disease cell models where sensitive detection of endogenous protein modulation is critical.

Application of CRISPR Technology to Quantify Endogenous Protein Turnover

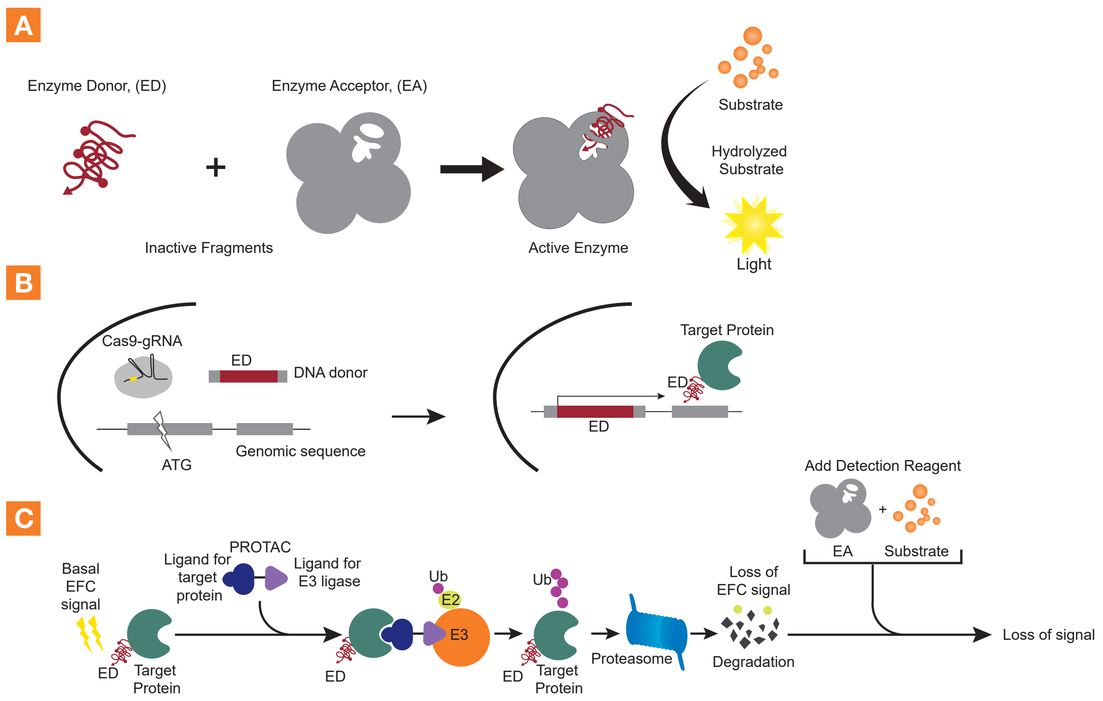


Figure 1. A. Enzyme Fragment Complementation (EFC) is a detection technology based on two recombinant β-galactosidase (β-gal) fragments – enzyme acceptor (EA) and enzyme donor (ED). Separately, the β-gal fragments are inactive, but when combined, they form an active β-gal enzyme that hydrolyzes substrate to produce a chemiluminescent signal. B. A schematic illustration of CRISPR mediated knock-in of ED tag into a target locus to create an ED-fusion protein for EFC assay. Cas9, gRNA and a donor DNA encoding the ED fragment are delivered into cells. Cell clones with robust EFC signal are isolated and optimized for protein turnover assay. C. A graphic representation of EFC detection of target protein degradation induced by PROTACs (trademark of Arvinas). The endogenous ED- target fusion protein (which produces a moderate to high basal EFC signal) is brought into close proximity to the protein degradation machinery by a PROTAC, a bi-functional small molecule that bridges the target protein and a specific endogenous E3 ligase. This proximity leads to the subsequent degradation of the target protein resulting in loss of EFC signal. Hence, the kinetics of protein turnover induced by PROTACs can be quantified using EFC, and is amenable to medium to high throughput screening modalities.

Development of Sensitive SPRINTer Cell Models for Quantitation of Endogenous BRD4 and c-Myc Degradation Induced by PROTACs

A Genetic engineering of BDR4 and its downstream effector c-Myc

B

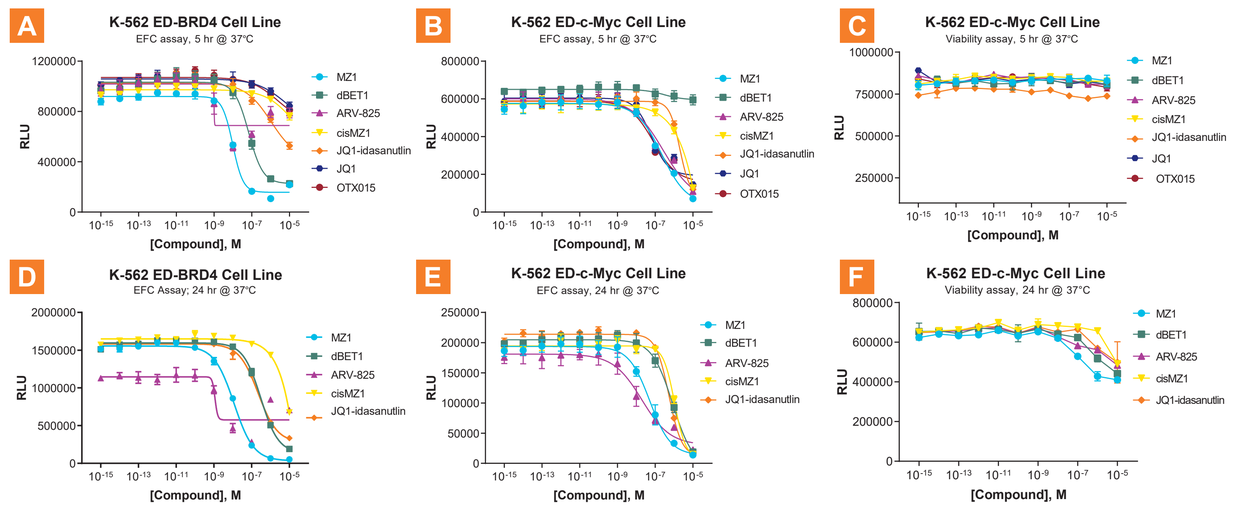
Relative expression of BRD4 and c-Myc in selected cancer cell lines			
Cell Lines	Origins	BRD4	c-Myc
K-562	Chronic myeloid leukemia (CML) cell line	++	+++
HCT116	Colorectal carcinoma cell line	+++	++++

C

BRD4 PROTACs and their E3 ligase targets		
PROTAC	Small molecule (target Brd4)	E3 ligand (target E3 ligase)
MZ1	JQ1	VHL-1 (VHL)
dBET1	JQ1	Thalidomide (cereblon)
JQ1-idasanutlin	JQ1	idasanutlin (MDM2)
ARV825	OTX015	pomalidomide (cereblon)

Figure 2. Sensitive SPRINTer cell models for quantitation of endogenous BRD4 and c-Myc protein degradation induced by PROTACs. A. Genetic engineering of BRD4 and its downstream effector c-Myc. An ED tag was introduced into the N-terminus of each target protein using CRISPR/Cas9 technology. B. For the POC, we wanted to choose a physiologically relevant cell line with good expression of both BRD4 and c-Myc. As shown in this list of cancer cell lines, the K-562 and HCT116 cell lines were initially chosen due to robust relative expression of BRD4 and c-Myc. C. Commercially available BRD4 targeted PROTACs used in this study are shown that are based on two different small molecules (JQ1 or OTX015) and recruit one of 3 different E3 ligases (VHL, cereblon and Mdm2).

Time Course of Endogenous ED-BRD4 and ED-c-Myc Protein Degradation Induced by BRD4-targeted PROTACs



G

Highlights of the kinetics of BRD4 and c-Myc protein degradation induced by MZ1							
	5 hr treatment		24 hr treatment		48 hr treatment		72 hr treatment	
	EC_{50}	S/N	EC_{50}	S/N	EC_{50}	S/N	EC_{50}	S/N
ED-BRD4 cell line	9.7 nM	5.8	4.3 nM	18	1.6 nM	28	1.5 nM	66
ED-c-Myc cell line	222 nM	15	51 nM	14.3	27 nM	32	13 nM	14

Figure 3. SPRINTer K-562 ED-BRD4 cells were treated with BRD4 inhibitors (JQ1 and OTX015), BRD4 PROTACs (MZ1, dBET1, ARV-825 and JQ1-idasanutlin) or control molecule (cisMZ1) for 5 hr A. and 24 hr D. and evaluated by EFC assay. A set of sister plates with identical treatments was also set up for cell viability assay using CellTiter Glo (Promega) (C. and F.). The degradation of c-Myc protein induced by the same treatments of molecules using the K-562 ED-c-Myc cell line are shown in B. and E. EFC assay quantifies PROTAC-mediated BRD4 protein turnover as early as 5 hours A., allowing rank ordering of PROTAC potency and efficacy (ARV825>MZ1>>dBET1>JQ1-idasanutlin). Rank order changes slightly with longer incubations. As previously reported, two small molecule BRD4 inhibitors, JQ1 and OTX015, are much less efficacious at mediating BRD4 degradation than the PROTACs (A. and D.). By contrast BRD4-targeting PROTACs mediated degradation of ED-c-Myc with slightly slower kinetics, producing more distinct profiles after 24 hr incubation (B. and E.). Nevertheless, the K-562 ED-c-Myc cell line provides equivalent rank order to that of K-562 ED-BRD4. These findings suggest the ED-c-Myc cell line may have utility for screening molecules that impact other cellular targets that regulate c-Myc stability. G. A table summarizing the kinetics of BRD4 and c-Myc protein degradation induced by MZ1 treatment. Overall, the ED-BRD4 cell line has an excellent assay window in response to PROTAC treatment.

In conclusion, our EFC-based SPRINTer BRD4 and c-Myc biosensors displayed high sensitivity and more rapid kinetics than the commonly used phenotypic endpoint assay of cell proliferation C. and F., and are simpler to run (and more amenable to high throughput) than traditional Westerns.

Selected PROTACs Displays Distinct Efficacies Among Different Cell Models

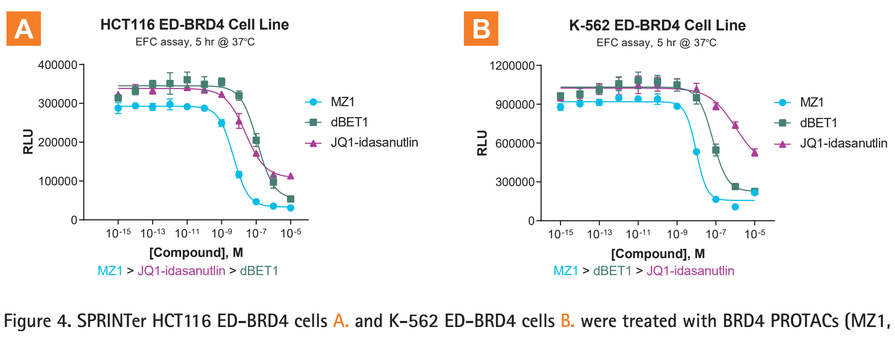


Figure 4. SPRINTer HCT116 ED-BRD4 cells A. and K-562 ED-BRD4 cells B. were treated with BRD4 PROTACs (MZ1, dBET1, and JQ1-idasanutlin) for 5 hr and evaluated by EFC assay. Differences in rank order (and potency) of BRD4-targeted PROTACs that engage different E3 ligases were observed between the two cell models. JQ1-idasanutlin is more efficacious in the HCT-116 cell model. C. Gene expression analysis reveals differential expression levels of MDM2 (target E3 ligase of JQ1-idasanutlin) between HCT116 and K-562 cell models, suggesting the abundance of the target E3 ligase is a key factor that influences the efficacy of JQ1-idasanutlin. VHL is the E3 ligase target for MZ1. Cereblon is the E3 ligase target for dBET1. POLR2A (RNA polymerase II subunit A) is a gene expression control.

Summary

- We have generated an EFC-based assay platform to quantify changes in endogenous protein levels in disease relevant cell models.
- As a proof-of-concept, we have applied this assay platform to detection of drug-induced changes in endogenous BRD4 levels and it's downstream target, c-Myc in a blood cancer cell models.
- The high sensitivity of the SPRINTer EFC-based assay platform allows for the detection of target protein turnover induced by PROTACs with more rapid kinetics than phenotypic endpoint assays, such as cell proliferation.
- These SPRINTer protein turnover biosensors provide a screening platform to identify new molecular entities that modulate oncogenic protein levels for therapeutic development.
- Learn more about the SPRINTer protein turnover biosensor assays by visiting discoverx.com/turnover.