SPRINTer Platform: Detection of Endogenous Protein Turnover and Target Engagement of Targeted Degraders

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Abstract

An assay platform that robustly and sensitively quantifies the kinetics of endogenous protein turnover and target engagement 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 the CRISPR technology and the well-established Enzyme Fragment Complementation (EFC) system, we introduced a small β-galactosidase fragment into the targeted loci, for instance, BRD4, c-Myc and BTK, 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 targeted protein levels. As proof of concept, 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. Then we expanded the utility of this cell-based platform, SPRINTer™ protein turnover biosensor assays, to test on E3 inhibitors that stabilize a targeted protein, CDKN1A (p21), and molecular glues that degrade a specific neo-substrate (IKZF1 of cereblon). Currently, we are adapting this platform for InCELL Pulse™ target engagement assays and our initial results have demonstrated a great promise of its feasibility. Specifically, inhibitors for BRD4 and BTK have shown similar rank orders of potencies between SPRINTer platform and other target engagement platforms. These findings suggest that discovery of new molecular entities that modulate the endogenous levels of targeted proteins is feasible using this assay format.

Application of CRISPR Technology to Quantify Endogenous Protein Turnover

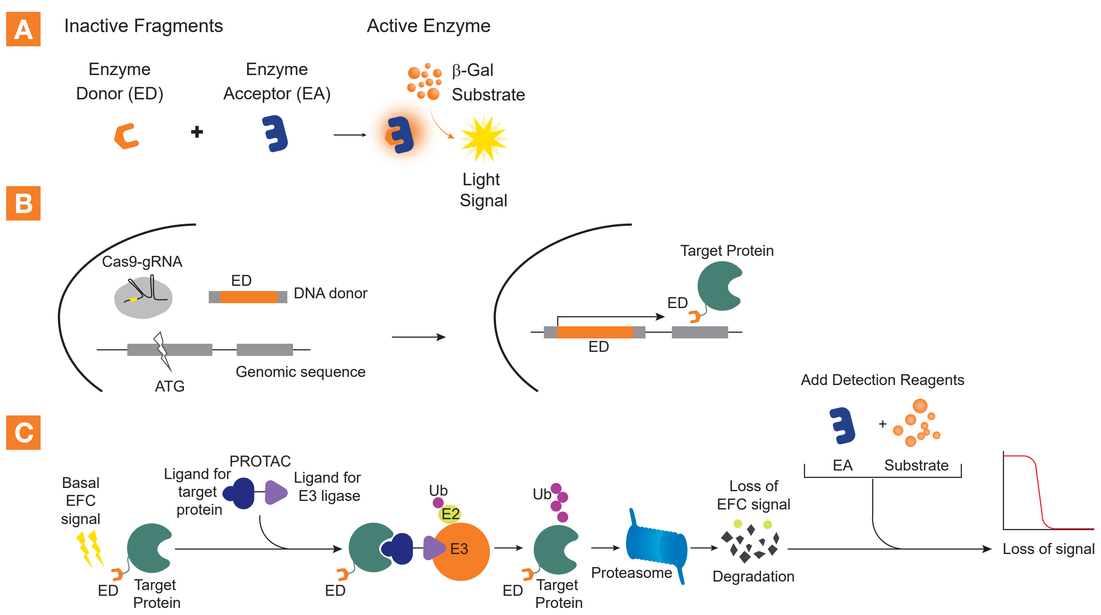
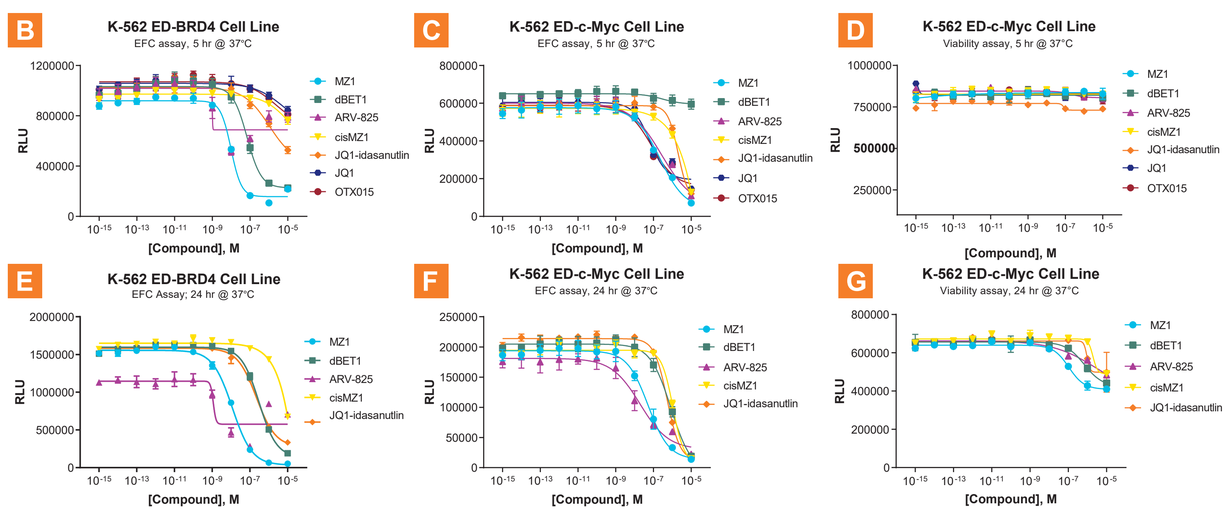


Figure 1. EFC and SPRINTer platform assay principles. A. Enzyme Fragment Complementation (EFC) is a detection technology based on two recombinant β-galactosidase (β-gal) fragments – enzyme acceptor (EA) and enzyme donor (ED). Separately, the β-gal fragments are inactive, but when combined, they form 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.

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

A

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)



H

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 2. Time course of endogenous protein degradation. SPRINTer K-562 ED-BRD4 cells were treated with BRD4 inhibitors (JQ1 and OTX015), BRD4 PROTACs (MZ1, dBET1, JQ1-idasanutlin and ARV-825 shown in A.) or control molecule (cisMZ1) for 5 hr B. and 24 hr E. 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) (D. and G.). 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 C. and F. EFC assay quantifies PROTAC-mediated BRD4 protein turnover as early as 5 hours B., 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 (B. and E.). In contrast, BRD4-targeting PROTACs mediated degradation of ED-c-Myc with slightly slower kinetics, producing more distinct profiles after 24 hr incubation (C. and F.). 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. H. 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 D. and G., and are simpler to run (and more amenable to high throughput) than traditional Westerns.

Selected PROTACs Display Distinct Efficacies Among Different Cell Models

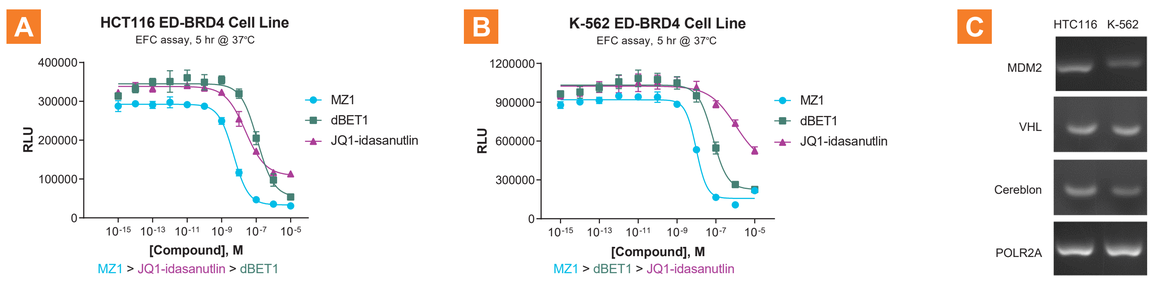


Figure 3. PROTACs display distinct efficacies for different cell models. SPRINTer HCT116 ED-BRD4 cells A. and K-562 ED-BRD4 cells B. were treated with BRD4 PROTACs (MZ1, dBET1, and JQ1-idasanutlin) for 5 hr and evaluated by EFC assay. Differences in rank order (and potency) of BRD4-targeted PROTACs that 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.

The Wide Range of Applications of SPRINTer Cell Lines

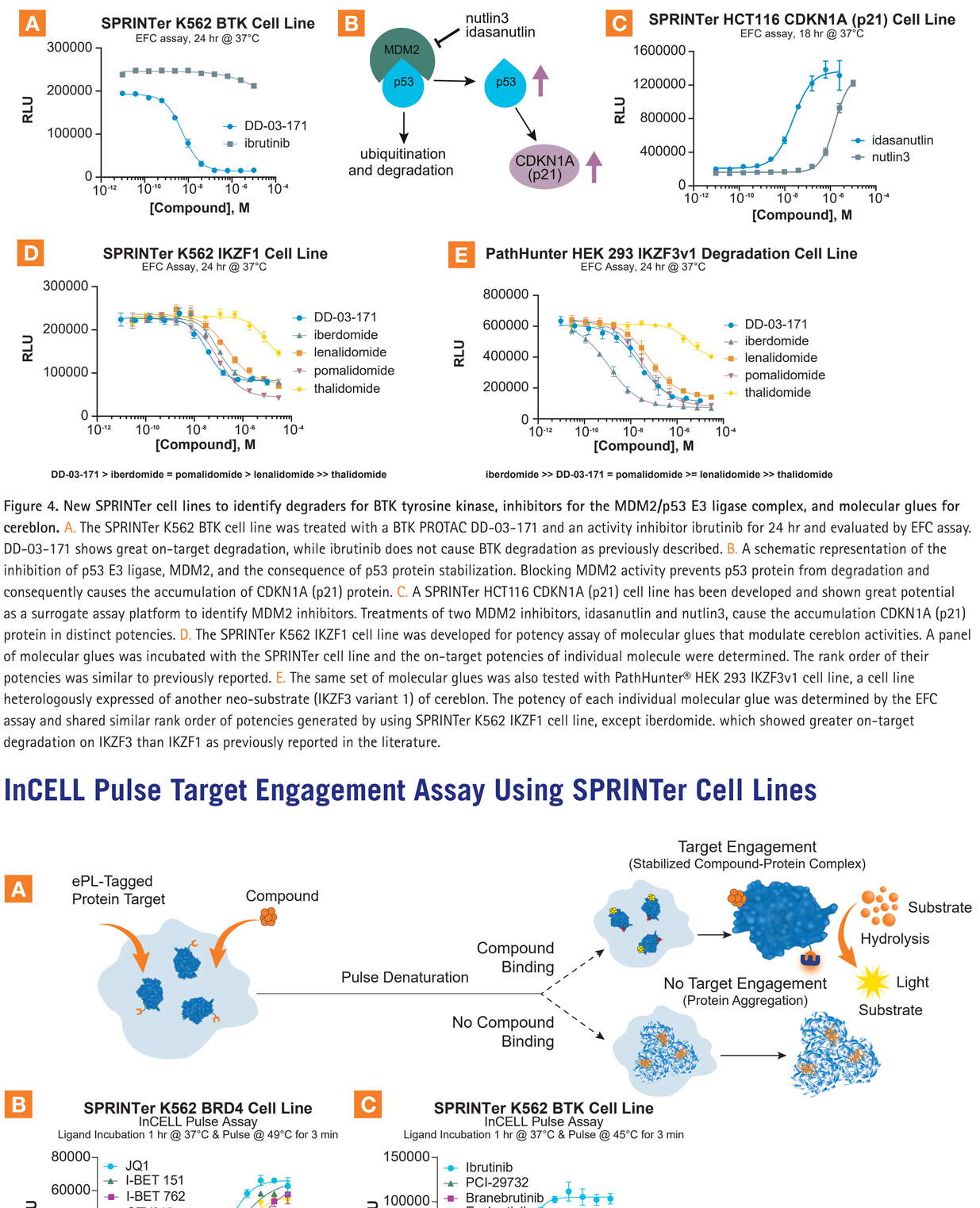


Figure 4. New SPRINTer cell lines to identify degraders for BTK tyrosine kinase, inhibitors for the MDM2/p53 E3 ligase complex, and molecular glues for cereblon. A. The SPRINTer K562 BTK cell line was treated with a BTK PROTAC DD-03-171 and an activity inhibitor ibrutinib for 24 hr and evaluated by EFC assay. DD-03-171 shows great on-target degradation, while ibrutinib does not cause BTK degradation as previously described. B. A schematic representation of the inhibition of p53 E3 ligase, MDM2, and the consequence of p53 protein stabilization. Blocking MDM2 activity prevents p53 protein from degradation and consequently causes the accumulation of CDKN1A (p21) protein. C. A SPRINTer HCT116 CDKN1A (p21) cell line has been developed and shown great potential as a surrogate assay platform to identify MDM2 inhibitors. Treatments of two MDM2 inhibitors, idasanutlin and nutlin3, cause the accumulation CDKN1A (p21) protein in distinct potencies. D. The SPRINTer K562 IKZF1 cell line was developed for potency assay of molecular glues that modulate cereblon activities. A panel of molecular glues was incubated with the SPRINTer cell line and the on-target potencies of individual molecule were determined. The rank order of their potencies was similar to previously reported. E. The same set of molecular glues was also tested with PathHunter® HEK 293 IKZF3v1 cell line, a cell line heterologously expressed of another neo-substrate (IKZF3 variant 1) of cereblon. The potency of each individual molecular glue was determined by the EFC assay and shared similar rank order of potencies generated by using SPRINTer K562 IKZF1 cell line, except iberdomide. which showed greater on-target degradation on IKZF3 than IKZF1 as previously reported in the literature.

InCELL Pulse Target Engagement Assay Using SPRINTer Cell Lines

Figure 5. InCELL Pulse target engagement assay using SPRINTer cell lines. A. A schematic representation of the principle of the InCELL Pulse target engagement assay. Cell lines expressed ED (ePL or ProLabel®) tagged fusion protein target is incubated with a test compound. In the absence of the compound binding (no target engagement), the protein target is denatured by a heat insult, resulting in the loss of EFC signal. In contrast, in the presence of the binding between the compound and the target protein (target engagement), the protein target remains stable upon a heat insult and consequently the EFC signal is detected. Two SPRINTer cell lines, BRD4 (B.) and BTK (C.), are adapted for the InCELL Pulse assay. Two sets of their specific inhibitors were tested as proof-of-concept and the rank orders of the resulting binding potencies were determined by EFC assay.

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 model.
- 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.
- The utilities of SPRINTer protein turnover cell lines have expanded to assays for E3 ligase inhibitors and molecular glues and these cell lines have also been adapted to InCELL Pulse Target Engagement Assay. This assay system provides 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.