Lysineless HiBiT and NanoLuc Tagging Systems as Alternative Tools Monitoring Targeted Protein Degradation

Hanfeng Lin,[a,b] Kristin Riching,[c] May Poh Lai,[d] Dong Lu,[a] Ran Cheng,[a,b] Xiaoli Qi,[a,b] Jin Wang,[a,b,e,*]

a. The Verna and Marrs McLean Department of Biochemistry and Molecular Pharmacology, Baylor College of Medicine, Houston, Texas 77030, United States.

b. Center for NextGen Therapeutics, Baylor College of Medicine, Houston, Texas 77030, United States.

c. Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin 53711, United States.

d. Malvern Panalytical Inc., 2400 Computer Drive, Westborough, Massachusetts 01581, United States.

e. Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030, United States.

* Correspondences can be emailed to wangj@bcm.edu

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Supplementary Materials

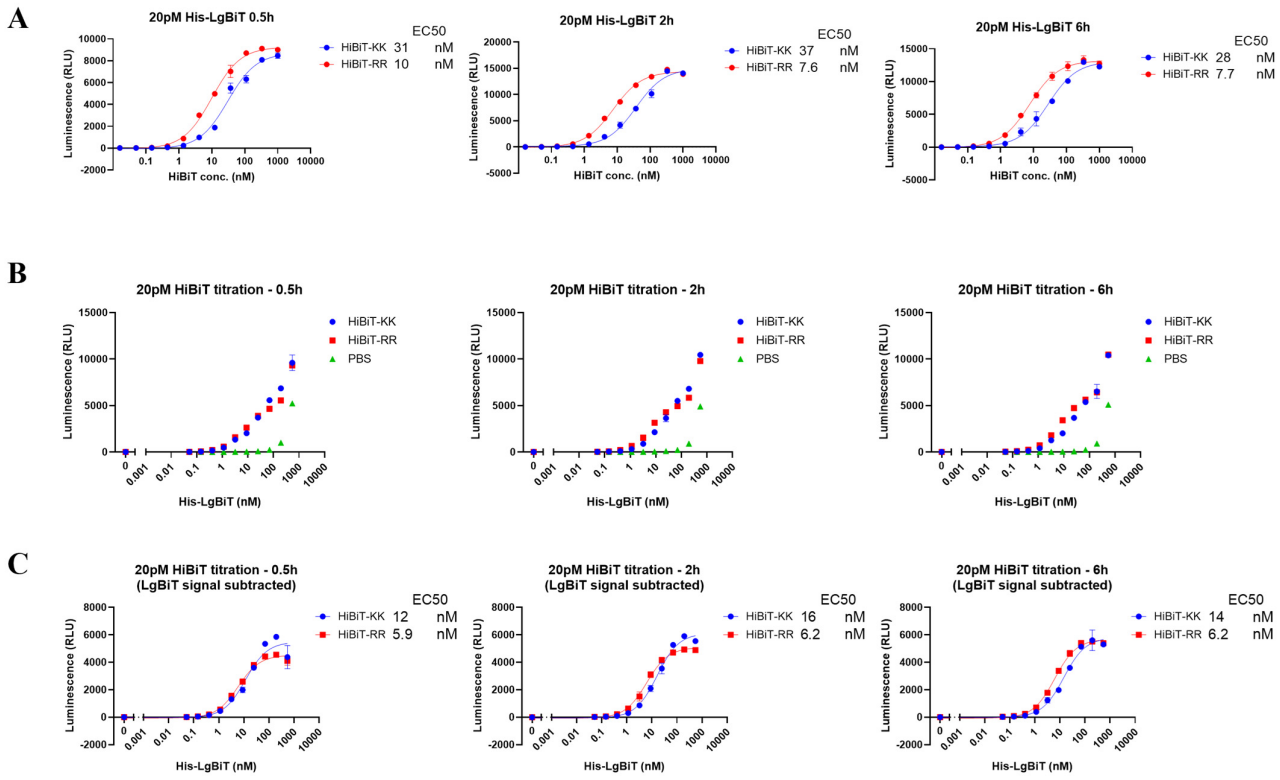


Figure S1. HiBiT-RR showed comparable LgBiT protein binding affinity and similar luminescence output with the original HiBiT-KK.

A. The luminescence signal from 20 pM LgBiT protein when titrating with various concentrations of HiBiT-KK or HiBiT-RR for 0.5h, 2h and 6h. **B.** The luminescence signal from 20 pM HiBiT variants or PBS when titrating with various concentrations of His-LgBiT recombinant protein for 0.5h, 2h and 6h. **C.** The background LgBiT luminescence from PBS group were subtracted from panel B.

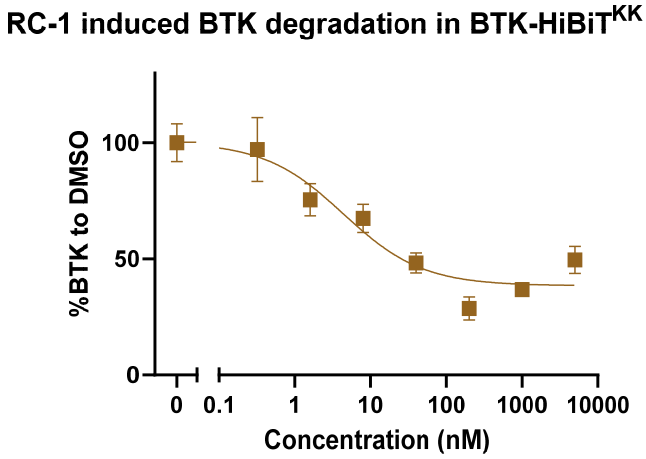


Figure S2. RC-1 degradation potency of BTK in Ramos BTK(full length)-HiBiT^{KK} knock-in cell line.
Cells were transferred into 96-well plate for 20,000 cells per well. Cells were incubated with DMSO or indicated compounds at 0.32, 1.6, 8, 40, 200, 1,000 nM, 5,000 nM in 1% DMSO for 24 hours. Bioluminescence signal was generated by adding furimazine substrate and LgBiT purified protein in the cell lysis buffer.

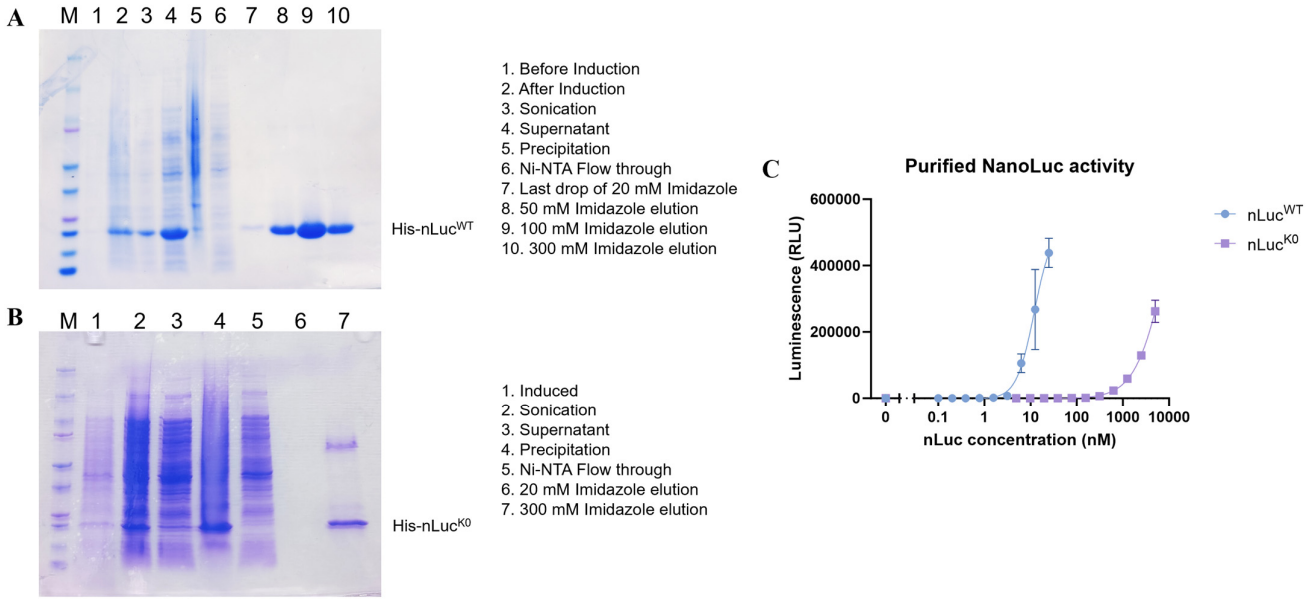
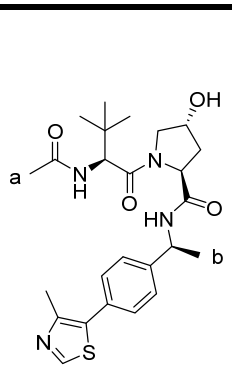


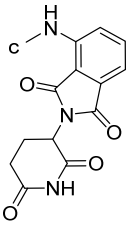
Figure S3. Protein purification and luciferase assay for purified nLucWT and K0.

A, B. Protein purification of His-nLuc^{WT} and His-nLuc^{K0}. **C.** Luciferase assay for purified nLuc WT and K0

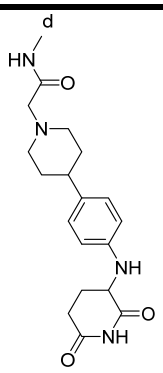
Table S1. RIPK1 degrader common structures



VHL binder



CRBN binder 1



CRBN binder 2

Compound	E3	Exit vector	Linker type
LD4172	VHL binder	a	Flexible
LD4172NC	VHL binder (stereo-center at b flipped)	a	Flexible
LD5037	VHL binder	a	Rigid
LD5042	VHL binder	b	Flexible
LD5097	VHL binder	b	Rigid
LD5063	CRBN binder 1	c	Flexible
LD5077	CRBN binder 2	d	Flexible
LD5084	CRBN binder 2	d	Rigid

Methods

Reagents & Cell culture

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich. HiBiT-KK and RR peptides were synthesized by GenScript, USA. pcDNA3.1 vector backbone was from GenScript, USA. DH5α and BL21(DE3) *E. coli* strains were from Thermo Scientific (Cat. No. EC0111, EC0114).

HEK293T/17 cell line (hereinafter referred as HEK293T) was purchased from American Type Culture Collection (Cat. No. CRL-11268). HEK293T cells were maintained in DMEM (Corning, Cat. No. 10-013-CV) supplemented with 10% fetal bovine serum (FBS) (Corning, Cat. No. 35-011-CV) at 37°C with 5% CO_2. Transfections were carried out using the homemade calcium phosphate method. Generally, for a 10 cm dish, 2 million HEK293T cells in 10ml medium were plated the day before transfection. The desired amount of plasmid was mixed with 75 µL 2 M $CaCl_2$ and 550 µL sterile H_2O, then 625 µL 2× HBSS buffer (50 mM HEPES, 280 mM NaCl, 1.42 mM Na_2HPO_4, pH 7.05) was added and settled for 15min to allow precipitation formation. The mixture was dropwise added into the culture dish and incubated for 15-18 h. The cells were further incubated in fresh medium for 24-36 h to allow gene expression and then ready for assay purpose.

Plasmid cloning & Protein purification

$nLuc^{WT/K0}$-$RIPK1_{KD}$ (kinase domain 1-324) fusion gene with a 24-residue flexible linker in between (SGGRSSGSGSTSGSGTSLYRRVGT) was synthesized and codon optimized by GenScript and cloned into pcDNA3.1 backbone. For BTK_{KD}-$HiBiT^{KK/RR}$ plasmids, the BTK-nLuc template was purchased from Promega (Cat. No. N2441). The gene coding for BTK kinase domain (residue 382-659) was subcloned into pcDNA3.1 vector, followed by the site-directed mutagenesis (ClonExpress II One Step Cloning Kit C112, Vazyme) to fuse the HiBiT-KK or HiBiT-RR gene to the C-terminus of BTK kinase domain with a two-residue linker (VS).

Codon-optimized LgBiT insert was cloned into pET-28a(+) vector using NdeI/BamHI restriction sites to obtain pET28a-His_6-LgBiT plasmid. The plasmid was transformed into BL21(DE3) *E. coli* strain under kanamycin selection pressure. The transformed strain was inoculated into 1 L LB medium and induced by 0.5 mM IPTG when the OD600 value reached 0.8. After shaking the culture overnight at 18 °C, the cells were pelleted and resuspended in the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, pH 7.5) plus protease inhibitor cocktail pills (Roche). The cells were ruptured by sonication on ice for 30min (4s pulse + 8s rest cycle) with 30% amplitude, followed by 18,000×g 45 min centrifugation at 4°C. The supernatant was mixed and gently rotated for 1h with 5ml Ni-NTA beads (Qiagen) pre-equilibrated with lysis buffer. The beads were further washed using 100 ml lysis buffer and bound protein was eluted by elution buffer containing 50 mM Tris-HCl, 150 mM NaCl, 300 mM imidazole, pH 7.5. Protein purity was checked by SDS-PAGE and Coomassie staining. The elution fraction was buffer exchanged into 50 mM Tris-HCl, 150 mM NaCl, pH 7.5, concentrated to 402 uM, aliquoted and snap frozen for long-term storage at -80 °C. His-$nLuc^{WT}$ and His-$nLuc^{K0}$ was purified using the same protocol.

Luminescence-based binding affinity titration

For HiBiT titration against LgBiT, the HiBiT-KK or RR peptide was dissolved in ddH_2O as 1 mM stock solution. LgBiT protein either purified in-house (His-LgBiT) or purchased from Promega (N401A, 20 µM stock) was diluted to 20 pM in PBS pH 7.4 solution and dispensed into white 96 well non-binding surface plate (Corning #3600) with 90 µL/well. HiBiT-KK or RR peptides were 3-fold serial diluted as 10× stock solution and 10 µL was added into each well, giving a HiBiT working concentration ranging from 0.016 nM to 1,000 nM. After equilibrating at room temperature for 30 min, 5 µL 1:25 diluted Nano-Glo luciferase assay substrate (Promega, N113A) in PBS was dispensed into each well and quickly mixed. The luminescence signal was collected using SYNERGY H1 microplate reader (BioTek) with the PMT gain set as 120. EC50 value was calculated by fitting into agonist concentration - response model (Prism 10, GraphPad software, Inc.).

For LgBiT titration against HiBiT-KK or RR, a similar protocol was used except for that 90 µL HiBiT peptides (20

pM) were pre-dispensed into each well, followed by adding 10 µL 3-fold serial diluted LgBiT protein in PBS with a working concentration from 0.016 nM to 1,000 nM. Since high concentration LgBiT protein showed intrinsic catalytic effect even in the absence of HiBiT peptide, we subtracted the baseline luminescence signal of LgBiT-only samples from that of the HiBiT experimental groups and then performed the agonist concentration - response fitting.

Grating-coupled interferometry (GCI) characterization of peptide-protein binding kinetics

Grating-coupled interferometry experiments were conducted on a Creoptix WAVEdelta system. A PCP-NTA (Polycarboxylate Planar functionalized with **Nitriloacetic acid**) chip was used to immobilize His_{6x}-LgBiT. Specifically, the PCP-NTA was conditioned for 3 mins at 10 µl/min with 0.1 M borate/1M NaCl at pH 9.0 (Xantec Elution buffer). The surface was injected for 2 mins at 10 µL/min with 0.5 mM $NiCl_2$. The ligand His-LgBiT was diluted to 50 ug/mL concentration with PBS, then injected directly to the surface at 10 µl/min using target level function. Final surface densities of 3000 pg/mm^2 (high surface density) and 1000 pg/mm^2 (low surface density) His-LgBiT were reached on two flow channels, respectively. Traditional Kinetic experiments were performed using running buffer composed of 0.01 M HEPES, 0.15 M NaCl and 0.05% v/v Surfactant P20, pH 7.4. For Traditional Kinetic experiment, various concentrations of peptides HiBiT-KK and HiBiT-RR were injected over the chip surface at flow rate of 30 µL/min with an association time of 120 s followed by 240 s of dissociation. For data analysis, both reference subtraction and blank subtraction were performed to generate the final sensorgrams. The global fitting for kon and koff was achieved through “association then dissociation” non-linear regression model using Prism GraphPad v10.2.

In vitro protein degradation assay

Two million HEK293T cells were transfected with BTK_{KD}-$HiBiT^{KK/RR}$ or $nLuc^{WT/K0}$-$RIPK1_{KD}$ plasmid in a 10cm dish according to the general transfection protocol as mentioned above. The absolute amount of RIPK1 plasmids used has been optimized through immunoblotting to ensure the expression level does not exceed the endogenous RIPK1 level. The absolute amount of BTK plasmids did not undergo optimization since HEK293T cells do not express BTK at all, but the transfected plasmid amount for BTK_{KD}-$HiBiT^{KK}$ and BTK_{KD}-$HiBiT^{RR}$ was consistent.

After 24-36 hours expression, cells were trypsinized and resuspended in Opti-MEM I phenol red-free medium (Life Technologies, Cat. No. 11058021) supplied with 4% FBS. 20,000 cells in 99 µL medium were plated into each well of the 96 well plates (Corning, #3917). After settling down for 2-4 hours, cells were incubated with serially diluted BTK PROTAC compounds (from 0.064 to 1000 nM, 5-fold dilution, 1% DMSO) or RIPK1 PROTAC compounds (from 1.6 to 1,000 nM, 5-fold dilution, 1% DMSO) for 24 h at 37 °C with 5% CO_2 in an incubator.

For nLuc-based assay, we chose to use live cell-based readout to maximize the luminescence signals. The medium was completely aspirated from each well, followed by adding into each well 25 µL Nano-Glo luciferase assay substrate 1:100 freshly diluted in PBS. Luminescence signals were collected using SYNERGY H1 microplate reader (BioTek) after brief shaking.

For HiBiT-based assay, lysis protocol was used to make HiBiT-tagged protein accessible to LgBiT protein. Briefly, Nano-Glo luciferase assay substrate was 1:100 diluted in nLuc lytic buffer [1] (100 mM MES pH 6.0, 1 mM CDTA, 0.5% (v/v) Tergitol, 0.05% (v/v) Antiform 204, 150 mM KCl, 1

mM DTT, and 35 mM thiourea) containing 200 nM LgBiT protein to make 2× nLuc substrate lytic buffer. 100 µL 2× buffer was subsequently added into each 96-well. After brief shaking and waiting 5 min for complete cell lysis and equilibration, the luminescence signals were collected.

HiBiT-KK and HiBiT-RR BRD4 CRISPR knock-in pool generation:

Single-stranded ultramer DNA oligonucleotides (IDT) containing 80-bp homology to the regions flanking the BRD4 start codon and either the standard HiBiT sequence (Promega) or HiBiT-RR in which the two lysine residues were changed to arginine were used as the ssODN donor templates for CRISPR knock-in in HEK293 cells stably expressing LgBiT as previously described [2]. Briefly, ribonucleoprotein complexes were assembled combining Cas9 protein (IDT) and gRNA (IDT) targeting the BRD4 locus and electroporated with the ssODN templates into 2×10^5 cells. Immediately following electroporation, cells were incubated at ambient temperature for 10 min before transferring to a 12-well plate. Edited pools were analyzed for HiBiT insertion by assaying for luminescence on a GloMax Discover (Promega) 48–72 h post-electroporation.

HiBiT-KK and HiBiT-RR BRD4 kinetic degradation experiments:

BRD4 CRISPR knock-in pools were plated into white, opaque 96-well plates at a density of $2x10^4$ cell/well and allowed to adhere overnight. The following day, media was exchanged with CO2-independent medium (Gibco) containing a 1:100 dilution of Endurazine substrate (Promega). Following a 2.5-hr incubation, a 3-fold dilution series of 1uM MZ1 or dBET6 was added to the plate and immediately placed in a GloMax Discover luminometer pre-equilibrated to 37 degrees. Luminescence was monitored kinetically over a 24-hour period. Analysis of kinetic degradation profiles and quantitation of degradation rate and D_{max} was performed as previously described [2].

Immunoblotting

Transfected HEK293T cells were plated in 12-well plates. After 24h treatment, cells were harvested in 1x RIPA buffer (Thermo Scientific J62524.AE) with 1x Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Cat. No. PI78440) and Benzonase Nuclease (Sigma E1014) to reduce sample viscosity. Lysates were centrifuged at 15,000 × g for 10 min at 4 °C and the supernatant was quantified for total protein concentration using the Pierce BCA Protein Assay (Thermo Fisher Scientific, Cat. No. 23225). Total protein 20 ug were separated by SDS-PAGE and further transferred onto 0.2 um PVDF membrane (Millipore, Cat. No. IPVH00010) pre-soaked with ethanol. The membranes were probed overnight at 4 °C with the specified primary antibodies at the dilution of 1:1000 (Cell Signaling Technology: RIPK1 Rabbit mAb#3493)) or 1:4000 (β-actin Rabbit mAb#4970)), followed by the HRP-conjugated secondary antibody (Kindle Biosciences, #R1006, 1:1000) for 1 h at room temperature. Imaging was performed using ultra digital-ECL substrate solution (Kindle Biosciences, #R1002) in the digital imaging system.

In vitro nLuc enzyme activity assay

His-nLuc^{WT} and His-nLuc^{K0} purified protein were 2-fold serial diluted in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. A total of 99 µL was dispensed in each well of a 96-well plate. 1µL undiluted

Nano-Glo luciferase assay substrate (Promega, N113A) was dispensed into each well and quickly mixed. The luminescence signal was collected using SYNERGY H1 microplate reader (BioTek) with the PMT gain set as 120.

Data analysis

All the experiment data was from at least n=3 technical replicates and cell-based experiment contains at least two batches of biological replicates. Degrader dose-response data was fitted using inhibitor vs response three parameters model, luminescence-based binding affinity titration data was fitted using agonist vs response three parameters model in GraphPad Prism 10.

Safety Statement

No unexpected or unusually high safety hazards were encountered.

Reference

(1) Hall, M. P.; Unch, J.; Binkowski, B. F.; Valley, M. P.; Butler, B. L.; Wood, M. G.; Otto, P.; Zimmerman, K.; Vidugiris, G.; Machleidt, T.; Robers, M. B.; Benink, H. A.; Eggers, C. T.; Slater, M. R.; Meisenheimer, P. L.; Klaubert, D. H.; Fan, F.; Encell, L. P.; Wood, K. V. Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. *ACS Chem. Biol.* **2012**, *7* (11), 1848–1857. https://doi.org/10.1021/cb3002478.

(2) Riching, K. M.; Mahan, S.; Corona, C. R.; McDougall, M.; Vasta, J. D.; Robers, M. B.; Urh, M.; Daniels, D. L. Quantitative Live-Cell Kinetic Degradation and Mechanistic Profiling of PROTAC Mode of Action. *ACS Chem. Biol.* **2018**, *13* (9), 2758–2770. https://doi.org/10.1021/acschembio.8b00692.