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An ultrasensitive system for measuring the USPs and OTULIN activity using Nanoluc as a reporter

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ABSTRACT

The deubiquitinating enzymes (DUBs) are a family of isopeptidases responsible for removing the ubiquitin from the ubiquitinated proteins. Identification of inhibitors for DUBs is emerging as an efficient way for discovering potential medicines for disease treatment. However, the high throughput screening (HTS) assay is still not available for all USPs, especially OTULIN. Here, we described a novel steadily quantifiable DUBs assay platform using Nanoluc (Nluc) as reporter. We further demonstrated that the Ub-Nluc assay could be used for HTS of DUBs inhibitors. Moreover, we generated a sensitive system for OTULIN inhibitors screening using Nluc as a reporter. In summary, our data indicate that Ub-Nluc and the improved Ub-Ub-GS-Nluc assay are efficient systems for measuring activities and screening inhibitors of USPs and OTULIN.

1. Introduction

Ubiquitination, a process of covalent conjugation of ubiquitin to proteins, is one of the most important post-translational modifications. Ubiquitination plays pivotal roles in protein degradation, protein trafficking, transcriptional regulation, immune signaling and oncogenesis [1–4]. Ubiquitination includes monoubiquitination and polyubiquitination. Several kinds of polyubiquitin chains, including M1, K6, K11, K27, K29, K33, K48 and K63, can be formed in cells. Different polyubiquitination may differentially regulate the function of targeted proteins. For example, attachment of Lys48-linked polyubiquitin chain to the proteins usually targets protein for degradation by 26S proteasome [5]. The Lys63-linked polyubiquitin chain regulates cellular functions of the targeted proteins [6]. Linear polyubiquitin chain (head-to-tail) is as an important way to regulate NF-κB pathway [7,8].

Ubiquitination is a reversible process. The removal of ubiquitin is catalyzed by a large group of proteases generically named as deubiquitinating enzymes (DUBs) [9]. At least 95 DUBs have been found in human genome [10]. Based on their structures, the DUBs are divided into five subfamilies, including ubiquitin-specific proteases (USPs), ubiquitin carboxy-terminal hydrolases (UCHs), Machado–Joseph disease protein domain proteases (MJDs), otubain proteases (OTUs) and JAMM motif metallo proteases (JAMM DUBs) [9]. USP is the largest subfamily which can cleave K48 Ub chains-substrate or K63 Ub chains [3,11,12]. Most DUBs show weak or no activity towards linear ubiquitin chains [13]. Yet, only OTULIN was identified as a specific DUB for linear ubiquitin chains [8,14]. Accumulating evidence shows that DUBs play a crucial role in various pathological or physiological functions, such as innate immunity and cancer, are emerging as potential drug targets [11,12,15]. Identification of DUB inhibitors may have significant social benefits.

Various assays have been developed to measure DUBs activity [16–18]. For example, DUBs activity could be simply analyzed by gel electrophoresis for small-scale analysis. Ub–AMC and Z-LRGG-AMC are the most frequently-used high throughput screening (HTS) assay for monitoring the deubiquitinase activity [16,19]. Ub-PLA2 assay is a recently developed assay with high sensitivity for HTS [19]. FRET-based assays have also been developed for HTS of

Abbreviations: Nluc, Nanoluc; AMC, 7-amino-4-methylcoumarin; DTT, dithiothreitol; DMSO, dimethylsulfoxide; HTS, high-throughput screening; USP, ubiquitin-specific protease; GST, glutathione s-transferase; EGFP, enhanced green fluorescence protein; FRET, fluorescence resonance energy transfer; RLU, relative luminance unit; RFU, relative fluorescence unit; CBB, Coomassie brilliant blue; BSA, bovine serum albumin; SD, standard deviation.

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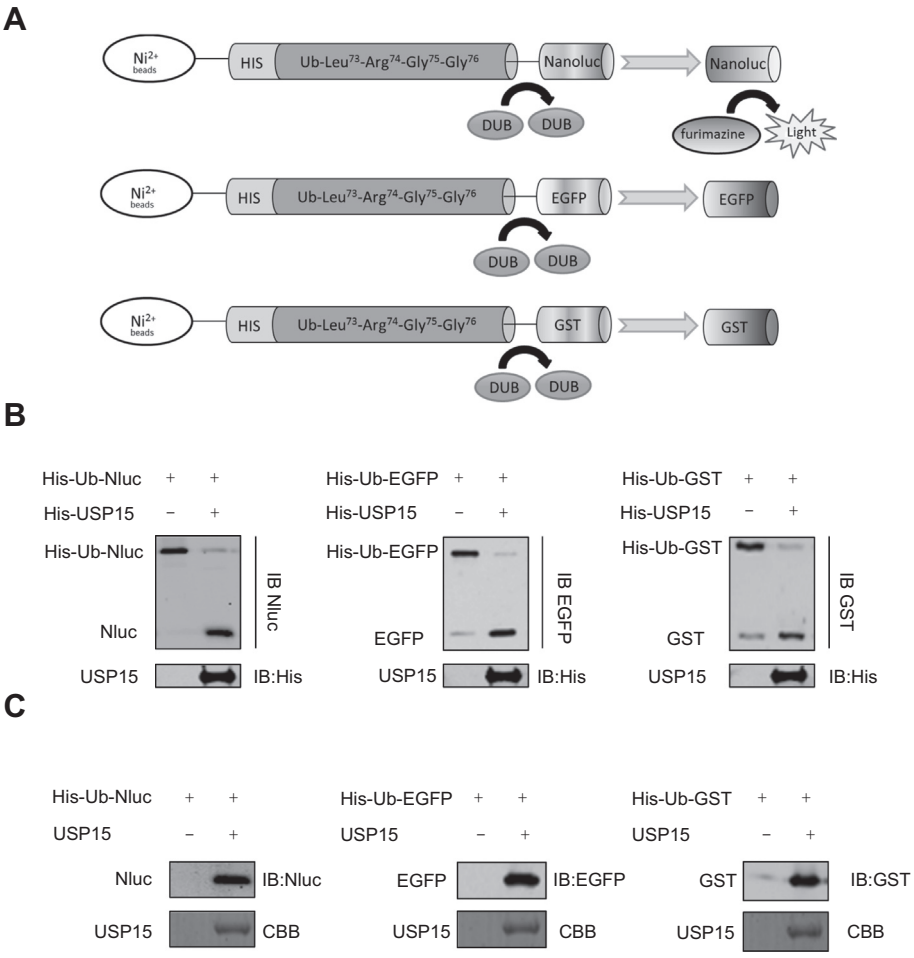

Fig. 1. USPs cleave Ub-reporter in vitro. (A) Schematic diagram shows Ub-reporters construct, specific cleavage by the DUBs at the carboxy-terminal glycine of the Ub releases Nluc reporter, which can turn over its substrate, giving a cold light, EGFP/GST as an indicate reporter. (B) Western blot analysis of 20 ng of Ub-Nluc, Ub-EGFP, and Ub-GST ± 100 ng of USP15. Almost complete cleavage of Ub-reporter is observed at 30 °C in 30 min. As expected reporter proteins lack from the Ub amino-terminal fusion. (C) Western blot analysis of reporter in supernatant which cleaves from Ni^{2+} agarose by USP15. 100 ng His-Ub-Nluc, His-Ub-EGFP, His-Ub-GST ± 1000 ng USP15 for every reaction, then Nluc/EGFP/GST were tested by specific antibody, CBB stains USP15 protein.

DUBs inhibitors [20]. However, the potential drawbacks are associated with these assays. For example, AMC based assay may lead to 20% false ratio in screening [21,22]. Importantly, none of these assays are suitable for efficiently measuring the activity of all DUB, especially OUTLIN. Given the potential shortages, we aimed to develop a highly sensitive assay to study USPs and OTULIN activities.

Nluc luciferase is a recently identified enzyme (19.1 kDa), that catalyzes furimazine into furimamide with carbon dioxide and light in an ATP-independent manner [23]. Importantly, Nluc activity can be easily examined using a luciferase reporter reader. Compared with other luciferases such as Firefly or Renilla luciferase, Nluc has smaller size, higher stability, stronger signal and higher sensitive quantitation with broad linearity [23]. In this study, we developed a highly sensitive assay to study deubiquitinating enzymes activity using Nluc as reporter. Moreover, we developed an efficient HTS assay to monitor the activity of OTULIN.

2. Materials and methods

2.1. Reagents

PR619 (Lifesensors), Ub-AMC (BostonBiochem), furimazine substrate/pNL1.1 plasmid (Promega), NBD-C6-HPC (Invitrogen), 96-well-solid Black/White Microplates (Greiner Bio-One), Ni-NTA agarose (QIAGEN), Coomassie brilliant blue R250 (Amersco), Anti-ubiquitin (P4D1) Santa cruz, Anti-GST/EGFP (Abcam), Nluc antiserum was made using purified GST-Nluc protein.

2.2. Expression and purification of proteins

The Nluc sequence was amplified from PNL1.1 plasmid and cloned into pEGX4T-2 and pET28a. The PLA2 sequence was amplified from mouse cDNA and cloned into pET28a vector; USP15, USP14, OTULIN (amino acids 80–352), USP21 catalytic domain (amino acids 196–565) and SENP1 (amino acids 415–643) were cloned through standard molecular biology techniques and purified from BL21 (DE3) bacteria. All vectors were confirmed by DNA sequencing. His tag was removed by Thrombin (Sigma).

2.3. Ub-AMC assay and Ub-PLA2 assay

Recombinant USP15 protein was incubated with 200 nM Ub-AMC and the activity was measured by using a Lumistar fluorescence plate reader with the excitation and emission filter pair of 355 nm and 460 nm. The experiment was conducted in black-96-well-plate. All dilutions were performed in Ub-AMC buffer (50 mM Tris–Cl, pH 7.5, 10 μg/ml BSA and 2 mM DTT). RFU values in the initial linear range were calculated by the following equation: RFU = fluorescence unit [(USP15 + Ub-AMC) –

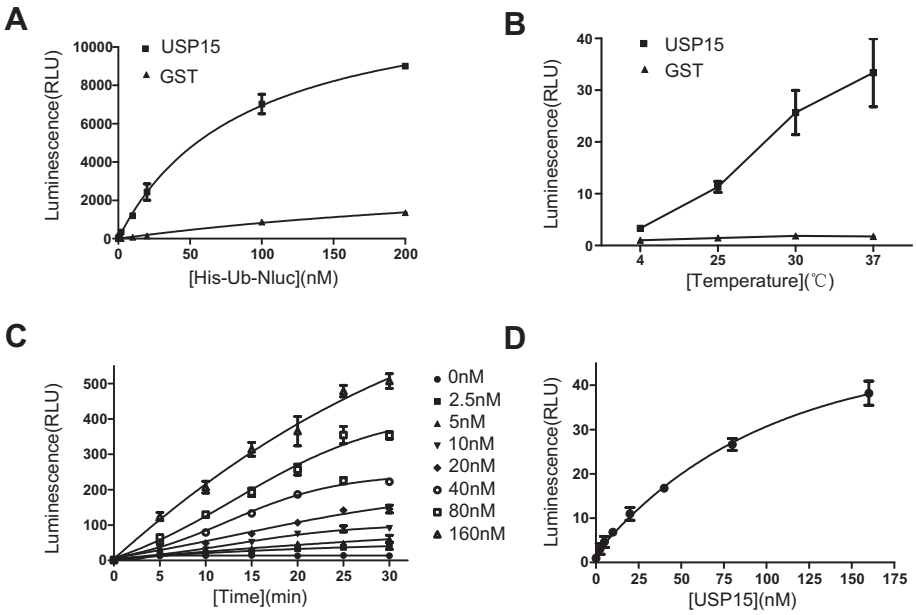

Fig. 2. Optimization for Ub-Nluc assay. (A) The luminescence signal in supernatant is dependent on the USP15. His-Ub-Nluc (0–200 nM) incubated with 40 nM USP15 at 30 °C for 30 min. (B) Temperature dependence of USP15 DUB activity. His-Ub-Nluc (20 nM) was incubated either with or without USP15 (40 nM) at the indicated temperature. (C) Different concentrations of USP15 were incubated with 20 nM Ub-Nluc in a time dose. (D) The data were fit to regression analysis revealed a tight correlation between RLU value and USP15. All data shown are mean ± SD of three wells.

(BSA + Ub-AMC)]/fluorescence unit (BSA + Ub-AMC). Ub-PLA2 assay carried as previously described, recombinant isopeptidase was mixed with Ub-PLA2 and NBD-C6-HPC to final concentrations of 20 nM, 30 nM, and 20 μM in a total volume of 100 μl in a well in a black-walled 96-well-plate. RFU values in the initial linear range were calculated by the following equation: RFU = fluorescence unit [(USP15 + Ub-PLA2) − (BSA + Ub-PLA2)]/fluorescence unit (BSA + Ub-PLA2)]. All dilutions were performed in PLA2 assay buffer (20 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$ and 2 mM β-mercaptoethanol).

2.4. Ub-Nluc and Ub-Ub-Nluc assay

Recombinant isopeptidase were mixed with 20 nM Ub-Nluc coated on 2 μl beads in a total volume of 100 μl in a mircotube for 30 min at 30 °C. After centrifuged in 4000 rpm at 4 °C, 50 μl supernatant was transferred into white-96-well-plate. After diluted Nluc substrate furimazine solution (50 μl/well, 1 μl of furimazine stock solution diluted in 49 μl of reaction) was added, the luminescence was immediately measured by using a Lumistar luciferase plate reader with luciferase emission filters. Ub-Ub-Nluc assay carried out as Ub-Nluc assay except different substrate His-Ub-Ub-Nluc and different enzyme OTULIN.

2.5. Immunoblotting and CBB staining

Proteins were separated by SDS-PAGE in 15% gel and were transferred onto nitrocellulose membranes (Millipore), followed by immunoblotting with antibodies. Immunoblots were analyzed in the Odyssey system (LI-COR Biosciences). 12% gel was stained using Coomassie Blue, destained and scanned in Odyssey system.

2.6. PR619 inhibition assay

10 nM USP15 protein was incubated with PR619 for 30 min at room temperature in a 96-well-plate and an equal volume of 20 nM Ub-Nluc coated beads or 500 nM Ub-AMC was added into 96-well-plate. The fluorescence was monitored using LUMIstar OPTIMA plate reader. The RLU/RFU values in the initial linear range were normalized: reporter alone equaled to 100% inhibition and USP15 + reporter equaled to 0% inhibition. Then data was plotted using GraphPad Prism 5.0. EC50 values were determined by fitting the data to a sigmoidal dose response (variable slope) equation. PR619 inhibited OTULIN activity in Ub-Ub-Nluc assay was tested as described.

2.7. Z′ determination

The reaction proceeded in a 96-well-plate, 48 wells containing 40 nM GST and other wells containing 40 nM USP15 preincubate with 1% DMSO in a volume of 50 μl for 30 min before the addition of 20 nM Ub-Nluc binding agarose beads. RLU was determined 30 min later. The Z′ value was calculated as Zhang's paper [24].

2.8. Statistical analysis

All data were presented as mean ± SD of three parallel wells. Data were analyzed by Student's *t*-test and all tests were considered statistically extremely significant at $p < 0.001$.

3. Results

3.1. Ub-Nluc can be used as a reporter for deubiquitinating activity

To set up an assay for measuring the DUBs activity, we fused Nluc, EGFP or GST to the C terminus of His-tagged ubiquitin respectively (Fig. 1A). Theoretically, the fusion protein can be easily attached to the Ni^{2+} agarose beads and deubiquitinating enzymes can release the reporter from the beads into the supernatant by cleaving the α-peptide linkage. Therefore, the DUB activity can be detected by measuring the activity of the released enzyme or fluorescent proteins in the supernatant.

The fusion protein was expressed and purified in *Escherichia coli* (BL21). The purified proteins were incubated with the purified deubiquitinating enzyme USP15 at 30 °C for 30 min. The cleavage was monitored by Western blotting. As shown in Fig. 1B, USP15

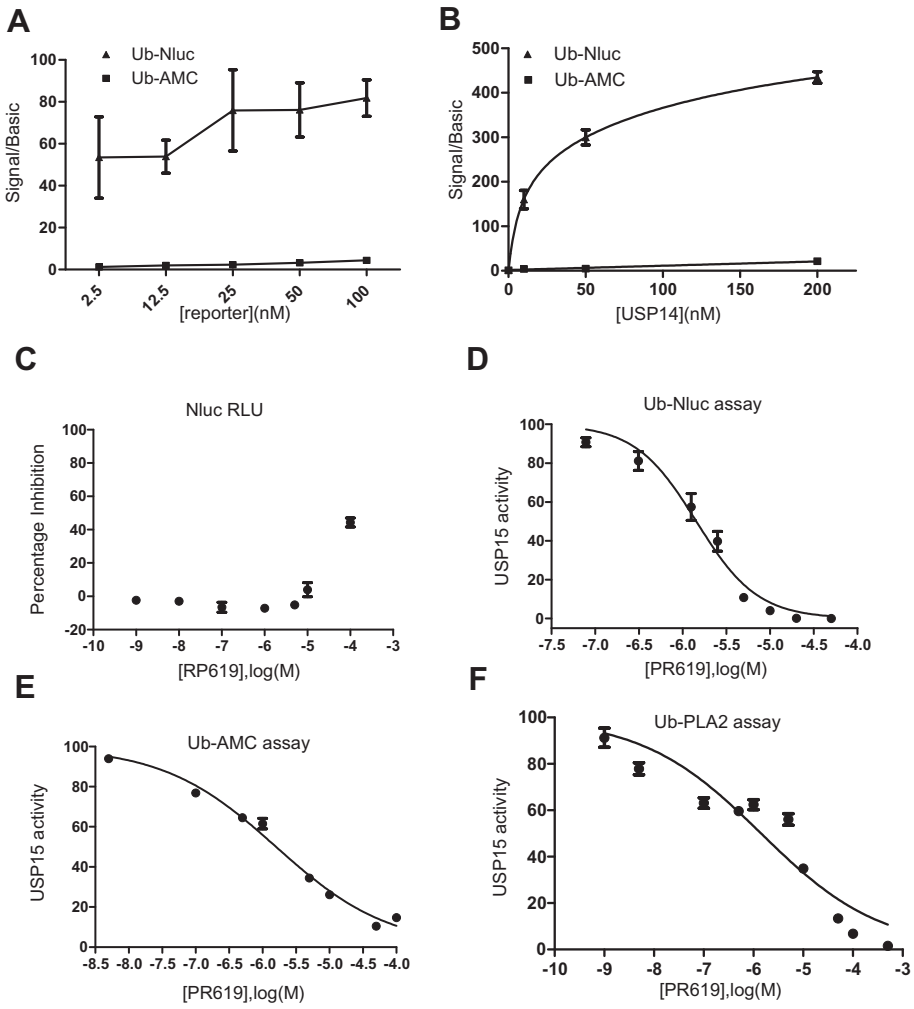

Fig. 3. Ub-Nluc Assay system was compared with other DUB activity monitor system. Ub-Nluc Assay system compares favorably with Ub-AMC and Ub-PLA2. With respect to USP15/USP14, Ub-Nluc is more sensitive than Ub-AMC. (A) 40 nM USP15 was incubated with increasing concentrations (2.5–100 nM) of Ub-Nluc or Ub-AMC. Data are mean ± SD of quadrupled wells. The signal/basic shows a higher ratio in Ub-Nluc assay for USP15. (B) Ub-Nluc is sensitive than Ub-AMC for measuring USP14 activity. Ub-Nluc concentration is 20 nM, and Ub-AMC concentration is 200 nM. Data are mean ± SD of quadrupled well. (C) 20 nM Ub-Nluc was incubated with varying concentrations of PR619 for 30 min. The inhibitory activity of PR619 was determined against free Nluc. (D) 10 nM USP15 was incubated with varying concentrations of PR619 for 30 min before addition of Ub-Nluc. EC50 for Ub-Nluc assay was calculated. EC50 = 1.433 ± 1.09 μM. (E) 10 nM USP15 was incubated with varying concentrations of PR619 for 30 min before addition of Ub-AMC. EC50 for Ub-AMC assay was calculated. EC50 = 1.591 ± 1.08 μM. Data are mean ± SD of quadrupled well. (F) 10 nM USP15 was incubated with varying concentrations of PR619 for 30 min before addition of Ub-PLA2. EC50 for Ub-PLA2 assay was calculated. EC50 = 1.415 ± 1.32 μM. Data are mean ± SD of triplicate wells. EC50 was calculated using GraphPad prism 5.0 by using sigmoidal dose response (variable slope) equation.

almost completely cleaved all the Ub-reporters, indicating that these fusion proteins can be used to monitor deubiquitinating activity. As expected, USP15 also efficiently released the reporter proteins from the Ni^{2+}agarose beads attached with fusion reporters (Fig. 1C). Because Nluc activity is easily monitored and much more sensitive than EGFP or GST, we chose Nluc as a reporter for further study.

3.2. Ub-Nluc assay optimization

To examine whether Ub-Nluc assay can be used for HTS DUB inhibitors, we first optimized the concentration (0–200 nM) of His Ub-Nluc using USP15 as candidate. Our data showed that the signal became saturated when 200 nM Ub-Nluc was used (Fig. 2A). We chose 20 nM of His-Ub-Nluc in the following study as a sensitive signal could be obtained with minimal reagents using this concentration. We also tested the effect of temperature on the USP15 activity in this system. As expected, we found a significant elevation of luminance signal with increasing temperature (Fig. 2B), suggesting that deubiquitinating activity is temperature-sensitive. Our data also showed that both time and concentration of enzyme affected the luminance signal (Fig. 2C and D). Moreover, this system can be used to measure the enzyme activity of other USPs, such as USP14 (Fig. 3B) and USP21 (Fig. S1).

3.3. The Ub-Nluc reporter compares favorably with other reporter systems

Next, we compared the sensitivity of the Ub-Nluc system with other deubiquitinating enzyme system. When equimolar concentrations of Ub-Nluc and Ub-AMC [16] were used, small amount (2–100 nM) of Ub-Nluc produced significantly higher signal-to-background (S/B) ratios than Ub-AMC (Fig. 3A). Consistent with previous study [25], USP14 showed little enzyme activity towards Ub-AMC without proteasome. However, USP14 shows much higher activity towards Ub-Nluc than Ub-AMC (Fig. 3B), suggesting that Ub-Nluc is a sensitive assay for measuring USP14 activity.

Then we examined whether the Ub-Nluc reporter system could be used to identify inhibitor of DUBs. PR619 is a well-characterized DUB inhibitor that can inhibit the activity of most DUBs [26]. Our

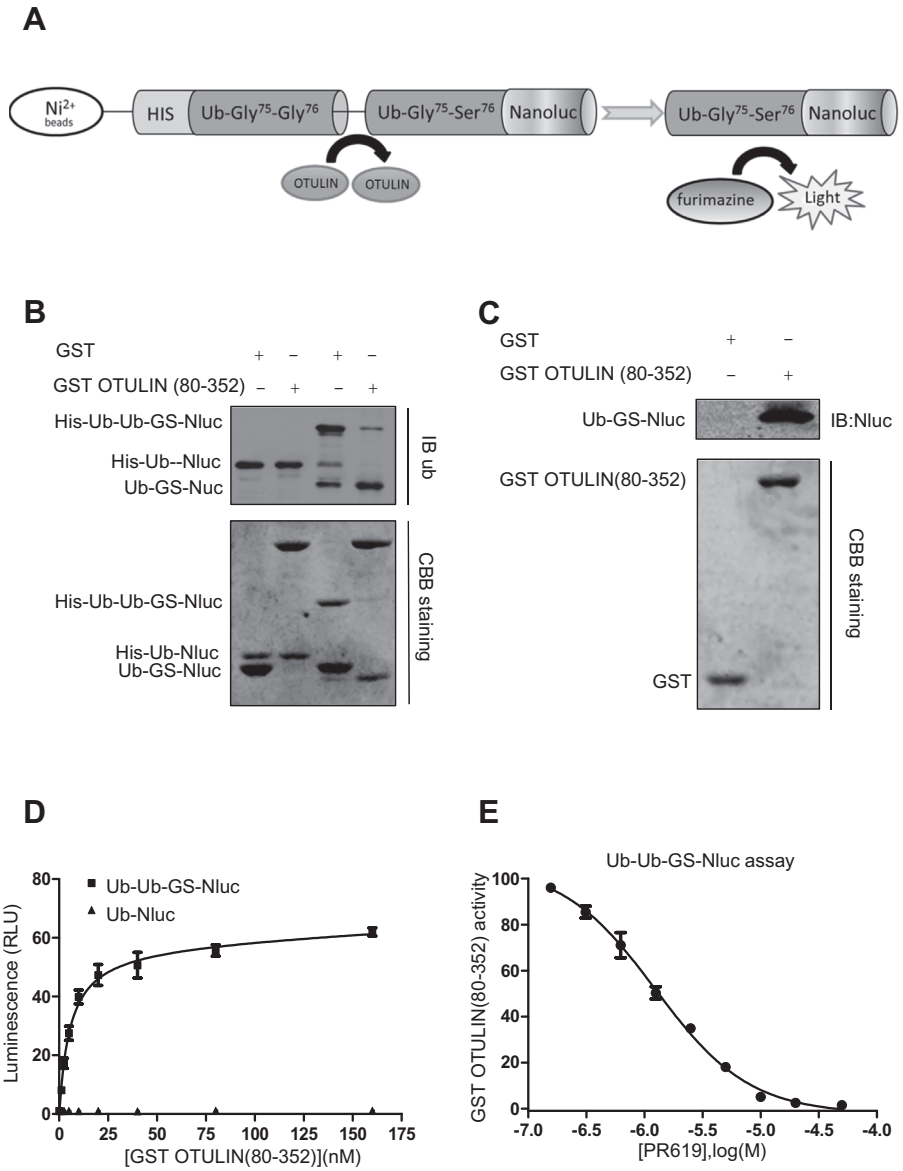

Fig. 4. An improved assay was established for measuring OTULIN activity. (A) Schematic diagram shows Ub-Ub-GS-Nluc construct. (B) Western blotting analysis of OTULIN activity. 500 ng of Ub-Ub-GS-Nluc were incubated with 500 ng of OTULIN (80–352). The cleavage of Ub-GS-Nluc was analyzed. CBB stains GST, GST OTULIN (80–352) protein. (C) Release of Ub-Nluc from Ub-Ub-GS-Nluc bound Ni^{2+} agarose beads by OTULIN. CBB stains test GST, GST OTULIN (80–352) protein in supernatant. (D) GST OUTLIN (80–352) can cleave Ub-Ub-GS-Nluc, but not Ub-Nluc, in a dose dependent manner. (E) PR619 inhibits GST OTULIN (80–352) activity in a dose dependent manner. All data are mean ± SD of triplicate wells. EC50 = 1.237 ± 1.23 μM. EC50 was calculated using GraphPad prism 5.0 by using sigmoidal dose response (variable slope) equation.

data showed that PR619 had little effect on the enzyme activity of Nluc even at 10 μM (Fig. 3C). Results from Ub-Nluc, Ub-AMC, and Ub-PLA2 assay showed comparable EC50 values of PR619 against USP15 (Fig. 3D–F).

We next used USP15 to determine the assay robustness and reproducibility in a HTS format, which is designated by the Z′′ value [24]. We found that Z′ value (0.65 ± 0.04) of Ub-Nluc assay was higher than the Z′ value required for a usable HTS assay (0.5) [24] (Fig. S2). These results indicated that the Ub-Nluc system could be used for testing deubiquitinating activity in HTS manner.

3.4. Specificity of ubiquitin like-Nluc reporter towards different isopeptidase

Except ubiquitin, a number of ubiquitin-like proteins, such as SUMO, NEDD8, and ISG15, can be conjugated to proteins to regulate the functions of their targets in a similar manner as ubiquitin [27]. Moreover, the modification of proteins by these ubiquitin-like proteins can also be reversed by isopeptidase. For example, SUMOylation can be reversed by SENPs [28]. USP21 can cleave Neddylation in vitro and UBP43 can remove ISG15 from substrate [29,30]. Our data indicate that the Nluc reporter system showed high specificity towards the specific isopeptidase (Fig. S3). For example, USP15 showed much higher activity towards Ub-Nluc than SUMO-Nluc, while SENP1 showed higher activity towards mSUMO1-Nluc. These data indicate that Nluc could be used to monitor the activity of both ubiquitin and ubiquitin-like isopeptidases.

3.5. Detection of OTULIN activity using the improved Nluc reporter system

OTULIN was recently identified as a specific DUB that can cleave linear ubiquitin chain. The mainly reported assay for measuring

the OTULIN activity is Western Blotting, which is not suitable for HTS. We measured whether our Ub-Nluc reporter can be used to monitor the OTULIN activity or not. We found that OTULIN showed little activity towards Ub-Nluc reporter system (Fig. 4D). This finding is consistent with the reports that OTULIN activity towards linear polyubiquitin is given by the 'Glu-16' residue in ubiquitin chain [14]. To solve this problem, we generate an Ub-Ub-GS-Nluc reporter as indicated (Fig. 4A–C) and the activity of OTULIN was measured. As shown in Fig. 4D, OTULIN, a C65 type peptidase shows high activity towards Ub-Ub-GS-Nluc. Moreover, our data showed that PR619 could significantly inhibit the OTULIN activity (Fig. 4E). Together, our data showed that Ub-Ub-GS-Nluc assay could be used to detect the OTULIN activity with high sensitivity.

4. Discussion

In this study, we generated a highly sensitive HTS system to monitor deubiquitinating enzyme activity using Nluc as reporter. Most DUBs could hydrolyze Ub linked conjugations with α-peptide linkage similar capacity like ε-peptide linkage. Thus, we fused the Nluc, a luciferase with higher enzyme activity than other luciferase [23], to the C terminus of ubiquitin. Our data showed that the fused Nluc could be efficiently released from ubiquitin by deubiquitining enzymes, under the circumstances in which Nluc activity was not affected. Thus, Ub-Nluc is a highly sensitive system for measuring the activity of deubiquitining enzymes, especially USPs efficiently.

Except Ub-Nluc, we also fused Nluc to the C terminus of mSUMO1. As expected, mSUMO1-Nluc reporter can be efficiently cleaved by isopeptidase SENP1, a specific enzyme of deSUMOylation. Together, our data indicated that Nluc is a suitable reporter that can measure the activity of both ubiquitin and ubiquitin-like isopeptidases.

Although Ub-AMC is the most widely used assay to screen inhibitors for deubiquitining enzyme, it has several potential defect [21,22]. Our data showed that Ub-Nluc assay may overcome the shortage of Ub-AMC assay. By Using PR619, we could obtain a comparable EC50 for the inhibition of USP15 detected by Ub-Nluc, Ub-AMC and Ub-PLA2 (Fig. 4). Moreover, the average Z′-score of HTS platform for Ub-Nluc assay was higher than the required Z′-score for HTS. To sum up, we conclude that the Ub-Nluc assay is reproducible, robust, and reliable assay and thus is pot high throughput compatible assay, and the improved Ub-Ub-GS-Nluc reporter assay is the first reported HTS assay to monitor OTULIN activity.

In summary, the Ub-Nluc reporter assay and the improved Ub-Ub-GS-Nluc reporter assay are robust and sensitive systems for testing USPs and OUTLIN activity in vitro.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.139.

References

[1] K. Wickliffe, A. Williamson, L. Jin, M. Rape, The multiple layers of ubiquitin-dependent cell cycle control, Chem. Rev. 109 (2009) 1537–1548.
[2] N. Liu et al., The Fbw7/human CDC4 tumor suppressor targets proproliferative factor KLF5 for ubiquitination and degradation through multiple phosphodegron motifs, J. Biol. Chem. 285 (2010) 18858–18867.
[3] X. He, Y. Li, C. Li, L.J. Liu, X.D. Zhang, Y. Liu, H.B. Shu, USP2a negatively regulates IL-1beta- and virus-induced NF-κB activation by deubiquitinating TRAF6, J. Mol. Cell Biol. 5 (2013) 39–47.
[4] D. Chauhan et al., A small molecule inhibitor of ubiquitin-specific protease-7 induces apoptosis in multiple myeloma cells and overcomes bortezomib resistance, Cancer Cell 22 (2012) 345–358.
[5] V. Chau, J.W. Tobias, A. Bachmair, D. Marriott, D.J. Ecker, D.K. Gonda, A. Varshavsky, A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein, Science 243 (1989) 1576–1583.
[6] L. Deng et al., Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain, Cell 103 (2000) 351–361.
[7] F. Tokunaga et al., Involvement of linear polyubiquitylation of NEMO in NF-κB activation, Nat. Cell Biol. 11 (2009) 123–132.
[8] F. Ikeda et al., SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis, Nature 471 (2011) 637–641.
[9] A.Y. Amerik, M. Hochstrasser, Mechanism and function of deubiquitinating enzymes, Biochim. Biophys. Acta 1695 (2004) 189–207.
[10] S.M. Nijman, M.P. Luna-Vargas, A. Velds, T.R. Brummelkamp, A.M. Dirac, T.K. Sixma, R. Bernards, A genomic and functional inventory of deubiquitinating enzymes, Cell 123 (2005) 773–786.
[11] Y. Fan et al., USP21 negatively regulates antiviral response by acting as a RIG-I deubiquitinase, J. Exp. Med. 211 (2014) 313–328.
[12] E.K. Pauli, Y.K. Chan, M.E. Davis, S. Gableske, M.K. Wang, K.F. Feister, M.U. Gack, The ubiquitin-specific protease USP15 promotes RIG-I-mediated antiviral signaling by deubiquitylating TRIM25, Sci. Signal. 7 (2014). ra3.
[13] A.C. Faesen et al., The differential modulation of USP activity by internal regulatory domains, interactors and eight ubiquitin chain types, Chem. Biol. 18 (2011) 1550–1561.
[14] K. Keusekotten et al., OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin, Cell 153 (2013) 1312–1326.
[15] P.J. Eichhorn et al., USP15 stabilizes TGF-beta receptor I and promotes oncogenesis through the activation of TGF-beta signaling in glioblastoma, Nat. Med. 18 (2012) 429–435.
[16] C.N. Larsen, B.A. Krantz, K.D. Wilkinson, Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases, Biochemistry 37 (1998) 3358–3368.
[17] R.T. Baker, S.A. Smith, R. Marano, J. McKee, P.G. Board, Protein expression using cotranslational fusion and cleavage of ubiquitin. Mutagenesis of the glutathione-binding site of human Pi class glutathione S-transferase, J. Biol. Chem. 269 (1994) 25381–25386.
[18] J.I. Lee, S.K. Woo, K.I. Kim, K.C. Park, S.H. Baek, Y.J. Yoo, C.H. Chung, A method for assaying deubiquitinating enzymes, Biol. Proced. Online 1 (1998) 92–99.
[19] B. Nicholson et al., Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities, Protein Sci. 17 (2008) 1035–1043.
[20] R.A. Horton, E.A. Strachan, K.W. Vogel, S.M. Riddle, A substrate for deubiquitinating enzymes based on time-resolved fluorescence resonance energy transfer between terbium and yellow fluorescent protein, Anal. Biochem. 360 (2007) 138–143.
[21] A. Tirat et al., Synthesis and characterization of fluorescent ubiquitin derivatives as highly sensitive substrates for the deubiquitinating enzymes UCH-L3 and USP-2, Anal. Biochem. 343 (2005) 244–255.
[22] Y. Liu et al., Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line, Chem. Biol. 10 (2003) 837–846.
[23] M.P. Hall et al., Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate, ACS Chem. Biol. 7 (2012) 1848–1857.
[24] J.H. Zhang, T.D. Chung, K.R. Oldenburg, A Simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen. 4 (1999) 67–73.
[25] B.H. Lee et al., Enhancement of proteasome activity by a small-molecule inhibitor of USP14, Nature 467 (2010) 179–184.
[26] M. Altun et al., Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes, Chem. Biol. 18 (2011) 1401–1412.
[27] R.L. Welchman, C. Gordon, R.J. Mayer, Ubiquitin and ubiquitin-like proteins as multifunctional signals, Nat. Rev. Mol. Cell Biol. 6 (2005) 599–609.
[28] D. Bailey, P. O'Hare, Characterization of the localization and proteolytic activity of the SUMO-specific protease, SENP1, J. Biol. Chem. 279 (2004) 692–703.
[29] M.P. Malakhov, O.A. Malakhova, K.I. Kim, K.J. Ritchie, D.E. Zhang, UBP43 (USP18) specifically removes ISG15 from conjugated proteins, J. Biol. Chem. 277 (2002) 9976–9981.
[30] L. Gong, T. Kamitani, S. Millas, E.T. Yeh, Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins, J. Biol. Chem. 275 (2000) 14212–14216.