Methods to Detect Small Molecule Inhibition of RING E3 Ligase Activity

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Protein ubiquitination is an essential post-translational modification that regulates a large number of cellular processes. This reaction is facilitated by the consecutive action of three central enzymes, i.e., E1 activating enzyme, E2 conjugating enzyme, and the E3 ligase. More than 600 E3 enzymes guarantee the specificity and selectivity of these reactions and thus represent an exciting, while highly underrepresented, class of drug targets. Specific methods can be employed to monitor their activity and thus query compound libraries for inhibitory small molecules. Here, we describe two protocols—one high-throughput and one low-throughput method—to detect E3 ligase activity and test small molecule modulation. © 2022 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: AlphaScreen assay to measure TRAF6-Ubc13 interaction
Basic Protocol 2: Gel-based *in vitro* ubiquitination assay (K63-linked chains)

Keywords: alpha screen • RING E3 ligase • small molecules • TRAF6 • Ubc13 • ubiquitination

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INTRODUCTION

Ubiquitination of proteins is a reversible post-translational modification that regulates the function of essential regulators of almost all cellular signaling pathways including protein homeostasis, cell death, transcription, cell cycle, trafficking, DNA damage response, and many more. The enzyme cascade consisting of an ubiquitin-activating E1 enzyme (Schulman & Harper, 2009), an ubiquitin-conjugating E2 enzyme (Ye & Rape, 2009), and an ubiquitin-ligating E3 enzyme is needed to covalently attach ubiquitin to the substrate proteins. This process is reversible through the action of de-ubiquitinating enzymes (DUBs; Fig. 1A). The E3 ligases contain domains that determine the substrate specificity and/or the localization to specific sites in the cell and mediate the transfer of ubiquitin from the E2 to the specific protein substrate. E3 ligases are the largest and most heterogeneous components in the ubiquitination reaction and are divided according to the catalytic structure into three subfamilies: Really interesting new gene (RING) E3 ligases, HECT E3 ligases, and RBR E3 ligases (Buetow & Huang,

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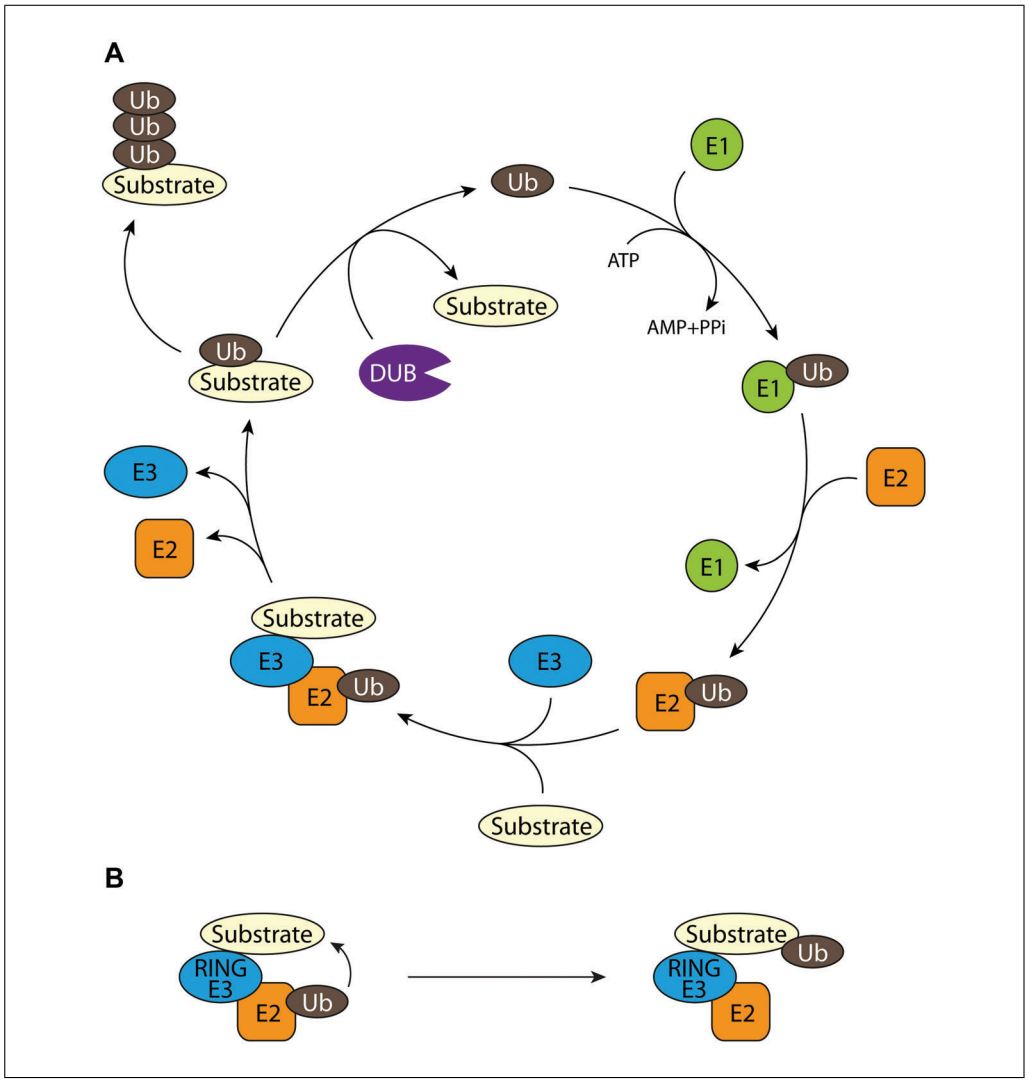

Figure 1. (**A**) Ubiquitination cycle involving E1, E2, and E3 enzymes. The process is reversible as ubiquitination can be hydrolyzed by deubiquitinases (DUBs). (**B**) Substrate ubiquitination mediated by RING E3 ligases: The transfer of the ubiquitin (Ub) occurs directly from the E2 enzyme to the respective substrate, with the E3 ligase being a scaffold that brings the E2 and substrate in close proximity to maintain specificity of the reaction.

2016; Li et al., 2008). The RING E3 ligases are characterized by the zinc-binding RING domain. Two groups of RING E3 ligases exist: Mono-subunit enzymes (e.g., TRAF6) and the multi-subunit RING ligase complexes (cullin-RING ligases, CRL). The RING domain mediates the binding of the ubiquitin-charged E2 and stimulates the direct ubiquitin transfer from E2 to the substrate (Fig. 1B). Here, the function of the E3 ligase is to bring the E2 protein in close proximity to its substrate protein and thereby guarantee substrate specificity. In contrast, HECT and RBR E3 ligases comprise an internal cysteine to first take up the ubiquitin and subsequently transfer it to the substrate.

Dysregulation of the ubiquitin system leads to a variety of diseases, including cancer, autoimmune diseases, and neurodegeneration (Popovic, Vucic, & Dikic, 2014). As the ubiquitination via RING E3 ligases critically requires the protein-protein interaction (PPI) with the E2 enzymes, these types of interactions can be utilized to identify small molecule disruptors. So far there are only a few FDA-approved drugs targeting the ubiquitin system and these are mainly modulating the proteasome. As each of the ~600 E3 ligases regulates a distinct set of targets, these enzymes are getting more attention as valuable drug

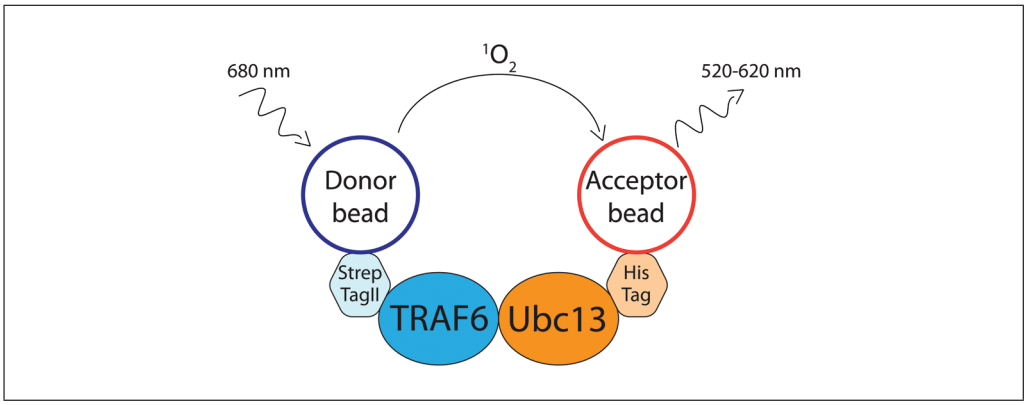

Figure 2. AlphaScreen technology. Assay principle of the AlphaScreen binding assay.

targets with advanced selectivity and potentially fewer side effects compared to, e.g., proteasome inhibitors. A possible mode of action for small molecules that inhibit E3 ligases is the inhibition of the E3 ligase interaction with its respective E2 conjugating enzyme (Brenke et al., 2018; Scott et al., 2017).

TRAF6 belongs to the RING-type E3 ligases and does not contain an internal cysteine (Yin et al., 2009). Hence, the ubiquitin transfer occurs, upon E2 enzyme (Ubc13 in the case of TRAF6) binding, directly from Ubc13 to the substrate. This is conceptually visualized in Figure 1B. Importantly, as Ubc13 binds to the RING domain, compounds inhibiting TRAF6-Ubc13 binding do not interfere with the TRAF6-substrate binding. The affinity of TRAF6 (RING-Z1)-Ubc13 binding is ~1.5 μM (Yin et al., 2009); thus, this PPI is favorable for compound interference even at mid-nM concentrations. In line with this, targeting TRAF6-Ubc13 interaction has yielded effective inhibitors with good selectivity across a variety of tested E3 ligases and *in vivo* activity (Brenke et al., 2018). TRAF6-mediated ubiquitination processes are dependent on activation of receptor signaling processes (e.g., IL-1β or Toll-like receptors) and in turn lead to downstream activation of the NF-κB or MAP kinase pathways (Walsh, Lee, & Choi, 2015).

Here, we describe two protocols that can be used to detect small molecule inhibition of RING E3 ligase activity, exemplified by the inhibition of the E3 ligase TRAF6. The fundamental difference between the two protocols is their throughput capacity.

The first protocol is a high-throughput amendable method—AlphaScreen (amplified luminescent proximity homogeneous assay)—that can be utilized to search for PPI inhibitors (Basic Protocol 1, see Fig. 2). This technology was adapted to measure the TRAF6-Ubc13 PPI and has already been successfully used in a screening campaign for the discovery of a TRAF6 E3 ligase inhibitor (Fig. 3; Brenke et al., 2018).

The second tool to identify RING E3 ligase inhibitors is an *in vitro* ubiquitination assay (Basic Protocol 2), here again described to monitor TRAF6 activity. In a biochemical enzyme reaction with addition of E1 and E2 (Ubc13) enzymes, ubiquitin (Ub), and ATP, the ability of TRAF6 to form ubiquitin chains as well as auto-ubiquitination is analyzed. This assay is suitable to validate identified small molecule inhibitors in a low-throughput, western blot format and has also been demonstrated to be effective and accurate (Fig. 4; Brenke et al., 2018).

ALPHASCREEN ASSAY TO MEASURE TRAF6-UBC13 INTERACTION

This protocol is used to identify small molecule inhibitors of TRAF6-Ubc13 interaction. The assay is based on AlphaScreen, which is a bead-based assay for studying biomolecular interactions in a microplate format. Here, Strep-Tactin donor beads bind to

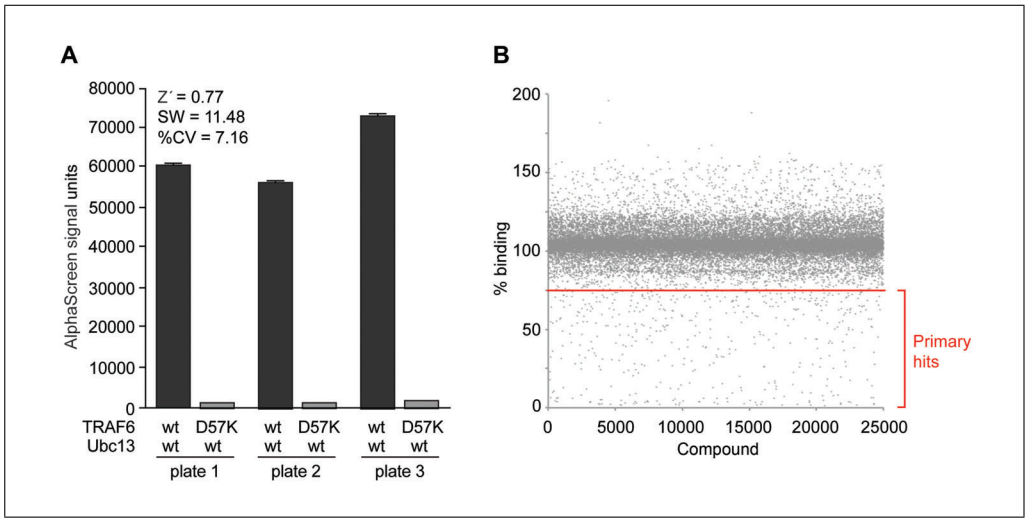

Figure 3. (**A**) AlphaScreen assay using TRAF6 wt or TRAF6 D57K mutant proteins each in combination with Ubc13 wild type (WT). Assay was tested on three different plates to evaluate plate-to-plate variations. Statistical parameters—Z′, signal window (SW), and coefficient of variation (%CV)—are depicted. (**B**) AlphaScreen was used to quantify inhibition by small molecules in a high-throughput screen (HTS). Data and graphs in A and B are from Brenke et al. (2018).

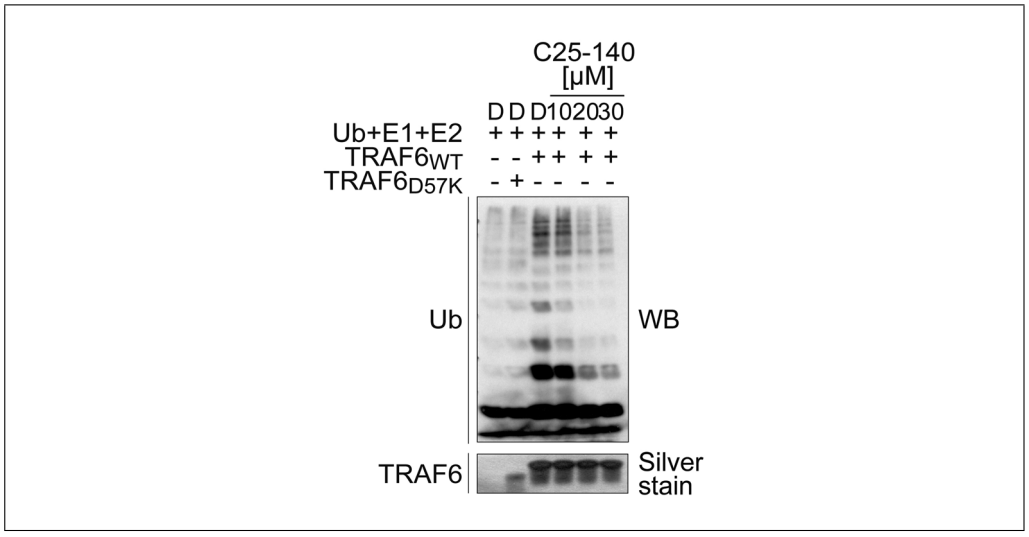

Figure 4. *In vitro* ubiquitination assay. Western-blotting based analysis of protein ubiquitination in a biochemical setting. ATP is present in all conditions. This assay is used to detect the inhibitory potential of small molecule inhibitors. Data and graph from Brenke et al. (2018). WB, western blot.

StrepII-TRAF6-RZ1 (RZ1 domain; present as dimer) and nickel-chelate acceptor beads bind to Ubc13-FlagHis. Upon interaction of the two protein partners, beads get into close proximity and singlet oxygen is transferred from donor to acceptor beads generating a luminescent signal that can be detected in a suitable microplate reader (Fig. 2). Small molecule inhibitors interrupting this interaction diminish the AlphaScreen signal. We describe the reaction conditions that were used in a screening campaign to identify small molecule inhibitors of TRAF6-Ubc13 (Brenke et al., 2018). Reaction conditions for other E3 RING ligases can be altered accordingly to meet specific requirements. Besides TRAF6-Ubc13, AlphaScreen has also been shown to be suitable to detect, e.g., RNF8-Ubc13 interaction (Weber, Rothenaigner, Brandner, Hadian, & Schorpp, 2017).

Materials

Recombinant StrepII-TRAF6-RZ1 protein, only RING-zinc finger1 (RZ1) domain: Wild-type (WT) or D57K mutant (Brenke et al., 2018)

Recombinant Ubc13-FlagHis protein
AlphaScreen buffer (see recipe)
Strep-Tactin Alpha Donor beads (AS106, PerkinElmer)
AlphaScreen Histidine (Nickel Chelate) Detection Kit containing nickel chelate (Ni-NTA) AlphaScreen Acceptor beads (6760619C, Perkin Elmer)
Small molecules in DMSO (specific to the research question)
Dimethyl sulfoxide (DMSO of high purity)

384-well white OptiPlates (6007290, Perkin Elmer)
Multiplate Dispenser (e.g., Flexdrop or Multidrop)
Liquid Handling (e.g., Sciclone G3, PerkinElmer)
Cover foil (to seal plate; DMSO resistant)
Plate reader (e.g., PerkinElmer Envision)

NOTE: The TRAF6 D57K mutant does not bind Ubc13 and acts as a control for the low signal. This mutant is a mimic of full compound inhibition.

1. Purify recombinant proteins in bacterial systems: StrepII-TRAF6-RZ1 (WT or D57K mutant) and Ubc13-FlagHis.

   Proteins are expressed in bacterial systems and purified using StrepTagII or HisTag affinity chromatography followed by gel filtration. More detailed protocols for protein purification are published in Brenke et al. (2018).

2. Prepare protein stocks: (1) StrepII-TRAF6-RZ1 (WT or D57K mutant) at 200 nM in AlphaScreen buffer and (2) Ubc13-FlagHis at 450 nM in AlphaScreen buffer. For master mixes, calculate 30 µl per sample for StrepII-TRAF6-RZ1 (WT or D57K mutant) and 10 µl per sample for Ubc13-FlagHis.

   The D57K, a binding mutant, will provide the negative control (low) signal. If no binding mutant is available, either TRAF6 alone with both beads or the two beads alone can be used as negative controls. For preparation of AlphaScreen buffer use protease free, fatty acid free, essentially globulin free BSA with purity >98%. In set-up experiments, cross-titrations of TRAF6 and Ubc13 revealed the optimal protein concentrations (robust signal at minimal protein concentration). Such a matrix titration experiment needs to be performed for any other E2-E3 pair. Place the diluted proteins on ice.

3. Dispense 30 µl StrepII-TRAF6-RZ1 (WT or D57K mutant; 200 nM in AlphaScreen buffer, final concentration: 100 nM) into each well of a 384-well Optiplate.

   When pipetting the different solutions into 384-well plates, try to avoid air bubbles. Appropriate assay controls should include wells without both or either of the proteins as well as binding mutant proteins if available. The use of a multiplate dispenser or multichannel electronic handheld pipet is advisable.

4. Add 0.3 µl compound (stock concentration: 1 mM; final concentration: 5 µM in 60 µl) to TRAF6-RZ1 protein (WT or D57K mutant) to each well of the 384-well plate using liquid handling and incubate 30 min at room temperature.

   The DMSO concentration of dissolved compounds should be kept below 1%; in the case of TRAF6-Ubc13 it should be at 0.5%. This is necessary as DMSO can affect the proteins as well as the StrepII donor beads of the AlphaScreen technology. This incubation step will allow the compounds to bind to StrepII-TRAF6-RZ1.

5. Dispense 10 µl Ubc13-FlagHis (450 nM in AlphaScreen buffer, final concentration: 75 nM) into each well of the 384-well plate.

   Again, try to avoid air bubbles.

6. Seal the 384-well plate with foil and incubate 1 hr at room temperature.

This step is needed to avoid liquid evaporation and thus change in overall concentrations. This incubation step will allow Ubc13-FlagHis to bind to StrepII-TRAF6-RZ1.

7. Prepare working solutions for the AlphaScreen beads (stock is 5 mg/ml). Dilute both beads (Donor and Acceptor) separately to 24 µg/ml in the AlphaScreen buffer.

   Calculate how many samples you will need and prepare a master mix for all samples. Always prepare fresh working dilutions of beads. The Donor beads used in AlphaScreen assays are light sensitive, therefore working with the beads should be done under subdued lighting conditions. AlphaScreen beads mix must be prepared on ice and used immediately to avoid increased background signals.

   The optimal concentration of Donor and Acceptor beads needs to be determined for any other E2-E3 pair. After cross-titration to determine ideal protein concentration, the next step will be to check different concentrations of beads to choose a concentration that is cost effective but gives enough of a signal-to-noise ratio (minimum of 5 fold of the positive signal over the negative signal).

8. Dispense 10 µl Donor beads and 10 µl Acceptor beads into each well of the 384-well plate containing the proteins/compound mix.

   When designing the plate layout, include the bead only control (10 µl of each of the beads and 40 µl of AlphaScreen buffer). This control, or ideally a mutant control, will give you the background signal.

9. Seal plate with foil and incubate 1 hr at room temperature.

   This step is needed to avoid liquid evaporation and thus change in overall concentrations. Working in the dark is very important. This incubation step will allow the AlphaScreen beads to bind to the respective proteins.

10. Read out the plate with an AlphaScreen-compatible plate reader (settings: laser excitation at 680 nm, emission at 520-620 nm).

    Usually, a 20- to 30-fold signal induction is observed compared to the bead control.

Sample data

See Figure 3.

BASIC PROTOCOL 2

GEL-BASED *IN VITRO* UBIQUITINATION ASSAY (K63-LINKED CHAINS)

This *in vitro* assay is used to examine the activity of purified E3 ligases to form K63-linked poly-ubiquitin chains after small molecule treatment. This protocol describes how to measure the ability of the E3 ligase TRAF6 in cooperation with the E2 ligase Ubc13 to build free K63-linked ubiquitin chains. Recombinant E1 and E2 enzymes are commercially available. The result of the reaction is checked by western blot analyses using an anti-ubiquitin antibody. This protocol is a low-throughput method that is suitable to validate a shortlisted number of molecules from a screening campaign using, e.g., Basic Protocol 1.

Materials

10× K63 assay buffer (see recipe)
Recombinant His6-ubiquitin E1 enzyme (UBE1; E-304-050, R&D Systems)
Recombinant His6-UBE2N(Ubc13)/Uev1a complex (E2-664, R&D Systems)
Recombinant E3-ubiquitin ligase TRAF6, only RING-zinc finger1 (RZ1) domain (Brenke et al., 2018)
Recombinant ubiquitin protein (U-100H-10M, R&D Systems)
1 mM $ZnCl_2$ in 1× K63 assay buffer
Small molecules in DMSO (specific to the research question)
Dimethyl sulfoxide (DMSO of high purity)

2 mM ATP (MilliporeSigma)
SDS loading buffer (4×)
10× SDS buffer (see recipe)
5× separation gel buffer (1.88 M Tris·HCl, pH 8.8)
Separation gel (see recipe)
4× stacking gel buffer (0.5 M Tris·HCl, pH 6.8)
Stacking gel (see recipe)
Pre-stained Protein Marker (Page Ruler)
Pierce™ Silver Stain Kit
Methanol
10× blotting buffer (see recipe)
Blocking buffer: 3% BSA (standard quality) in PBST
PBST: PBS (Applichem) with 0.1% Tween 20 (Carl Roth)
Anti-ubiquitin antibody (P4D1, 1:1,000; Santa Cruz)
Secondary HRP-conjugated anti-mouse antibody (1:7,500; Dianova)

1.5-ml microcentrifuge tubes
SDS-PAGE gel chamber
PVDF membrane
Whatman paper
Semi dry blotter
Amersham Hyperfilm ECL

1. Purify recombinant proteins in bacterial systems: Untagged TRAF6-RZ1 (WT or D57K mutant).

   Untagged TRAF6-RZ1 is expressed and purified as a GST-TRAF6-RZ1 and the GST is cleaved off during the purification procedure. More detailed protocols for protein purification are published in Brenke et al. (2018).

2. Thaw reagents and place them on ice: His6-ubiquitin E1 enzyme, His6-UBE2N (Ubc13)/Uev1a complex, purified E3-ubiquitin ligase TRAF6-RZ1, ubiquitin, $ZnCl_2$, and ATP. Compounds and DMSO should be placed at room temperature.

   If no binding mutant is available, TRAF6 WT without ATP can be used as a negative control. Remember to have a control reaction with no compound and DMSO instead. The final concentration of 1% for either DMSO or compound should not be exceeded.

3. Pre-incubate 0.125 µM TRAF6-RZ1 with the compound (amount dependent on concentration to be tested) or DMSO in 1× K63 assay buffer for 30 min. The total reaction volume is 50 µl.

   Remember to take a 25-µl aliquot of input sample for silver staining. Add SDS loading buffer (4×) to each input sample, vortex to mix, and boil the samples at 95°C for 5 min. Spin down the samples before storing at –20°C.

4. Prepare master mix of 0.01 µM His6-ubiquitin E1 enzyme, 0.2 µM His6-UBE2N (Ubc13)/Uev1a complex, 4 µM ubiquitin, 1 mM $ZnCl_2$, and 2 mM ATP in 1× K63 reaction buffer. Add 50 µl master mix to start reaction. Incubate reaction 120 min at 37°C.

   The total reaction mixture is 75 µl. Handle quickly to start parallel samples at similar times.

5. Stop reaction by adding 25 µl SDS loading buffer (4×), vortex to mix, and boil samples at 95°C for 5 min.

   Spin down and store samples at −20°C until further use.

6. Load the input samples on a 12.5% acrylamide gel and the ubiquitination samples on a 10% acrylamide gel for SDS-PAGE electrophoretic separation.

   Do not forget to load a molecular weight marker as reference. Loading the ubiquitination samples on a 10% acrylamide gel will allow better visualization of the poly-ubiquitination smear. While running the gel electrophoresis, be sure to stop the electrophoresis in time as the molecular weight of mono-ubiquitin is 8.5 kDa.

7. The input sample gel will be used directly for silver staining, whereas the ubiquitination sample gel will be used for transfer of the separated proteins onto a PVDF membrane via semi-dry blotting.

   Silver staining allows detection of various E3 ligases avoiding the need of E3 ligase specific antibodies. More detailed information for silver staining is available in the manufacturer's protocol for Pierce™ Silver Stain Kit. During semi-dry blotting, try to avoid bubbles as these will prevent protein transfer.

8. Block the membrane for 60 min in a blocking buffer at room temperature to cover unspecific binding sites and subsequently incubate the ubiquitination sample membrane with an anti-ubiquitin antibody (P4D1) overnight at 4°C.

   Use as much blocking solution as needed to cover the membrane. Dilute the primary antibody 1:1,000 in 1.5% BSA in PBST.

9. After washing the membrane three times for 10 min with PBST, add the horseradish-peroxidase-conjugated secondary anti-mouse antibody. Incubate 1 hr at room temperature.

   Dilute the primary antibody 1:7,500 in 0.75% BSA in PBST.

10. Wash membrane again three times with PBST and add enhanced chemiluminescence substrate according to manufacturer's instructions. Detect chemiluminescence using X-ray films for various time points and develop the films in a developer machine.

    Poly-ubiquitin chains will appear as smears on the western blot.

Sample data

See Figure 4.

REAGENTS AND SOLUTIONS

AlphaScreen buffer

1× PBS, pH 7.3 (Thermo Fisher Scientific, 14190-094); store at room temperature
0.5% BSA, purity ≥98% (MilliporeSigma, A7030-100G); store at 2°C to 8°C
0.01% Tween 20 (Carl Roth, 9127.2); store at room temperature
Store at 4°C for a few days

Blotting buffer, 10×

48 mM Tris (Carl Roth, A411.2); store at room temperature
39 mM glycine (Carl Roth, 3908.3); store at room temperature
0.03% SDS (Carl Roth, 2326.2); store at room temperature
20% methanol (Merck, 1.06009.2500); store at room temperature
Store at room temperature for several months

K63 assay buffer, 10×

250 nM Tris·HCl, pH 7.6 (Carl Roth, A411.2); store at room temperature
25 mM $MgCl_2$ (Carl Roth, KK36.2); store at room temperature
50 mM creatine phosphate, disodium salt, ≥97% (MilliporeSigma, 2380); store at −20°C

3 U/ml inorganic pyrophosphatase
3 U/ml creatine phosphokinase
Use immediately.

SDS buffer, 10×

250 mM Tris (Carl Roth, A411.2); store at room temperature
2 M glycine (Carl Roth, 3908.3); store at room temperature
1% SDS (Carl Roth, 2326.2); store at room temperature
Store at room temperature for months

Separation gel

375 mM Tris·HCl, pH 8.8 (Carl Roth, A411.2); store at room temperature
12.5% acrylamide-bisacrylamide (Rothiphorese® Gel 30; Carl Roth, 3029.1); store at 4°C to 8°C
0.1% SDS (Carl Roth, 2326.2); store at room temperature
0.075% ammonium persulfate (APS; Bio-Rad, 161-0700); store at room temperature
0.05% N,N,N′,N′-tetramethyl ethylenediamine (TEMED; Bio-Rad, 161-0800); store at 4°C to 8°C
Use immediately.

Stacking gel

125 mM Tris·HCl, pH 6.8 (Carl Roth, A411.2); store at room temperature
5% acrylamide-bisacrylamide (Rothiphorese® Gel 30; Carl Roth, 3029.1); store at 4°C to 8°C
0.1% SDS (Carl Roth, 2326.2); store at room temperature
0.1% ammonium persulfate (APS; Bio-Rad, 161-0700); store at room temperature
0.1% N,N,N′,N′-tetramethyl ethylenediamine (TEMED; Bio-Rad, 161-0800); store at 4°C to 8°C
Use immediately.

COMMENTARY

Background Information

The hierarchical enzyme cascade orchestrated by the E1, E2, and E3 enzymes facilitates ubiquitination of target proteins during post-translational modification processes. It is important to notice that E3 ligases are subdivided into three main categories, namely RING, HECT, and RBR E3 ligases. Their enzymatic functions differ a lot, as RING ligases do not possess an internal cysteine, while HECT and RBR ligases do. Here, we only concentrate on targeting RING E3 ligases with small molecules for therapeutic interventions.

TRAF6 (a RING E3 ligase) interacts with the heterodimeric E2 enzyme Ubc13/Uev1a to attach K63-linked ubiquitin chains to its target proteins. This process is essential for signal progression in numerous cellular signaling processes, such as (1) T-cell receptor signaling, (2) IL-17 signaling, and (3) innate immune responses (Walsh et al., 2015). Disease onset and progression of distinct autoimmune diseases such as rheumatoid arthritis (RA) are notably dependent on these signaling pathways (McInnes, Buckley, & Isaacs, 2016), thereby making TRAF6 an attractive target for therapeutic interventions. Using a high-throughput small-molecule screening approach, an inhibitor of TRAF6–Ubc13 interaction was discovered that reduces TRAF6 activity both *in vitro* and in cells (Brenke et al., 2018). Thus, the inhibition of TRAF6 activity by small molecules represents a promising novel strategy for targeting autoimmune and chronic inflammatory diseases.

Importantly, ubiquitin is directly transferred from ubiquitin-charged Ubc13 (E2) to the substrate, both proteins bound by the E3 ligase TRAF6. Thus, targeting TRAF6 E3 activity requires the identification of protein–protein interaction inhibitors, which disrupt the TRAF6–Ubc13 interaction because TRAF6 has no intrinsic catalytic activity. Therefore, we present Basic Protocol 1 that monitors TRAF6-Ubc13 binding and Basic Protocol 2 that detects TRAF6

activity generating poly-ubiquitin chains. The consecutive use of both methods provides a thorough understanding of the compounds' effects.

Critical Parameters

Critical parameters important for both protocols:

• Expression and purification of E3 ligases in bacterial systems can be very demanding. Thus, purification of the RING domain alone or the RING domain together with an adjacent domain may be much easier to generate. In most cases this will be sufficient to produce robust AlphaScreen binding signals or poly-ubiquitin moieties in *in vitro* ubiquitination assays.

• Purified proteins should always be snap-frozen in liquid nitrogen prior to long-term storage at −80°C. Importantly, prepare small aliquots to avoid multiple freeze and thaw cycles of recombinant proteins.

• A binding mutant form of the E3 ligase (point mutation in the RING domain, e.g., D57K in the case of TRAF6) should be generated that does not bind to the E2 protein anymore. This will represent the negative control, i.e., the low signal of the AlphaScreen reaction as well as no poly-ubiquitin smears in *in vitro* ubiquitination reactions.

• It might be necessary to test different tags attached to the respective protein as well as various tag combinations, such as the size of the tag as well as its location on the protein itself (N- versus C-terminus), because these may have an impact on activity in the assay. For example, one tag may cover the site of protein-protein binding or oligomerization of the tag may impact activity of the protein.

Critical parameters for AlphaScreen binding assay (Basic Protocol 1):

• When working with the AlphaScreen technology, it is important to consider the hook effect. As the beads only have a limited capacity of protein binding sites, these can get saturated. Thus, the hook effect describes the fact that extended amounts of either of the proteins, which is beyond the capacity of the beads to bind, will act as inhibitors of the reaction and reduce the signal.

• Both the protein solutions and the AlphaScreen bead mix must be freshly prepared on ice and used immediately to avoid increased background signals. This is a very critical consideration as this will remarkably affect the signal-to-noise ratio.

• AlphaScreen assays should always be carried out in subdued light conditions to ensure no bleaching of the beads due to photosensitivity.

• White opaque plastic plates are required for reading AlphaScreen; it is advisable to use white plates for a better outcome. Black plates will absorb light and lead to greatly reduced signals.

• Buffer choice can be very important. Choose pH, buffering capacity, and salt concentration that will facilitate the desired interactions between the PPI of your assay. In critical instances, a buffer screen might be needed to improve signal output. BSA concentration of 0.1% (w/v) is sufficient to minimize non-specific interactions. A highly pure version of BSA (>98%) is mandatory for good signal output. In non-critical instances, the AlphaScreen buffer can be a buffer of choice and a good starting point.

• Chemiluminescent reactions such as the ones involved in AlphaScreen signal generation are temperature dependent. For the best outcome always perform reactions at constant temperatures around room temperature.

• Always include important controls: (1) both beads without proteins; (2) both beads with either of the protein pairs; (3) one of the beads with both proteins.

Critical parameters for gel-based *in vitro* ubiquitination assay (Basic Protocol 2):

• Always include important controls: During the assay development phase, always run control reactions (1) without ATP (or if possible, as in our case, an E3 ligase mutant with ATP) and (2) without the E3 ligase.

• Reactions without the E3 ligase will also generate smaller amounts of poly-ubiquitin chains as the E2 enzyme together with the E1 enzyme in a biochemical reaction can assemble ubiquitin chains, although with much less efficiency. It is critical that there is a substantial increase in poly-ubiquitin smears in the presence of the E3 ligase.

• The time window for measuring the compound inhibition is limited and critical. As compounds are usually non-covalent, there will be an equilibrium of on/off rates, which over time, will lead to accumulation of covalent and irreversible ubiquitination. This will dilute the effect.

Troubleshooting

When using the AlphaScreen technology for testing inhibitory compounds, it is important to be aware that many compounds interfere with the underlying chemistry; both inhibitory compounds and interfering compounds may reduce the assay signal.

Frequent hitters (i.e., promiscuous compounds) will generate false positive hits that need to be eliminated from the hit list. Chemoinformatics filters can help identify these frequent hitters (Baell & Holloway, 2010; Brenke et al., 2016; Schorpp et al., 2014). Hit evaluation in orthogonal and counter screens is mandatory to confirm activity and specificity (Rothenaigner & Hadian, 2021; Weber et al., 2017).

A counter assay can include the analysis of a linear fusion of StrepII and His to test compound activity in an AlphaScreen assay that is not relying on the PPI. Another strategy of analyzing false positive compounds in an AlphaScreen assay is to apply different PPI pairs and identical tags or the same PPI pairs with different protein tags (e.g., StrepII/His versus GST/His). A genuine TRAF6-Ubc13 inhibitory compound should diminish signals of different tag combinations with the same PPI of interest (here TRAF6 and Ubc13). However, it should not reduce AlphaScreen signals with other PPI pairs or in assays using the linear fusion constructs (Rothenaigner & Hadian, 2021). A study by Brenke et al. (2016) used a GST-His construct to verify GST frequent hitters. This study exemplifies the benefit of using such linear fusions. The gel-based ubiquitination assay is also used as a confirmation assay. Due to the entirely different assay detection methods, AlphaScreen assay artifacts should not have any influence on the formation of poly-ubiquitin chains in the gel-based assay and could therefore be identified and eliminated from the hit list.

Gel-based *in vitro* ubiquitination reactions are generally straightforward. If enzyme reactions do not work, repeat reactions with fresh enzymes and ATP. Moreover, the percentage of acrylamide of the SDS gels may be an important factor in the visualization of a poly-ubiquitin smear and should be adapted if necessary. When establishing *in vitro* ubiquitination reactions for other E3 ligases, it might be advantageous to perform a time course first. Taking samples after various time points will help to determine the optimal incubation time for visualization of the poly-ubiquitin smear. In order to analyze the compounds selectivity, various E3 ligase ubiquitination assays could be run in parallel. Several E3 ligases are also commercially available and can be adopted to the described protocol.

Understanding Results

AlphaScreen signals are generally presented as raw signal measurement data that are extracted from plate readers. These numbers may vary from reader to reader. In general, it is important to compare positive and negative controls and aim to achieve a minimum of 5-fold increase of the positive signal over the negative signal as this will guarantee a more robust screening assay. Having these numbers, one can calculate Z' values, signal windows, and variation of plate-to-plate or day-to-day results (Hughes, Rees, Kalindjian, & Philpott, 2011). During high-throughput screening there will be only one measurement per compound. This limitation is based on the need to maximize throughput.

The output of a gel-based *in vitro* ubiquitination reaction is the detection of smears in ubiquitin-stained western blot analyses. These smears represent various lengths of ubiquitin linkages (i.e., di-Ub, tri-Ub, tetra-Ub). There are two types of poly-ubiquitin moieties possible: (1) auto-ubiquitination of TRAF6 or (2) free poly-ubiquitin chains. To be able to understand the results, a control without ATP (or if possible, as in our case, an E3 ligase mutant with ATP) as well as a control lacking the E3 ligase is mandatory to be able to visualize the smear.

Time Considerations

Assay development times for AlphaScreen assays are typically very short due to the rapid speed with which one can generate data. Once the purified components are available, the assay can be set up very quickly, typically within a few hours. The set-up experiment using cross-titrations of both binding partners to find the optimal protein concentrations can be performed on 384-well plates in 1 or 2 days, thereby running multiple iterations for refinement. When protein concentrations are optimized and robust signals with low background are available, the assay can be performed in high-throughput format (96- or 384-well plates, or even 1,536-well plates) in <1 day. For AlphaScreen assays, reagents are reasonably stable and the setup of the experiment can tolerate delays.

The *in vitro* ubiquitination assay takes 2 days to finalize, which is more time consuming than the AlphaScreen assay. On the first day, the enzyme reaction is carried out within 2 to 3 hr. Next, samples are loaded onto SDS gel and subsequently transferred to membranes for western blot analysis. Ubiquitin antibody incubation occurs overnight and the remaining steps of the western blot analysis happen on the second day. Alternatively, the ubiquitination reaction can be stored at –20°C

and the SDS PAGE and western blot analyses can be continued whenever possible. For the *in vitro* ubiquitination assay, the timing of the experiments is crucial. As this setup uses enzymes that will start running the reaction once the ATP is available, it is necessary to prepare all components before starting the reaction.

Author Contribution

Ina Rothenaigner: Formal analysis, investigation, methodology, visualization, writing original draft, writing review and editing; **Jara Brenke:** Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing review and editing; **Kenji Schorpp:** Conceptualization, investigation, methodology, writing review and editing; **Kamyar Hadian:** Conceptualization, funding acquisition, resources, supervision, visualization, writing original draft, writing review and editing.

Conflict of Interest

The authors have no conflicts of interest.

Data Availability Statement

Data sharing not applicable – no new data generated.

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Key References

Brenke et al. (2018). See above.

This paper was the first that identified a TRAF6-Ubc13 PPI inhibitor with a screening campaign.