Profiling UPP inhibitors for HTS, drug discovery, and target validation

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LifeSensors
from genomics to proteomics

Introduction:

The human genome encodes ~700 ubiquitin E3 ligases (E3s), the proteins involved in terminal transfer of ubiquitin (Ub) to substrate proteins, and 95 deubiquitylases (DUBs), the enzymes involved in removal of Ub. Conjugation requires synthesis of an isopeptide bond between the C-terminus of Ub and the ε-NH_2-group of a Lys (K) residue in the target protein. In addition, Ub can be attached to any one of the seven lysine residues in another Ub to generate poly-ubiquitin chains. It is now well established that poly-ubiquitin chains built on lysine 48 (K48) and lysine 63 (K63) of ubiquitin facilitate proteasomal degradation and protein trafficking, respectively. With the success of drugs like bortezomib, carfilzomib, and lenalidomide, which affect different aspects of the ubiquitin-proteasome pathway (UPP), there has been renewed interest in developing drugs that target the UPP.

One of the major hurdles in evaluating the efficacy of new drug candidates, as well as their specificity, is assessing their effects on substrates in a cell-based assay system. Such assays have traditionally relied on time consuming, laborious, semi-quantitative immunoprecipitation and Western blot analyses. There is great need for quantitative, higher throughput assays that also lower resource utilization. We have developed a series of substrate specific assays that can be used for multiple samples simultaneously in a microplate format. The basis of the assay is the capture of poly-ubiquitylated proteins using tandem ubiquitin binding entities (TUBEs) followed by detection of bound substrates using antibodies selective for those substrates. As a proof-of-principle experiment, we used pan-selective TUBEs absorbed in the wells of a microtiter plate and transiently expressed substrates carrying epitope tags. We have shown that the degree of ubiquitylation is increased dramatically by co-transfection of the appropriate E3 ligase. We have demonstrated that ligase inhibitors reduce ubiquitylation of the substrate in a dose dependent manner. Additional studies to determine poly-ubiquitylation of endogenous substrates will also be presented. The assay systems described in this presentation offer a powerful application to examine changes in ubiquitylation of endogenous substrates and help evaluate drug candidates that inhibit selective ubiquitylation and/or deubiquitylation.

Tagged, exogenous substrate ubiquitylation

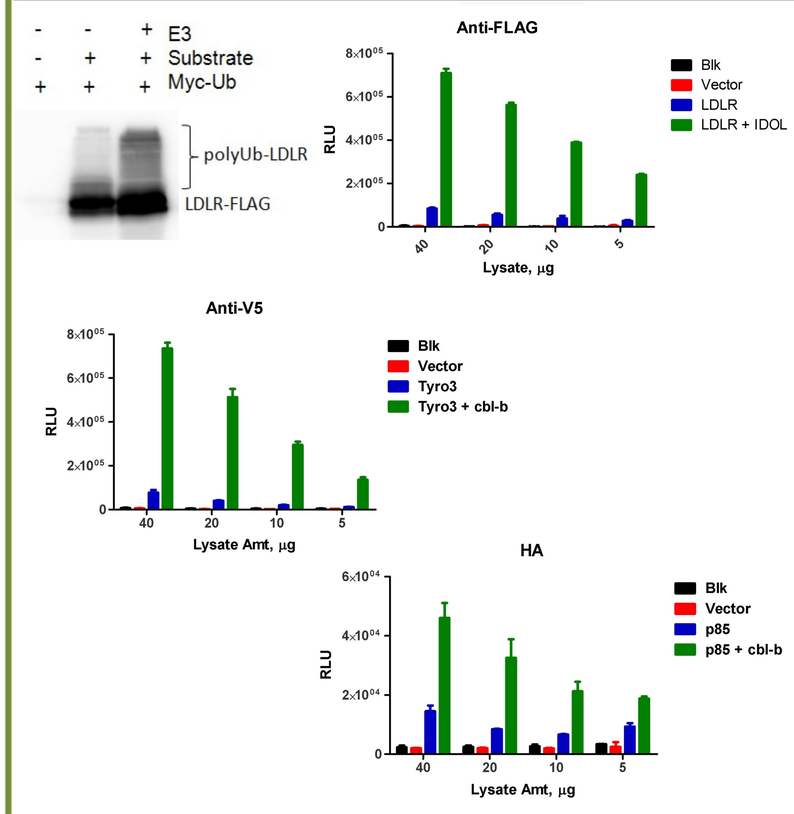

Figure 1. Detection of specific substrate ubiquitylation using panUb TUBE coated plates. HEK293T cells were transfected with different substrate/ E3 ligase pairs: Flag-LDLR/IDOL, HA-p85/cbl-b, and V5-Tyro3/cbl-b. Twenty-four hr after transfection, the cells were harvested and lysed in lysis buffer (1% Triton X100, 0.5% CHAPS, 0.1% NaDOC, 0.1% SDS, 200mM Tris-HCl, pH 7.5, 5mM EDTA, 150mM NaCl, 10% glycerol containing a 1:500 dilution of protease inhibitor cocktail, 50mM PR619, 5mM 1,10-phenanthroline (*o*PA) and 20mg/mL aprotinin.) The protein concentrations of the lysates were determined and each lysate was assayed in duplicate at 40, 20, 10, and 5µg/well. Controls included substrate alone and empty vector. Anti-tag and HRP-conjugated anti-IgG antibodies were used to detect bound substrates.

Detection of an untagged, exogenous protein

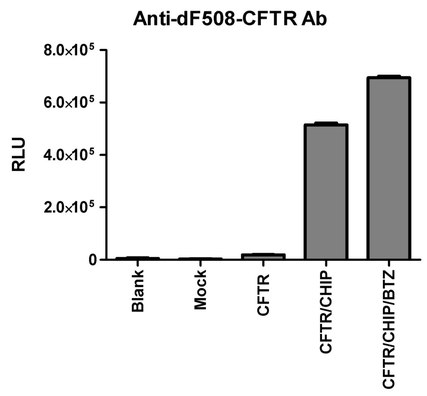

Figure 2. Detection of CFTR ubiquitylation using anti-CFTR antibody. HEK293T cells were transfected with a plasmid encoding dF508 CFTR ± the E3 ligase CHIP. A subset of the CFTR/CHIP transfected cells were also treated with the proteasome inhibitor PS341. Twenty-four hr after transfection, the cells were harvested and lysed with 1% Triton X100, 0.5% CHAPS, 0.1% NaDOC, 0.1% SDS, 200mM Tris-HCl, pH 7.5, 5mM EDTA, 150mM NaCl, 10% glycerol containing a 1:500 dilution of protease inhibitor cocktail, 50mM PR619, 5mM 1,10-phenanthroline (*o*PA) and 20mg/mL aprotinin. The protein concentrations of the lysates were determined and each lysate was assayed in duplicate. Controls included substrate alone and empty vector. Anti-CFTR and HRP-conjugated anti-IgG antibodies were used to detect bound substrate.

Ubiquitylation of endogenous substrates

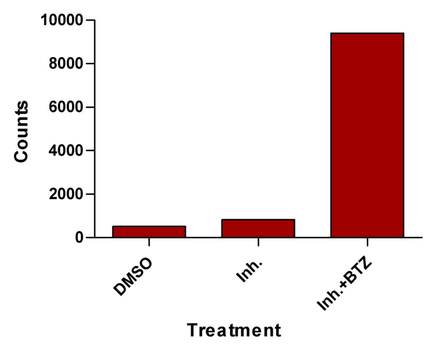

Figure 3. Effect of a USP7 inhibitor ± Bortezomib on the level of ubiquitylation of USP7. Jurkat cells were treated with DMSO, a USP7 inhibitor (Inh.) or the inhibitor+Bortezomib (Inh.+BTZ) for 2 hr. The USP7 inhibitor was used at 10µM and Bortezomib was used at 200nM. The cells were collected by centrifugation and frozen at -80°C. The cell pellets were thawed and lysed using RIPA buffer (Pierce) with vigorous agitation. Insoluble material was removed by centrifugation at 14,000 rpm for 10min. The supernatant solution was isolated and the protein concentration determined by micro-Bradford assay. The lysates were diluted to ~1mg/mL and 50µg of each was assayed in a His_6-TUBE1 coated plate. Bound USP7 was measured using a rabbit anti-USP7 mAb (Santa Cruz Biotechnology) as primary and HRP-conjugated anti-rabbit IgG (Rockland Immunochemicals) as the secondary.

Translation to HTS-friendly format

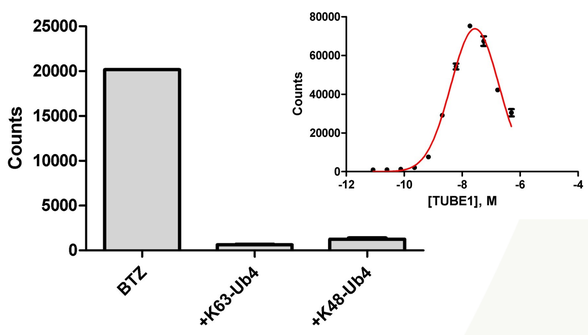

Figure 4. Detection of endogenous USP7 ubiquitylation with AlphaLISA beads. Jurkat cells were treated with 200 nM Bortezomib (Inh.+BTZ) for 2 hr. The cells were collected by centrifugation and frozen at -80°C. The cell pellet was thawed and lysed using AlphaLISA lysis buffer. The supernatant solution was isolated and the protein concentration determined by micro-Bradford assay. 10µg of each sample was assayed. Streptavidin coated donor beads were coated with biotinylated TUBE1 (40nM final) and Protein G coated acceptor beads were preincubated with anti-USP7 mAb. The assay was also run in the presence of 5µM K48-Ub4 or K63-Ub4. The inset shows a preliminary titration of donor beads versus biotinylated TUBE1.

Measuring inhibition of ubiquitylation in tissue culture

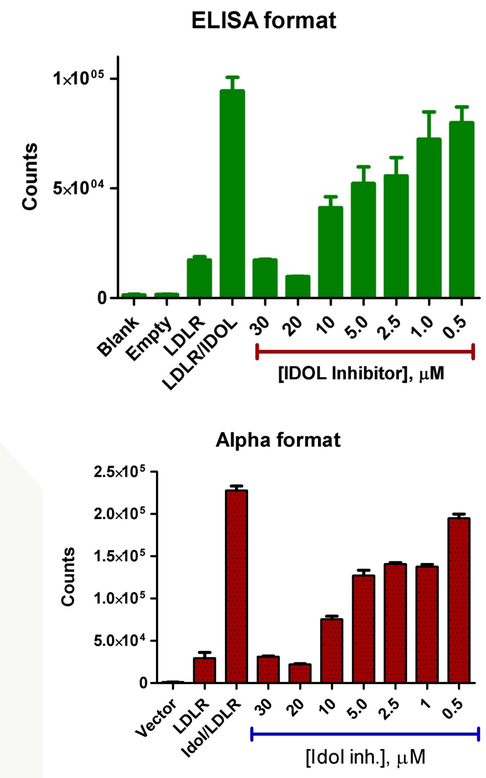

Figure 5. Inhibition of LDLR ubiquitylation in tissue culture with an inhibitor of the E3 Ligase IDOL. Cells were transfected with plasmids encoding Flag-LDLR and IDOL. The cells were then plated and individual cultures treated with the indicated concentrations of the IDOL inhibitor for 2 hr prior to lysis. The lysates were then assayed for the level of ubiquitylation of LDLR in either the ELISA format (**A**) or the AlphaLISA format (**B**) of the assay. In the ELISA format, the plates were coated with His6-TUBE1 and in the AlphaLISA format the donor beads were coated with biotinylated TUBE1. Anti-Flag and Flag-acceptor beads were used for detection, respectively. Transfection with the empty vector served as a control. N.B. the IDOL inhibitor was kindly provided by Progenra, Inc. and is proprietary to that company.

Conclusions

- We show the performance of two different formats of a new assay system for measuring changes in the ubiquitylation of specific substrate proteins.
- The assay relies on the use of tandem ubiquitin binding entities (TUBEs) for capture of ubiquitylated proteins and specific antibodies for detection of substrate proteins.
- The assay can be used for detection of either epitope-tagged exogenous proteins or endogenous substrates.
- For moderate through-put assays, the ELISA format is simple, straight-forward and economical.
- For higher through-put assays the homogeneous AlphaLISA format is rapid and reproducible.
- We are currently carrying out further characterization of these assays to demonstrate sensitivity and robustness.
- We are also investigating the use of linkage selective TUBEs to provide data on ubiquitin-chain linkage for the substrates.

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