Selective Detection and Enrichment of K63-linked Polyubiquitin with K63-TUBEs

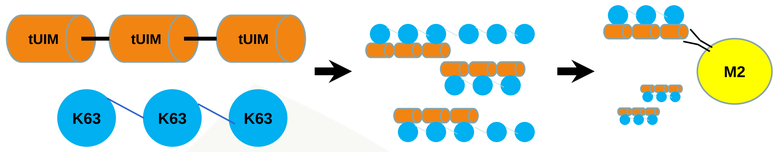
Steven J. Orcutt[1], Victoria Zhukareva[1],Suresh Kumar[2], Esteban Martinez[1], Kathryn Longenecker[1], and James E. Strickler[1]

[1]LifeSensors, Inc., Malvern, PA, USA. [2]Progenra, Inc. Malvern, PA, USA.

LifeSensors
from genomics to proteomics

Abstract:

The covalent modification of cellular (target) proteins through the addition of polyubiquitin (polyUb) chains has been shown to have profound effects on their function, compartmentalization, and stability. These polyUb chains are attached through isopeptide bond formation between the C-terminus of Ub to lysines of the target proteins. PolyUb chains themselves are formed through similar conjugation mechanisms between individual monomeric Ub units, and have been shown to be linked through all lysines present in Ub (K6, K11, K27, K29, K33, K48, and K63). Polymeric chain formation on target proteins linked specifically through K63 have been shown to play a role in the modulation of many cellular pathways and their cognate disease states including inflammation, the DNA damage repair response, signal transduction, and the regulation of endocytic recycling pathways. Recently, constructs consisting of tandem ubiquitin interaction motifs (tUIMs) have been designed by Sims, et. al. to maximize affinity for K63 polyUb chains, while maintaining relatively low affinity for other linkages (e.g. K48- and K11-), approximating those of the monomer UIM. This increased affinity (roughly 1000 to 10,000-fold) is likely due to both the structure and length of the artificially designed linkers separating each UIM. Rigid, helical spacing linkers position each UIM precisely for optimal interaction with each Ub in the extended polymer chain. These tUIMs have the ability to distinguish among these linkage types immobilized on membranes, in addition to acting as "sensors" to allow for *in situ* visualization of cellular processes considered to be dependent upon post-translation modification by K63-linked polyUb. We have developed these tUIMs into novel tools called K63 TUBEs that allow for specific *in vitro* investigation of K63 polyUb dependent processes. We demonstrate that these K63 TUBEs can be used to visualize K63 polyUb chains by Ligand blotting (far Western), demonstrating both sensitivity and selectivity that is comparable to or better than K63-specific monoclonal antibodies. K63 TUBEs have also been adapted for use in "pull down" reactions. Studies with either *in vitro* synthesized chains or HEK293T cell lysates demonstrated the ability of K63 TUBEs to enrich for K63 polyUb and proteins modified through this linkage, while being relatively ineffectual for similar enrichment for K48 polyUb. In addition, several cell lines were used to investigate the utility of these reagents to infer the status of K63 polyubiquitination through pull down studies. These reagents extend available TUBE reagents from pan-selective to K63-selective TUBEs and allow, for the first time, specific identification and enrichment of K63 polyUb modified proteins for proteomic analysis of these distinct cellular processes.



Detection of K63-polyUb by Ligand Blotting

Figure 1

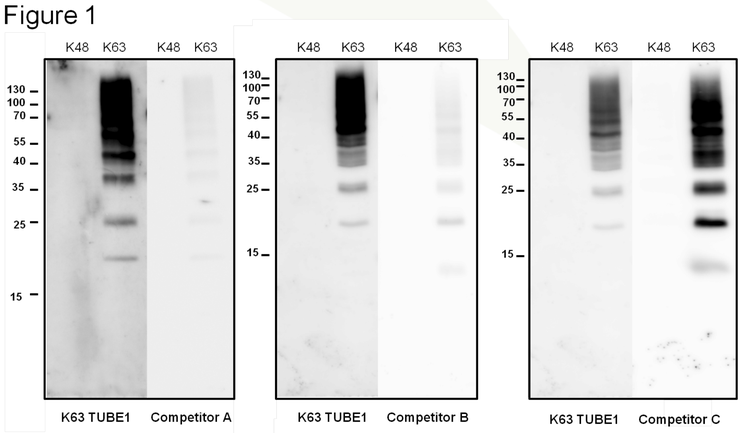


Figure 1: Polyubiquitin chains generated with either E2-25K (K48) or yMMS2/Ubc13 (K63) were subjected to non-reducing SDS-PAGE analysis and immunotransfer. Equivalent loading was verified by Ponceau (not shown). Membrane sections were immunoprobed overnight with various commercial K63-specific monoclonal antibodies (according to manufacture's instructions) or incubation for 1h with Biotin K63-TUBE1. Blots were then incubated with either species appropriate HRP-conjugated secondary antibodies, or with HRP-conjugated avidin. Sectioned blots were then extensively washed and subjected to simultaneous ECL development and imaging (FUJI LAS3000).

Figure 2

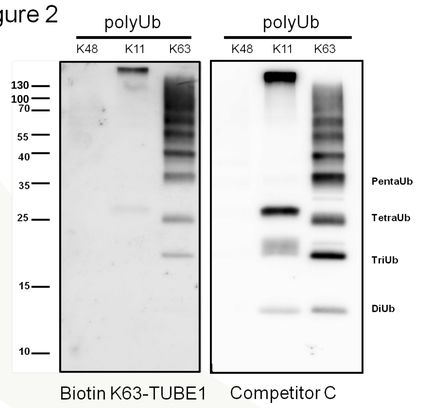
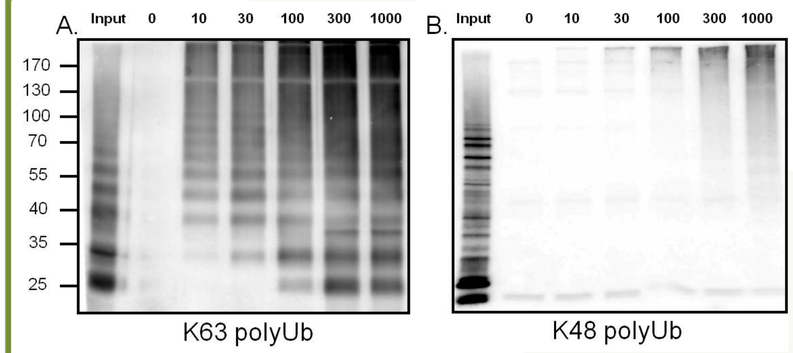


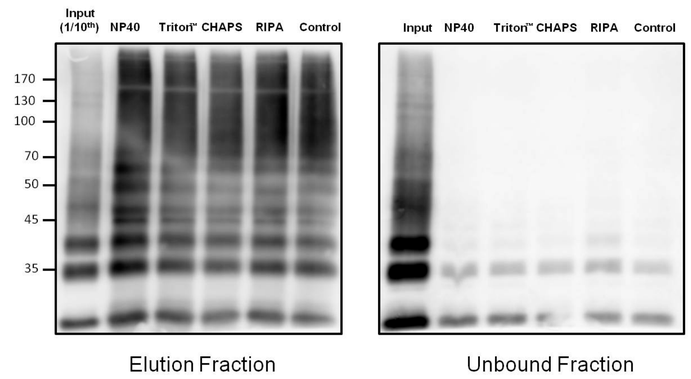
Figure 2. Polyubiquitin chains generated with either E2-25K (K48), yMMS2/Ubc13 (K63), or UBE2S (K11) were subjected to duplicate loading, SDS-PAGE analysis and immunotransfer. Equivalent loading was verified by Ponceau (not shown). The blot was sectioned and probed as described above with either Biotin K63-TUBE1 or a commercial monoclonal antibody specific for K63 polyUb chains to further investigate sensitivity and specificity for these two reagents.

Enrichment for *in vitro* PolyUb with K63-TUBE1



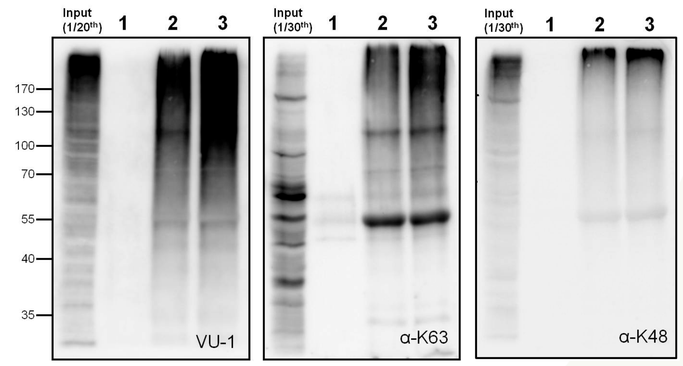
PolyUb chains (10µg) generated by E2-25K **(Panel A)** or yMMS2/Ubc13 **(Panel B)** were diluted into 0.25ml of 200mM Tris, pH 8.0, 0.15M NaCl, 5mM EDTA (TBSE), 0.1% NP-40 and incubated with indicated amounts of FLAG® K63-TUBE1 (nM) for 1.5h (4°C). After addition of M2 Affinity Resin (10µl, Sigma-Aldrich), reactions were incubated an additional 2h with rotation. Resin was collected by low speed centrifugation, washed three times in buffer containing 0.6M NaCl, and heat treated in SDS-PAGE sample buffer for protein elution. Eluted proteins (1/5th of total) were subjected to non-reducing SDS-PAGE, transfer to PVDF, and immunoprobing for total ubiquitin (VU-1, LifeSensors). **Input** (1/50th of total) samples are shown for comparison. Input samples were also analyzed by SDS-PAGE and silver staining to verify equivalent loading (not shown).

Detergent Compatibility of K63-TUBE1



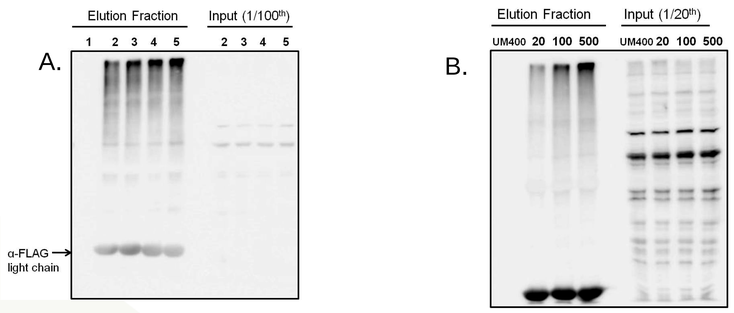
PolyUb chains (10µg) generated by yMMS2/Ubc13 (K63) were incubated (TBSE) with 400nM FLAG® K63-TUBE1 in the presence of 1% indicated detergent or 0.1% SDS, 0.5% deoxycholate (**RIPA**) for ~1h (4°C), including a high speed centrifugation step to mimic cellular lysis. PolyUb/TUBE complexes were diluted 1:5, ensuring M2 resin (10µl) compatibility. Reactions were rotated overnight (4°C). Resin was processed as above. **Elution Fraction**: Samples (1/4th of total) were probed with VU-1, with Inputs being 1/10th of load. **Unbound Fraction:** Equivalent amounts of Input and Unbound Fraction samples were probed with VU-1 to infer depletion efficiency. No significant loss in efficiency was observed when comparing detergents commonly used for cell lysis.

Enrichment for Cellular PolyUb with K63-TUBE1

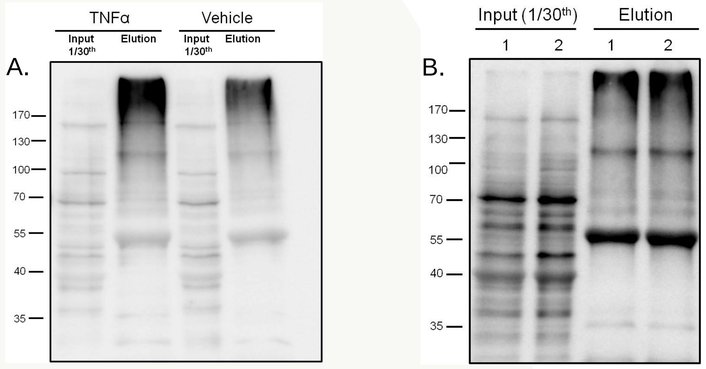


HEK293T cells were lysed in TBSE, 1% NP-40, 0.5% Triton X-100, 5mM NEM, 50µM PR-619 (LifeSensors), 5mM 1,10-phenanthroline (LifeSensors), protease inhibitor cocktail (Calbiochem) and either 0.5µM **(2)** or 1µM **(3)** FLAG® K63-TUBE1. Pre-cleared samples (~2mg total protein) were clarified and rotated with M2 resin (10µl) for 3h (4°C). Resin was washed in TBSE containing 600mM NaCl. Protein was eluted in SDS-PAGE (reducing) buffer with heating. **IB:** For eluted protein, ~6.5e5 cells (1/4th) was immunoprobed with VU-1. The remaining fraction (~2e6 cells) was probed individually with commercial monoclonal antibodies specific for either K63- or K48-polyUb (Cell Signaling). **Inputs** were scaled as indicated to infer relative pulldown efficiency. While K63-TUBE appears to be enriching for a fraction of total Ub (**VU-1**), results indicate that K63 polyUb species are clearly enriched **(α-K63),** while the enrichment of K48 polyUb is relatively poor **(α-K48)**.

Application of K63-TUBE1



Isolation of polyUb from Jurkat cells: Cells were cultured in RPMI-1640 supplemented with 10% FBS and 1% L-glutamine. **Panel A:** Cells (~2.5e7) were treated with either **2)** PBS, **3)** lipid polysacchride (LPS, 100ng/ml), **4)** a combination of phorbol 12-myristate 13-acetate (PMA) and Ionomycin at 40ng/ml and 300ng/ml, respectively, or **5)** α-CD3 IgG (2µg/ml) for 30min at 37°C in order to elicit an increase in total cellular K63-linked polyUb. Cells were washed in PBS, and lysed in TBSE, 10% glycerol, 1% Triton™ X-100, 0.5% CHAPS, 5mM NEM, 50µM PR-619, 5mM 1,10-phenanthroline, protease inhibitor cocktail and FLAG® K63-TUBE1 (0.5µM). Lysates (~2mg total protein) were clarified, pre-cleared, and rotated with M2 resin (10µl) overnight (4°C). Resin was then processed as above and eluted protein was immunoprobed for K63 polyUb. **Panel B:** α-CD3 IgG treated cells were harvested and subjected to pull down as above, with incubation at indicated concentrations of FLAG® K63-TUBE1.



Stimulation of macrophages (Panel A): Macrophages were isolated from murine bone marrow of 8 week old pups. Cells were cultured for 8 days in DMEM supplemented with a combination of 10% FCS and conditioned media from the cultured murine L929 fibroblasts. Macrophages were then harvested and treated with either vehicle control or TNFα (20µg/ml) for 15 min to induce up regulation of K63-specific ubiquitylation. **Stimulation of leukocytes (Panel B):** Murine leukocytes (from spleen and lymph nodes) were isolated and either treated **(2)** with PMA/Ionomycin for 4h or left untreated **(1)** prior to being washed, aspirated, and snap frozen for storage at -80°C. **Isolation of polyUb:** Cells (~1.5e8) were lysed in inhibitor containing TBSE plus 500nM FLAG® K63-TUBE1. Lysates (~2mg total protein) were pre-cleared, incubated with M2 resin (10µl) with rotation (2h, 4°C), analyzed by SDS-PAGE and immuno-probed for K63 polyUb.

Conclusions:

K63-TUBEs provide for the application of K63-selective tUIMs to the detection of K63 polyUb by ligand (far Western) blotting, as well as the *in vitro* enrichment of K63 polyUb and K63 polyUb-modified proteins from cell lysates and tissue homogenates. These reagents show minimal reactivity with immobilized K48- and K11 polyUb chains, as well as demonstrating selectivity for *in vitro* synthesized K63 polyUb in solution in a manner consistent with binding studies previously established for these constructs. Complex formation between K63-TUBEs and K63 polyUb appears to tolerate a wide range of lysis conditions. Pulldown reactions with K63-TUBEs and cell lysates demonstrate that enrichment of K63-linked polyUb is extremely efficient compared to observed enrichment of K48 polyUb. In addition, using three different experimental models, total detected K63-polyUb recovered from cell lysates by FLAG® K63-TUBE1 appears to increase under conditions typically associated with stimulation of K63-dependent processes. K63-TUBEs provide a means to further elucidate cellular mechanisms that are regulated by K63-polyUb, through the investigation of specific target proteins as well as proteome wide analysis.

Acknowledgements:

LifeSensors is would like to gratefully thank the generous contributions of TNFα-stimulated macrophages from Dr. Paula Oliver (Perelman School of Medicine at the University of Pennsylvania), and PMA-stimulated leukocytes from Dr. Wayne Hancock (School of Medicine at the University of Pennsylvania). LifeSensors would also like to recognize the invaluable consultations of Drs. Joshua J. Sims (Harvard Medical School) and Robert E. Cohen (Colorado State University), whose intellectual contributions throughout the development of K63-TUBEs were crucial to adapting this technology to its intended application.