Ubiquitin code: Accelerating the discovery of poly-ubiquitylated proteins by novel chain-selective affinity matrices

Apurva Chaturvedi[1], Peter Foote[1], Dominic Vasturia[1], Rajesh Singh[1]

[1]Research and Development, LifeSensors Inc, Malvern, PA,

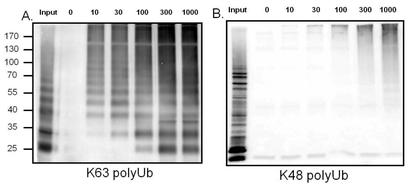
LifeSensors
from genomics to proteomics

Abstract

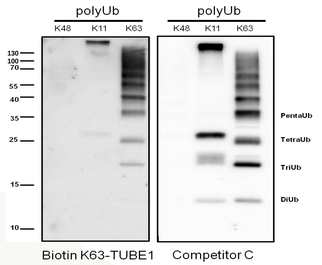
***Purpose of study*: The work presented here focuses on developing easy to use tools to distinguish between specific ubiquitin chain linkages, which are involved in a multitude of cellular functions.**

Ubiquitin (Ub) is attached via isopeptide bonds to lysine residues in the target protein or to another Ub to form poly-Ub chains. The reversibility, heterogeneity, and diversity of these modifications combined with the lack of suitable tools have made it difficult to properly isolate and characterize poly-ubiquitylated cellular proteins. However, in 2009, a novel technology called TUBEs (Tandem-repeated Ub-Binding Entities) was developed. TUBEs have revolutionized the Ub field by allowing poly-ubiquitylated proteins to be enriched/purified from cellular extracts. Structure-based approach to engineer unique high affinity Ub binding domains(UBDs) for Ub-linkages has been implemented to design highly selective affinity matrices. Development of TUBEs that selectively bind M1-, K48- and K63-linked poly-ubiquitylated proteins has helped understand the role of modified proteins in cell physiology. Biochemical and biophysical experiments demonstrate highly selective interactions of the matrices to distinct lysine-linked poly-Ub chains. Power of these highly selective affinity matrices has made it possible to perform Ub proteomics bypassing SILAC mass spectrometry. Application of these affinity matrices in far-Western detection and pull-down will be described in the poster. Development of chain selective affinity matrices (TUBEs) that selectively bind to poly-ubiquitylated proteins will dramatically accelerate the pace of discovery in this important area of biology.

Enrichment of Cellular PolyUb with K63 TUBE

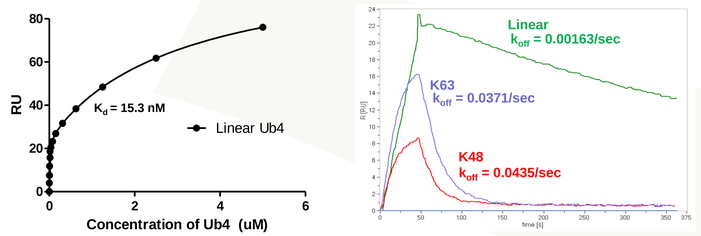


Pull-down Experiments using FLAG K63 TUBE. *K63 linked PolyUb chains (10 µg) were generated by yMMS2/Ubc13* ***(Panel A)*** *and K48 chains were generated by E2-25K* ***(Panel B).*** *The ubiquitin chains were pulled down with indicated amounts of FLAG® K63-TUBE1 (nM) for 1.5 hours (4°C) and M2 FLAG Affinity Resin (10 µl, Sigma-Aldrich).*



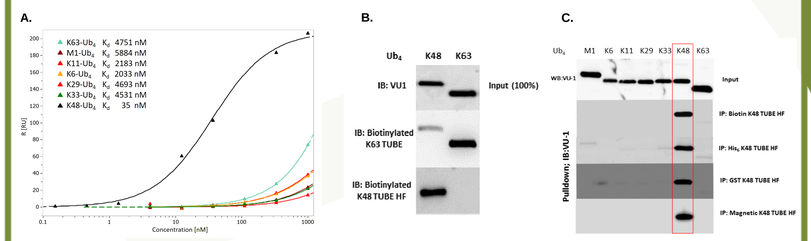
Comparison of K63 TUBE Specificity to Monoclonal Antibody. *PolyUb chains generated with either E2-25K (K48), yMMS2/Ubc13 (K63), or UBE2S (K11) were subjected to duplicate loading, SDS-PAGE analysis and western-blot. The blot was sectioned and probed as described above with either Biotin K63-TUBE1 or a commercial monoclonal antibody specific for K63 polyUb chains to further investigate sensitivity and specificity for these two reagents.*

Binding and Kinetics of M1 (Linear) TUBE



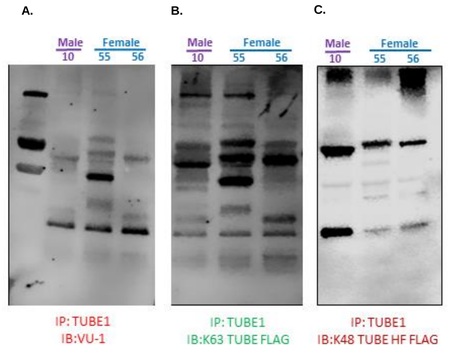
Determining Binding Affinity of M1 (Linear) TUBE. *His_6-tagged TUBEs were immobilized on a His Capture chip. His_6-SUMO was loaded onto the control surface. Injections of increasing amounts of tetra-ubiquitin (Ub_4) were analyzed. The binding curve and K_d was generated in PRISM. (Right) Injections of 9.5 nM tetra-ubiquitin were performed on the same surface of Linear (M1) TUBE. Kinetic analysis was performed using Sierra Sensors SPR software.*

Ubiquitin Binding Properties of the Newly Developed Highly Specific K48 TUBE HF



Determination of Binding Affinity and Specificity of K48 TUBE HF. *(A) SPR experiments using increasing amounts of Ub_4 were performed using His_6-K48 TUBE and His_6-M1-Ub_2 on the control surface. The binding curves and Kds were generated using Sierra Sensors analyzer software. (B) The detection of K48- and K63-Ub_4 by ligand blotting (far-Western) using biotinylated K63-TUBE and K48-TUBE HF. (C) 1 µg of the indicated Ub4 were pull-down with 1.05 nmol of either biotinylated-, His_6-, GST- or Magnetic-K48 TUBE HF for 2 hours at 4°C. 20 % of the pulled-down samples were loaded onto SDS-PAGE followed by western blot. The blots were probed with anti-Ub (VU-1) antibody.*

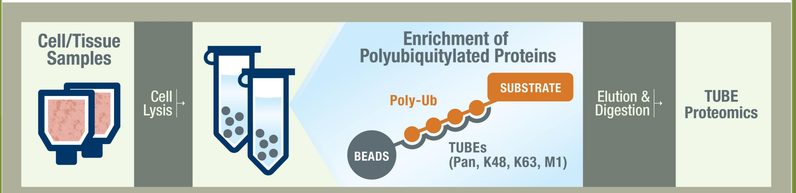
Pulldown and Far Western Detection in Serum



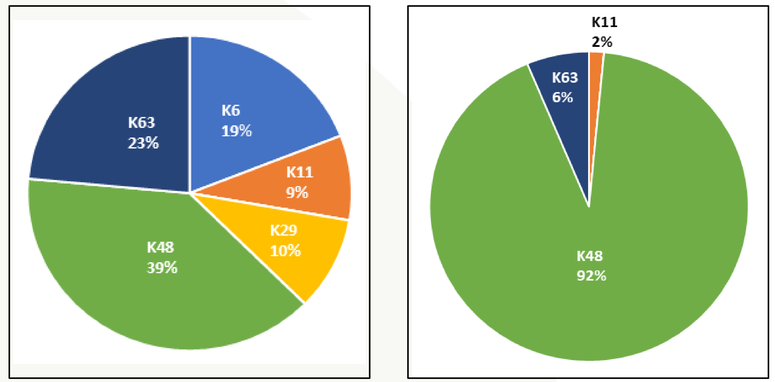
Pulldown experiments and far-Western detection in serum. *TUBE1 IP of polyUb proteins from human serum, analyzed by (A) anti-Ub (VU-1), (B) K63 TUBE or (C) K48 TUBE HF. The multitude of high molecular weight proteins indicate the presence of conjugates of K48- and K63-linked polyUb.*

Ubiquitin Proteomics using TUBEs

A.



B.



Ubiquitin proteomics utilizing TUBEs A. **Workflow schematic of TUBE proteomics.** *Treated cells were enriched using TUBEs (TUBE1 and K48). The eluted polyubiquitylated proteins were pixelated by SDS-PAGE and digested with trypsin. Individual ubiquitylated proteins were identified by the presence of at least 2 peptides in at least 2 of 3 replicates. Ub linkage types were identified by K-ε-GG peptides corresponding to covalent Ub-Ub modification at a particular lysine. (B)* **Linkage distribution of polyubiquitin chains based on LC-MS/MS identification of ubiquitin remnant peptides.** *Ubiquitylated proteins were enriched from cell lysates using Magnetic TUBE1 (left), and K48 TUBE HF Biotin (right).*

Conclusions

A collection of linkage-specific TUBEs will dramatically improve current methods for studying ubiquitin chain linkages and their regulation/prevalence in disease states. Using a structure-based method, we were able to design highly specific UBDs. Domains that display selective binding specificity were utilized to construct linkage-specific TUBEs. Our data show how these TUBEs can be used to perform far-Western, pull-downs and ubiquitin proteomics. The expansion of the TUBE toolkit to include recombinant proteins that can discriminate between each of the Ub linkages would dramatically accelerate the pace of discovery in this important area of biology.