Deciphering the Ubiquitin Code with Poly-Ubiquitin Chain Selective Affinity Matrices

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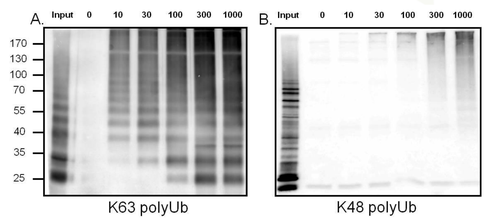
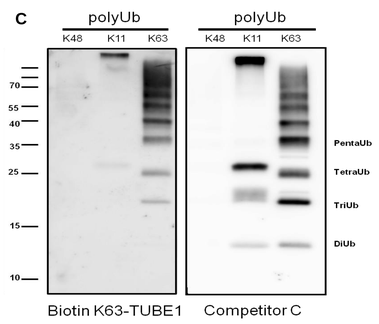
Abstract:

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.**

Post-translational modification of proteins by ubiquitin (Ub) is a versatile process, highly dynamic, and involved in nearly all aspects of biological functions in eukaryotes. Dysregulation of ubiquitylation has been implicated in plethora of pathological conditions such as developmental abnormalities, neurodegenerative diseases, and cancers. 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. Development of TUBEs that selectively bind K48-, K63-, and M1-linked poly-ubiquitylated proteins has helped understand the role of modified proteins in cell physiology. However, the roles of other Ub-linkages remain obscured mainly due to the lack of tools that specifically recognize them. To overcome this, we recently developed a novel microarray platform that facilitates the identification of unique Ub binding domains (UBDs) for rare Ub-linkages. This array contains GST-tagged versions of 140 known and predicted UBDs from over 8 different Ub interacting families. Our initial screen with mono-Ub as well as K48-linked diUb revealed several unique binders. For example, Ub-binding motif (UBM) of REV1L bound uniquely to mono-Ub. On the other hand, Ub-associated (UBA)-like domain of NSFL1C bound preferably to K48-linked diUb. Moreover, UBA domains of RAD23A and RAD23B interacted uniquely with K48-linked diUb, which is concordant with the published data. We also identified several proteins that non-selectively bind mono-Ub and K48-linked diUb, such as the UBA domain of NBR1.

Enrichment of Cellular PolyUb with K63 TUBE

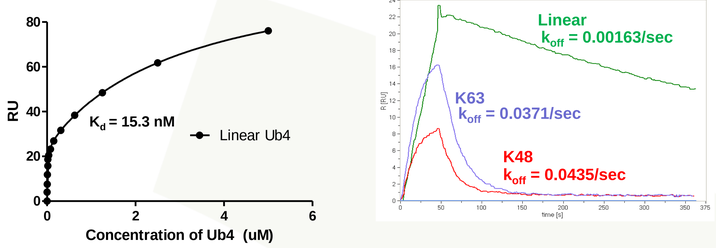




A-B. Pull-down Experiments using FLAG K63 TUBE. *K63 linked PolyUb chains (10 µg) were generated by yMMS2/Ubc13* ***(Panel A)*** *and K48 chains were generated by E2-25K* ***(Panel B).*** *The ubiquitin chains were pulled-down with indicated amounts of FLAG® K63-TUBE (nM) for 1.5 hours (4°C) and M2 FLAG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

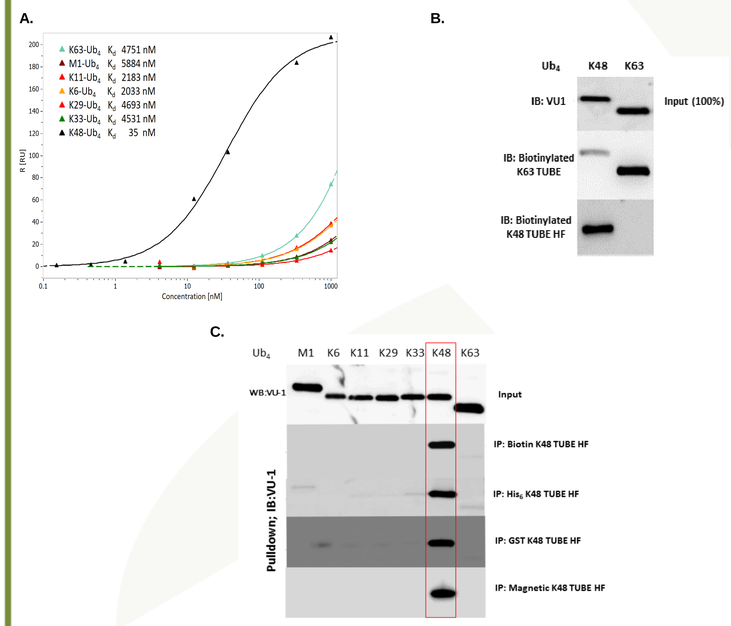
C. Comparison of K63 TUBE Specificity to Monoclonal Antibody. *PolyUb chains generated with either E2-25K (K48), yMMS2/Ubc13 (K63), or UBE2S (K11) were subjected to duplicate loading, SDS-PAGE analysis and Western blot. The blot was sectioned and probed as described above with either Biotin K63 TUBE 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



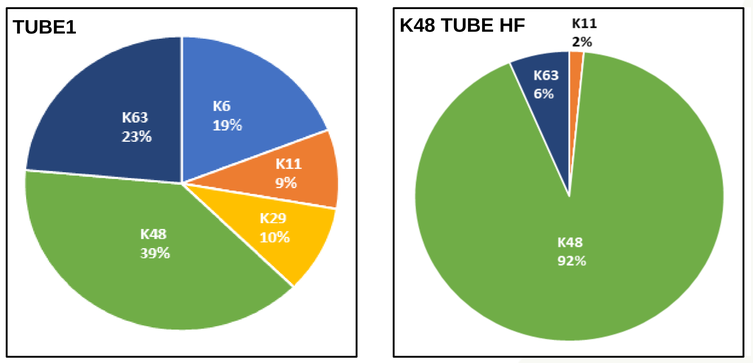
Determining Binding Affinity of M1 (Linear) TUBE. *His_6-tagged TUBEs were immobilized on a His Capture chip. His_6-SUMO was loaded onto the control surface. Injections of increasing amounts of tetra-ubiquitin (Ub_4) were analyzed. The binding curve and K_d was generated in PRISM. (Right) Injections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



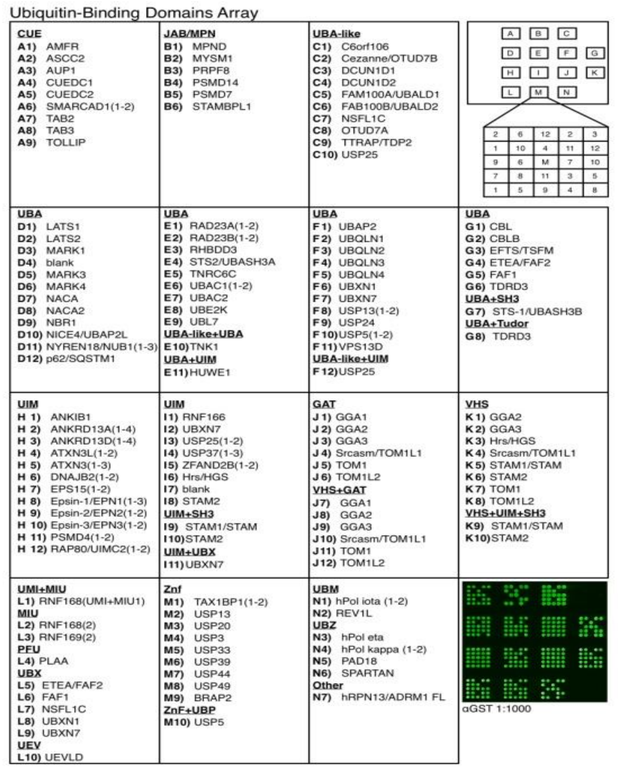
Determination of Binding Affinity and Specificity of K48 TUBE HF. *(A) SPR experiments using increasing amounts of Ub_4 were performed using His_6-K48 TUBE and His_6-M1-Ub_2 on the control surface. The binding curves and Kds were generated using Sierra Sensors analyzer software. (B) The detection of 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.*

Ubiquitin Proteomics using TUBEs



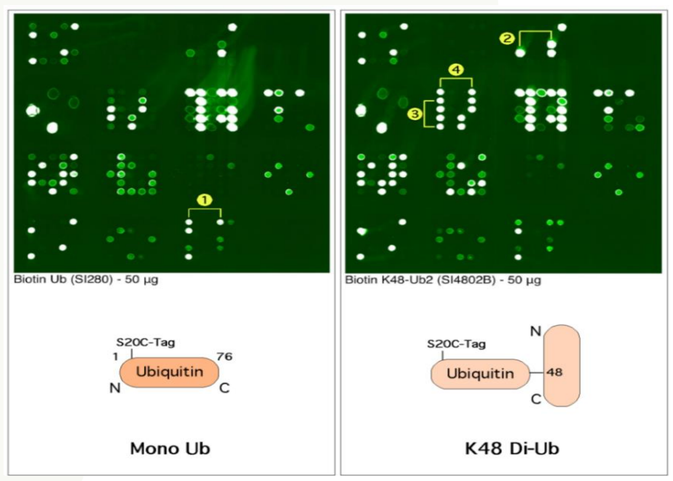
Ubiquitin proteomics utilizing TUBEs. *Treated cells were enriched using magnetic TUBEs (TUBE1 and K48 TUBE HF). The eluted polyubiquitylated proteins were separated by SDS-PAGE and pixelated into 4 sections followed by digestion with trypsin. Individual ubiquitylated proteins were identified by the 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.*

Ubiquitin Binding Domains Array



Microarray layout of ubiquitin readers. *GST-tagged ubiquitin binding domains are spotted in duplicate. The arrangement is such that no two duplicates are immediately next to each other. Bottom right: anti-GST was used to ensure equal spotting of each domain. Note that different grids have different number of proteins as described in the family of proteins in the table. These domains are selected after thorough searches and analysis of families of proteins that contain UBDs.*

Screening of UBD Microarray with Mono-Ub and K48-linked diUb



Selective binding of mono-ubiquitin and K48-linked di-ubiquitin to UBD Protein Microarray. *Monoubiquitin (left) and K48-linked di-ubiquitin (right) were incubated with the microarray of UBDs and tested for proof-of-concept experiments. The microarray grid is the same as shown in the middle panel. Numbered spots (yellow dots) indicate ubiquitin binding domains that show preliminary specificity (each domain is spotted in duplicate and shown by connected line).*

Conclusions:

A collection of linkage specific TUBEs will dramatically improve current methods for studying ubiquitin chain linkages and their regulation/prevalence in disease states. The array allowed us to identify unique binding partners for mono-Ub and K48-linked diUb, and we strongly believe that our future screens with other Ub-linkages will identify several selective binding partners for rare Ub-linkages. Domains that display selective binding specificity will be utilized to construct linkage-specific TUBEs. 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.