Bioengineering More Functional Enzymes One Amino Acid at a Time

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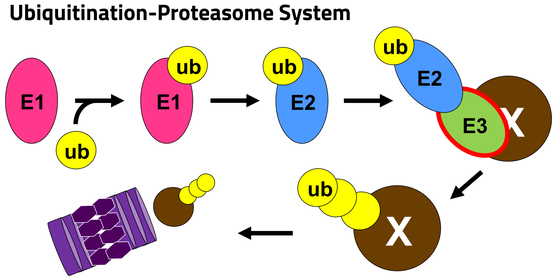
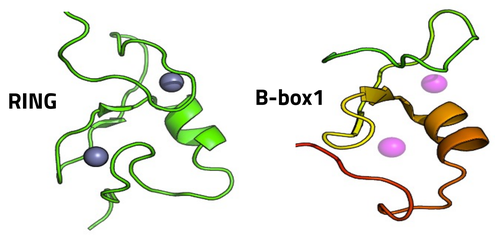
Introduction

To maintain cellular homeostasis, protein concentration are maintained in cell. Ubiquitination is the process where a protein, called Ubiquitin (Ub), is attached to proteins, Ubiquitinated proteins are degraded by the proteasome. The pathway is controlled by the E1, E2, and E3 ligases. The E3 enzyme target the specific proteins to be regulated or degraded by ubiquitination, RING and B-box1 E3 ligases are linked to cancer, neurodegenerative disorders, and developmental disorders.

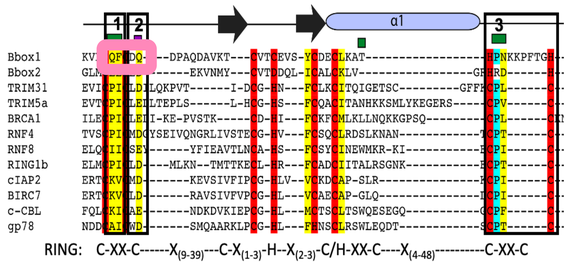
Ubiquitination-Proteasome System



TRIM-MID1 Domain



Comparing of B-box1 Domain Amino Acid Sequences



Location	RING Interaction Points	Bbox1 Wild-Type	Changes to Bbox1
Loop1	-C-XX-C-X**D/E**-	-C-QF-C-**DQ**-	-C-QF-C-**QD**-
Loop1	-C-X**I/V**-C-	-C-Q**F**-C-	-C-**QI**-C-

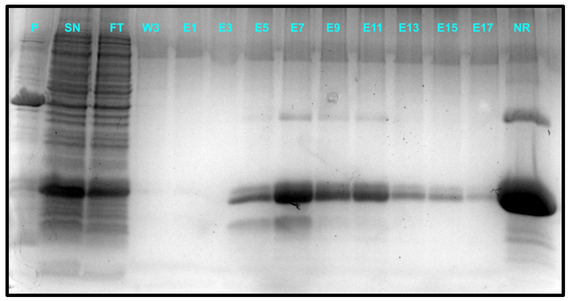
Methodology

Domain Mutating, Cloning, and Insertion in Bacteria

- Genes of interest are cloned into a plasmid vector, transformed into Escherichia coli (E. Coli) bacteria, BL21DE3
- Plasmids included: B-box1 wild-type, B-box1-D122Q, B-box1-F120I, B-box1-D122Q-F120I, RING wild-type, RB1 wild-type, RB1-D122Q, RB1-F120I, and RB1-D122Q-F120I

Isolating the Proteins of Interest

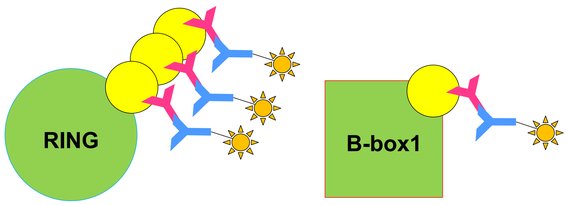
- Grow bacteria in Luria Broth and induce with IPTG (Isopropyl ß-D-1-thiogalactopyranoside)
- Lyse cells by sonication
- Purify target proteins via nickel-resin affinity purification
- Use gel electrophoresis to confirm successful protein purification and purity



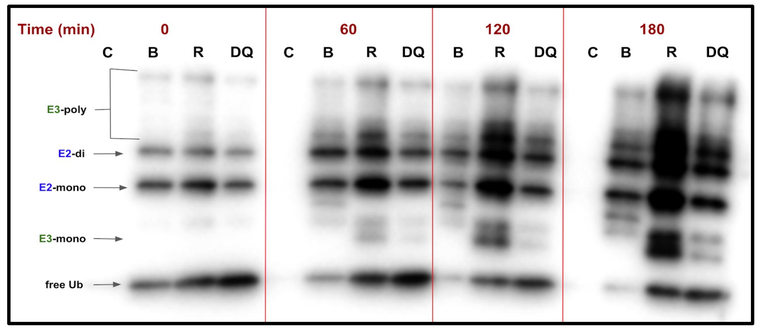
- Proteins are concentrated to dialyze out imidazole
- Flash-freeze proteins with liquid N for later use

Ubiquitination Assays

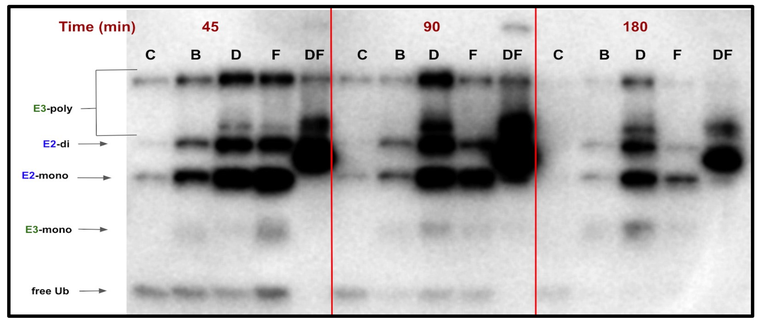
- For in vitro ubiquitination: MID1 E3 enzyme constructs were added to an assay with the E1 and E2 enzymes, mgATP, Ub, and allowed to incubate at 37
- Small aliquots of reaction were collected at specific times and analyzed by SDS-PAGE and Western Blot
- Membrane is blocked with BSA solution
- Anti-mouse IgG primary antibodies tareting HA-tag Ub
- Anti-mouse IgG secondary antibodies bind to primary antibody
- Chemiluminescence solutions are used to image the fluorescence-emitting secondary antibodies



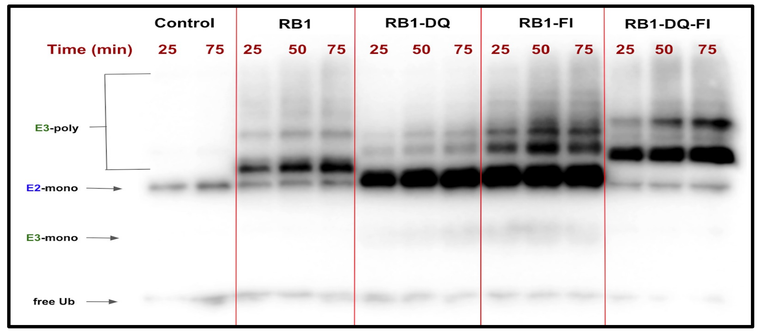
Results & Discussion



- **Western Blot of Ub assay of RING (R) B-box1 (B), and the D122Q mutant (DQ).**
- D122Q and RING domain show increased mono-ubiquitination and poly-ubiquitination activity compared to wild-type B-box.
- Wild-type RING still displays significantly more ubiquitination activity than D122Q.
- D122Q B-box1 is more active than wild-type B-box1 indicating mutation works.



- **Ubiquitination results of D122Q mutant (D), F120I (F) and D122Q-F120I double mutant (DF).**
- F122Q and D122Q/F120I display more ubiquitination activity than wild-type B-box and F120I.
- D122Q/F120I displays significantly more ubiquitination activity than D122Q



- **Ubiquitination results using the RING-B-box1 constructs with B-box1 mutations.**
- All mutants show enhanced ubiquitination activity compared to wild-type RB1.
- RB1-F120I and the double mutant display significantly more activity.
- Mono-ubiquitination is limited and all reactions display lower than expected ubiquitination activity.

Conclusions

Although the experiments need to be repeated for D122Q and F120I single mutants, the D122Q-F120I double mutants displays increased ubiquitination activity compared to wild-type B-box1 and RB1, as well as the other mutants studied in this project.

The increased activity highlighted by this double mutant can inform future studies on the impact of RING's amino acids at the 120 and 122 positions. These studies show that proteins have altered functionality from differences in just a few amino acids. Mutating the B-box1 domain with a few conserved amino acids found in specific locations in RING domain confirm the importance of E3 ligase activity.

References

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