Introduction

- NF-κB essential modulator (NEMO) and Optineurin (OPTN) are two proteins which rely on Ubiquitin to carry out the degradation of other macromolecules
- NEMO, a regulatory subunit, is ubiquitinated, beginning a signal pathway leading to transcription of immune-response genes
- OPTN binds to ubiquitinated substrates, leading to autophagy of degraded proteins or structures (prominently in nervous tissue)
- Mutations (OPTN and Ubiquitin), phosphorylation, and oligomerization state (Ubiquitin) have been shown to impact protein ubiquitination affinity
- The purpose of this project was to determine the effects of such variations on ubiquitination affinities, and explain effects structurally (via X-ray crystallography)

Keywords: NEMO, OPTN, Ubiquitin.

Methods

1. Transform E. coli mutants with plasmid encoding for protein of interest, GST or His tag, antibiotic resistance (lac operon)
2. Grow E. coli in LB + Antibiotic media, scale up to several liters (37 °C)
3. Express protein overnight with IPTG (25 °C)
4. Spin down cells, lyse
5. Attach tag-binding GS48 or TALON substrates to protein tags
6. Purify and concentrate (Nickel columns, FPLC, Nanodrop, SDS-PAGE)
7. Crystallize and collect structural data (X-ray diffraction, affinity data, Resonance)

Results

OPTN

Figure 1. Wild-type Optineurin (OPTN) binding affinity with mono-ubiquitin mutants (UbV) and linear di-ubiquitin dimer (LUb2). Bottom-right numbers correspond to dissociation constants. Data acquired using Bioptix Surface Plasmon Resonance machine. Error bars represent standard deviation. For each concentration, N = 3.

Figure 2. OPTN Mutants (S473D, S473E) binding affinities with phosphorylated and mono-ubiquitylated LUb2 dimer. Bottom-right numbers correspond to dissociation constants. Data acquired using Bioptix Surface Plasmon Resonance machine. Error bars represent standard deviation. For each concentration, N = 3.

Figure 3. OPTN-S473D + LUb2 crystals. Crystals were acquired via 1:1 molar co-crystallization in 96-well plates, using the Art Robbins Crystal Gryphon machine, and 1-week incubation in 20 °C conditions.

NEMO

Figure 4. NEMO binding affinity with UbV and LUb2. Bottom-right numbers correspond to dissociation constants. All differences are significant, except C-D. Data acquired using Bioptix Surface Plasmon Resonance machine. Error bars represent standard deviation. For each concentration, N = 3.

Figure 5. NEMO + UbV 229 crystals. Crystals were acquired via 1:1 molar co-crystallization in 96-well plates, using the Art Robbins Crystal Gryphon machine, and 1-week incubation in 20 °C conditions.

Conclusions

- OPTN mutants bind to LUb2 with higher affinity than WT
  - Negative charges on Asp and Glu are “phospho-mimetic”
- Phosphorylation of LUb2 enhances binding affinity in OPTN-WT and mutants, but not in NEMO
- Mono-ubiquitin mutants show higher affinity to NEMO than LUb2, also bind to OPTN
- OPTN-S473D + LUb2 structure will be first published structure involving OPTN mutant
  - OPTN-WT structure not yet published
- Crystals not obtained for OPTN-S473E + LUb2, nor NEMO + UbV 231

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