Characterizing the Catalytic Activity and Specificity of RNF168 using Targeted Mass Spectrometry and Ubiquitination Stoichiometry-Based Quantitative Analysis

Introduction

RNF168 is a site specific E3 ligase whose ubiquitination activity triggers the recruitment of particular DDR proteins. While previous research has revealed the mechanisms and principles of ubiquitination, the broad spectrum of DNA damage dependent ubiquitin substrates for RNF168 is still unknown.

Isotopically Balanced Quantification of Ubiquitination (IBAQ-Ub), is a method that quantify the absolute abundance of ubiquitination. IBAQ-Ub and in vitro ubiquitination enzymatic assays were conducted to characterize the site-specific ubiquitination dynamics and linkage specificity of RNF168 in DDR.

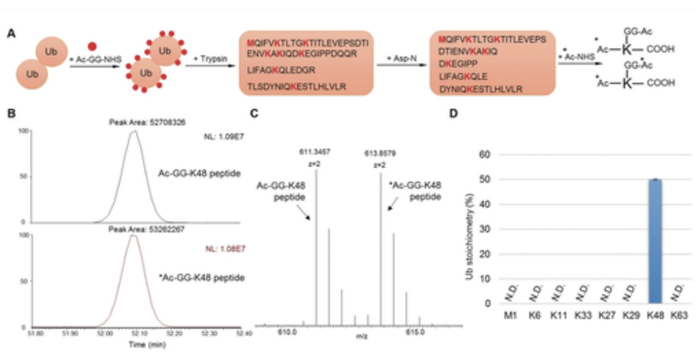

Figure1: Flow chart for mechanism of iBAQ-Ub.

Reference: Schwertman, P., Bekker-Jensen, S. & Mailand, N. Regulation of DNA double-strand break repair by ubiquitin and ubiquitin-like modifiers. *Nat. Rev. Mol. Cell Biol.* 17, 379– 394 (2016).
Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J. & Stahl, F. W. The double-strand-break repair model for recombination. Cell 33, 25–35 (1983).
Li, Y. et al. A Quantitative Chemical Proteomics Approach for Site-specific Stoichiometry Analysis of Ubiquitination. Angew. Chemie Int. Ed. 58, 537–541 (2019).
https://www.sinobiological.com/resource/protein-review/gst-tag-protein-expression

Method

1. *GST-tagged protein purification*
   - 1.1. EX-E2013-B03 (GST-RNF168) was acquired commercially and transfected to *E.coli*.
   - 1.2. IPTG induction and Sepharose 4B Beads purification with three wash and three elution steps
   - 1.3. Three wash and three elution for two different strains were analyzed using SDS-PAGE gel
   - 1.4. western-blot using sheep RNF168 antibody.
2. *AcGG-NHS tag*
   - 2.1. RNF168 ubiquitination reaction, one for 1.5 hour and another for overnight
   - 2.2. Light-tagged and trypsin digest overnight and heavy tagged the second day
   - 2.3. Verify the tag efficiency using tandom spectrometry

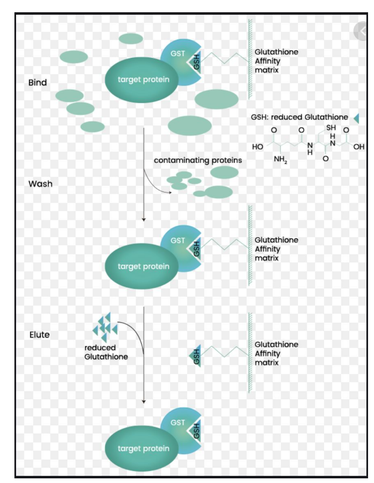

Figure2: Flow Chart for GST-Tagged purification.

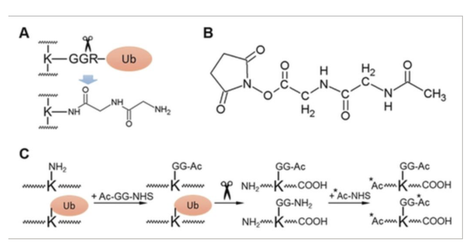

Figure3: Mechanism for AcGG-NHS heavy and light tagg

Result

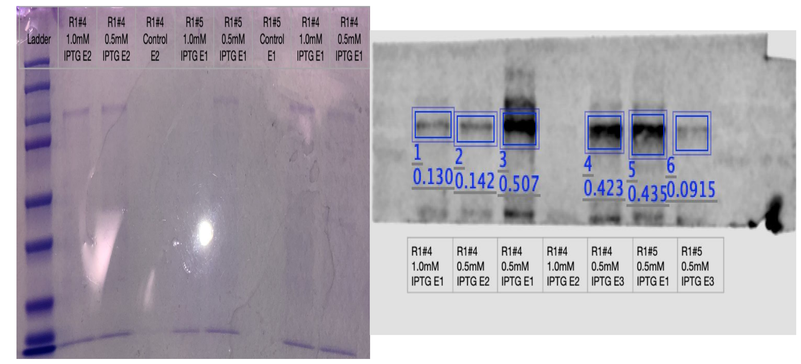

Figure4: The top figure shows the SDS-PAGE result for GST purification, the band should be 91kDa. The figure on the righ Showing the result for western blot using anti-RNF168

The the molecular weight for RNF168 protein is 65kDA, and the GST tag has 28 kDa, thus it should show a 91kDa on a SDS-PAGE gel. The signal can be seen in six different elution samples, thus verified these suspected bands in SDS-PAGE results as our target protein, RNF168.

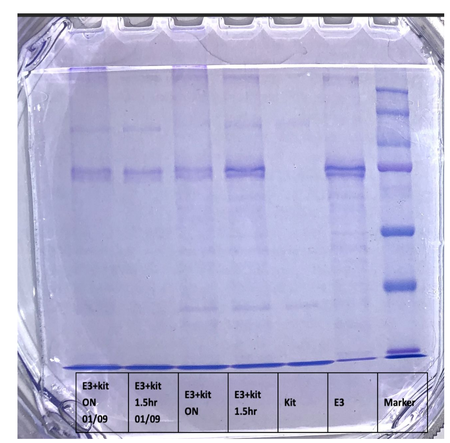

Figure5: Result of RNF168 ubiquitination reactions.

Stronger ubiquitination within overnight reaction trail, since smeariness of the overnight reaction band is more substantial.