In Vitro Reconstituted Ubiquitylation Assays for Mechanistic Studies of E3 Ligases

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Introduction

Ubiquitylation is a widespread post-translational modification that regulates protein stability and cell signaling. Substrate specificity is conferred by more than 600 putative E3 ubiquitin ligases spanning several mechanistically distinct families. RING and U-box ligases position a ubiquitin-charged E2 relative to the substrate for direct ubiquitin transfer, whereas HECT and RBR ligases form a transient E3-ubiquitin thioester intermediate before modifying the substrate.

Although CRBN- and VHL-based complexes remain the most widely used E3 ligase systems in targeted protein degradation, the range of ligases being explored continues to expand. Proximity assays can identify native or small-molecule-induced interactions between an E3 ligase and a protein of interest, but do not directly determine whether the resulting complex supports substrate ubiquitylation. Fully reconstituted biochemical assays measure this functional outcome and help distinguish protein recruitment from catalytic activity.

Reconstitution must be adapted to the mechanism and regulation of each E3 ligase. Appropriate E2 enzymes, activation steps, cofactors, and substrate formats can each influence observed activity. Here, we present *in vitro* systems for the U-box ligase CHIP, the RBR ligase Parkin, and the HECT ligase E6AP that recapitulate their distinct biological and catalytic requirements.

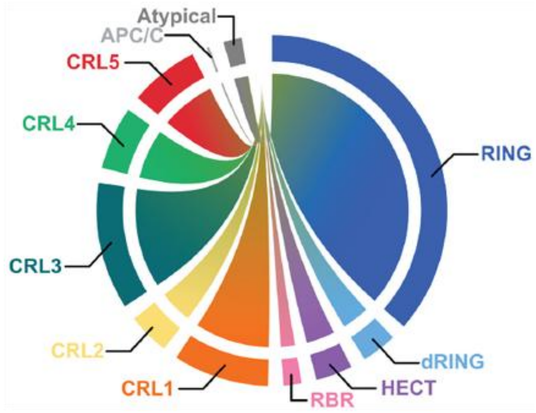
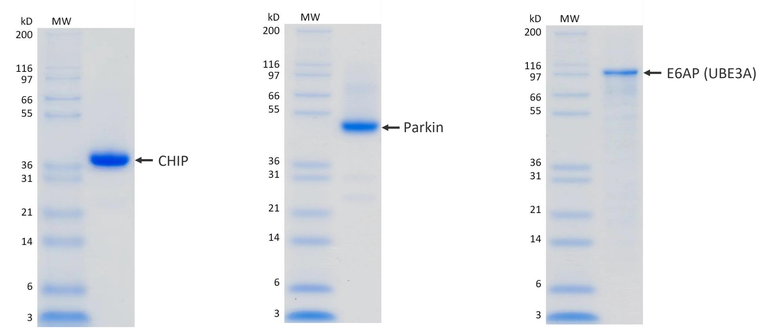


Image credit: N.K. Chua and R. Feltham, WEHI

Reagents

Every Trilogie reagent is designed for defined experimental applications. Our enzymes, proteins, and reagents are functionally validated using relevant biochemical assays, including ternary proximity and *in vitro* ubiquitylation assays.



Reconstituted Ubiquitylation Assays Require Mechanistic Understanding for Development and Optimization

CHIP is a dRING (U-box) E3 ligase that links molecular chaperones to ubiquitin-mediated protein quality control.

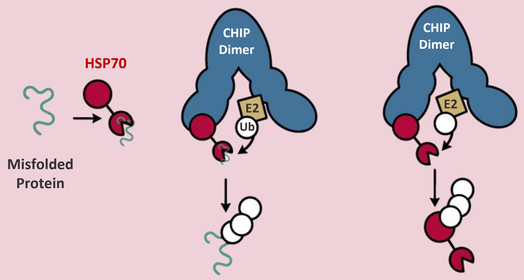


Figure 1. Chaperone-coupled CHIP activity.
The TPR domain of CHIP interacts with heat shock proteins, enabling ubiquitylation of chaperone-bound client proteins (*1*). CHIP can also ubiquitylate HSP70, potentially contributing to regulation of chaperone abundance following client processing.

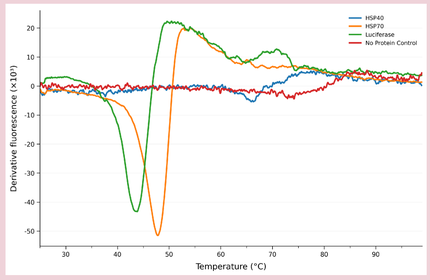


Figure 2. Thermal melt profiles of luciferase, HSP40, and HSP70.
DSF was used to determine thermal melt profiles and inform the denaturation temperature used in the CHIP ubiquitylation assay.

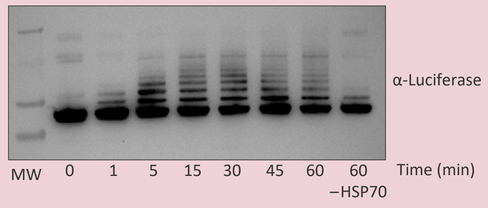


Figure 3. Efficient *in vitro* polyubiquitylation of denatured luciferase by CHIP requires HSP70.
All *in vitro* reactions contained CHIP, E2, E1, ubiquitin, HSP70, HSP40, and heat-denatured luciferase and were visualized by Western blotting with an anti-luciferase antibody.

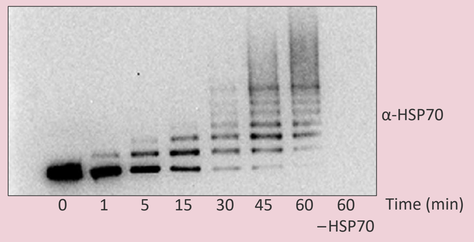


Figure 4. HSP70 is ubiquitylated by CHIP with delayed kinetics compared to the luciferase client protein.
Same experiment as Figure 3, visualized by Western blotting with an anti-HSP70 antibody.

Parkin is an RBR E3 ligase that regulates mitochondrial quality control through PINK1-dependent mitophagy and is a key protein in Parkinson's disease research.

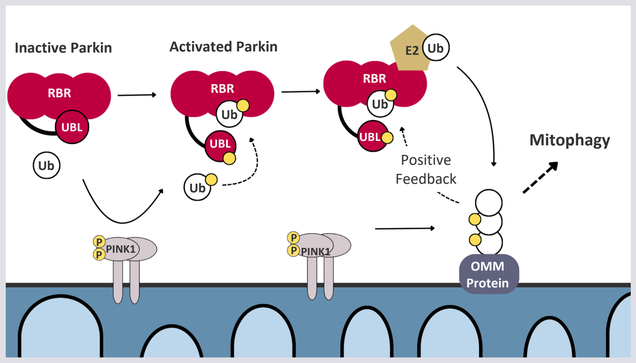


Figure 5. PINK1 regulates Parkin activity through direct phosphorylation and by increasing phospho-ubiquitin at the mitochondrial membrane.
PINK1 phosphorylates ubiquitin and Parkin's ubiquitin-like domain at Ser65, and these events cooperate to activate Parkin. A positive feedback loop of mitochondrial protein ubiquitylation and ubiquitin phosphorylation amplifies the phospho-ubiquitin signal, promoting OPTN- and NDP52-mediated mitophagy (*2*).

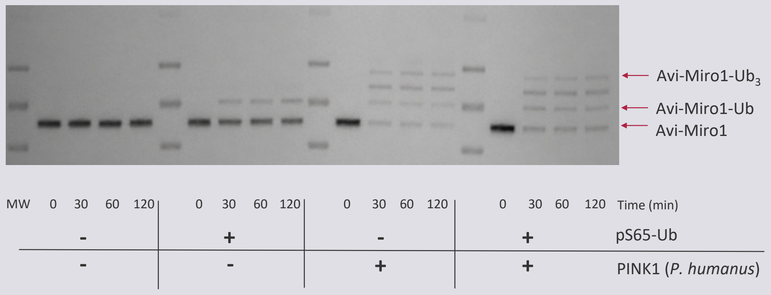


Figure 6. Phospho-Ub partially activates Parkin, while PINK1 kinase-mediated phosphorylation of Parkin further activates the ligase, enabling robust polyubiquitylation of Miro1.
Transmembrane domains of PINK1 and Miro1 are not included in the constructs. *P. humanus* PINK1 was used because recombinant human PINK1 did not provide sufficient kinase activity under *in vitro* assay conditions.

E6AP (UBE3A) is a HECT E3 ligase that plays essential roles in neuronal development, while also mediating HPV E6-dependent degradation of p53 in papillomavirus-associated cancers.

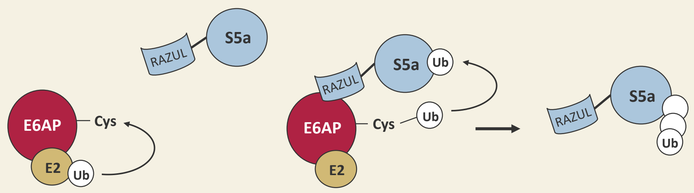


Figure 7. Mechanism of S5a ubiquitylation by HECT E3 ligase E6AP
HECT ligases accept Ub on a catalytic cysteine to form a transient E3-ubiquitin thioester intermediate. The Ub is then transferred to a lysine residue on a substrate. S5a (hRpn10) is a proteasome subunit that directly binds E6AP through its RAZUL domain (*3*). S5a can also serve as an *in vitro* ubiquitylation substrate for E6AP.

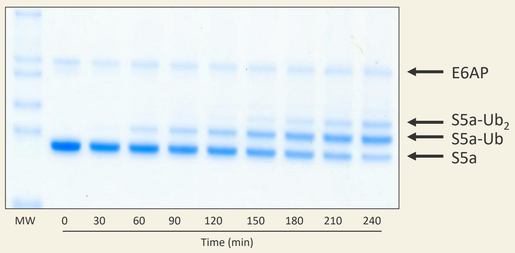


Figure 8. Ubiquitylation of S5a by E6AP
All *in vitro* reactions contained E6AP, E2, E1, ubiquitin, and S5a and were visualized by Coomassie staining.

Conclusions

- Functional validation of recombinant E3 ligases requires assays tailored to their distinct catalytic and regulatory mechanisms; protein purity alone is insufficient to demonstrate activity.
- In a chaperone-coupled system, HSP70 supported CHIP-mediated ubiquitylation of denatured luciferase and was itself ubiquitylated with delayed kinetics. Parkin and E6AP were evaluated using systems incorporating PINK1/phospho-ubiquitin-dependent activation and S5a ubiquitylation, respectively.
- These systems directly assess E3 ligase activity and provide platforms for investigating biological mechanisms and evaluating small-molecule degraders.

About Trilogie Bioscience

At Trilogie Bioscience, our mission is to accelerate research and discovery in ubiquitin biology, targeted protein degradation, and induced proximity. By providing high-quality and **functionally validated** recombinant proteins, *in vitro* assay development, and custom projects, we help researchers generate robust data.

Hardware and Additional Reagents
Second-generation AlphaLISA beads ("Omega" beads) including streptavidin-Sensibeads and anti-His_6-Chemibeads were from Biosignal2.

References:
1. Smith *et al*. 2013, *Biochemistry*
2. Pickrell and Youle 2016, *Neuron*
3. Buel *et al*. 2020, *Nature Communications*