In Vitro Reconstituted Ubiquitylation Assays for Mechanistic Studies of Cullin-RING Ligases

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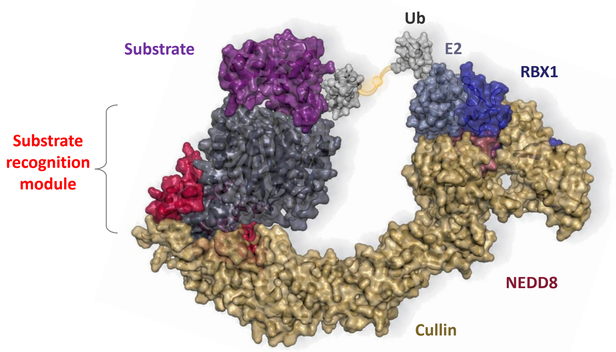
Introduction

Cullin-RING ligases (CRLs) comprise the largest family of E3 ubiquitin ligases and use hundreds of substrate receptors to regulate protein stability and cell signaling. Unlike HECT and RBR ligases, which form catalytic E3–ubiquitin intermediates, CRLs are modular, multi-subunit RING ligases assembled around one of seven canonical mammalian Cullin proteins. Each Cullin serves as a central scaffold that positions the E2-bound RING subunit relative to the substrate-recognition machinery. CRL activity therefore depends on proper complex assembly, Cullin neddylation, compatible ubiquitin-conjugating enzymes, and productive substrate recruitment.

CRLs are also widely exploited in targeted protein degradation. 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 assays measure this functional outcome while enabling controlled investigation of CRL regulation and substrate recognition.

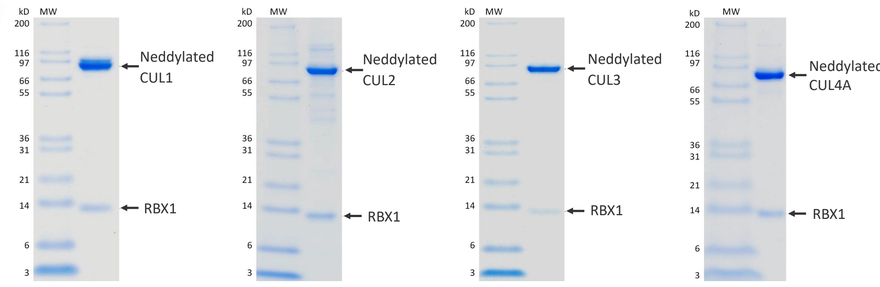
In vitro ubiquitylation assays minimally require a functional E3 ligase, an appropriate E2 (or E2 combination), E1, ubiquitin, recombinant (or IP'd) target of interest, and Mg-ATP solution. Readouts for the assays include Western analysis, direct gel staining, and in some cases, a proximity assay based on TUBE reagents.

Generic structural architecture and componentry of an active CRL



Reagents

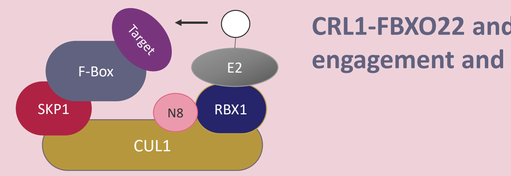
Every reagent from Trilogie is designed with specific applications in mind. Our enzymes, proteins, and reagents are not just pure; they are functionally tested in meaningful ways, including ternary proximity assays, *in vitro* ubiquitylation assays, and others. Cullins need to be properly folded to be enzymatically neddylated, a post-translational modification that activates CRLs and promotes productive ubiquitin transfer. The shift in molecular weight allows selective purification of neddylated Cullins.



Proximity assays report on complex formation while ubiquitylation assays report on functional activity.

Reconstituted assays reveal how E2 selection, cofactors, substrate format, and other factors influence productive Ub transfer, enabling degrader molecule screening and SAR.

CRL1-FBXO22 and CRL1-FBXO31: Emerging TPD recruiters through covalent engagement and C-terminal amide degron mimetics, respectively. [1,2]



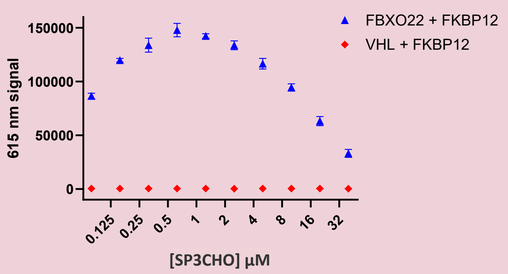


Figure 1. SP3CHO-mediated ternary complex formation of FKBP12 and FBXO22/SKP1.
Classic heterobifunctional hook effect observed. Second-generation AlphaLISA beads ("Omega" beads) were purchased from Biosignal2. VHL/EloB/EloC is a negative control, as it should not bind SP3CHO.

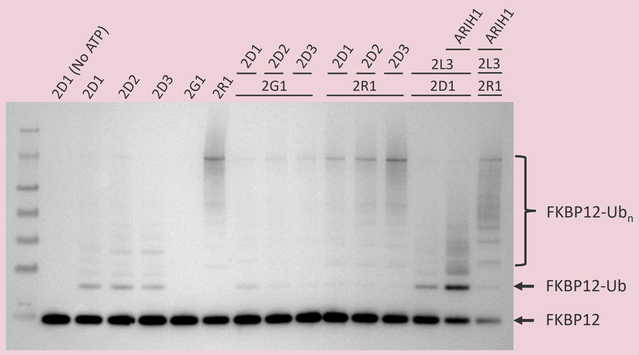
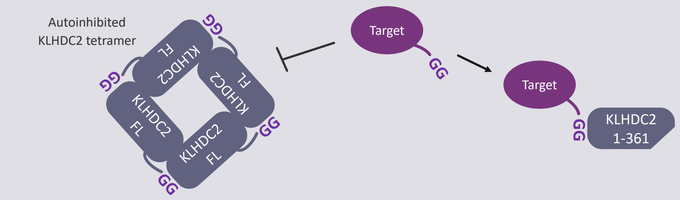
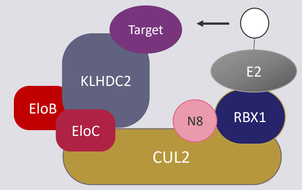


Figure 2. E2 scouting identifies combinations for efficient PROTAC-mediated ubiquitylation of FKBP12 by CRL1-FBXO31.
All *in vitro* reactions contained FBXO31/SKP1, (NEDD8)CUL1/RBX1, E2, E1, ubiquitin, and biotinylated FKBP12. ARIH1 is an RBR E3 ligase that cooperates with UBE2L3 and neddylated RBX1-containing CRLs to catalyze substrate priming. Samples were visualized with streptavidin-HRP.

CRL2-KLHDC2 recognizes C-terminal Gly-Gly degrons and has a complex mechanism of substrate-mimetic autoinhibition. [3]





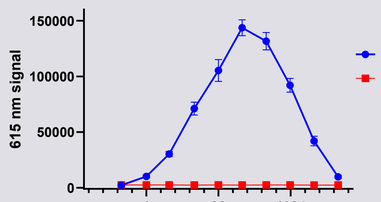




Figure 3. C-terminally truncated KLHDC2 relieves auto-inhibition and allows for measurement of binary binding of a biotinylated SELK peptide.
Second-generation AlphaLISA beads ("Omega" beads) were purchased from Biosignal2.

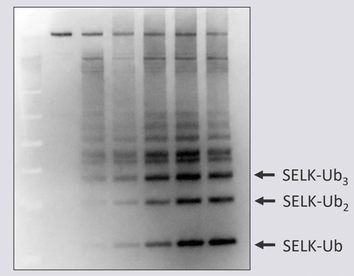
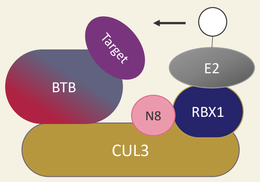


Figure 4. *In vitro* reconstitution of neddylated CRL2-KLHDC2 enables efficient polyubiquitylation of a SELK C-degron peptide.
All *in vitro* reactions contained KLHDC2/EloB/EloC, (NEDD8)CUL2/RBX1, E2, E1, ubiquitin, and biotinylated SELK peptide. Samples were visualized with streptavidin-HRP.

CRL3-SPOP is a regulator of many transcription factors, and SPOP mutations are associated with multiple cancers. [4]



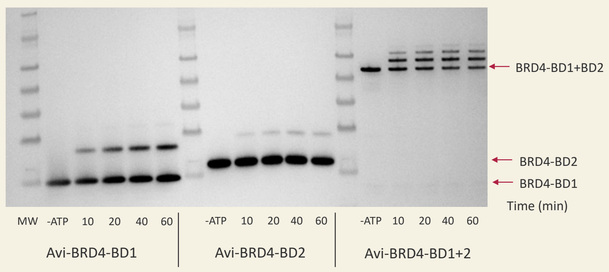


Figure 5. Linker region between BRD4-BD1 and BRD4-BD2 enhances ubiquitylation by SPOP.
All reactions contained SPOP, (NEDD8)CUL3/RBX1, E2, E1, biotinylated BRD4, and ubiquitin. Samples were visualized with streptavidin-HRP.

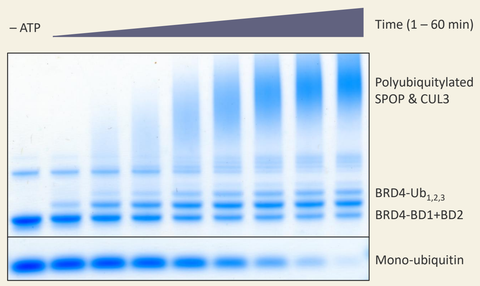
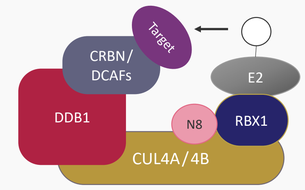


Figure 6. Robust CUL3 and SPOP autoubiquitylation observed even in the presence of BRD4-BD1+BD2 native substrate.
All reactions contained SPOP, (NEDD8)CUL3/RBX1, E2, E1, biotinylated BRD4, and ubiquitin. Samples were visualized by Coomassie staining.

CRBN and other DCAFs are substrate receptors for CRL4 and are most commonly utilized for targeted protein degradation.



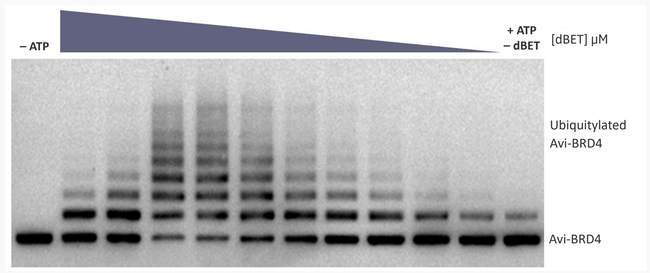


Figure 7. dBET1-dependent *in vitro* polyubiquitylation of BRD4 by CRL4-CRBN. [5]
All *in vitro* reactions contained CRBN/DDB1, (NEDD8)CUL4A/RBX1, E2, E1, ubiquitin, and biotinylated BRD4. Samples were visualized with streptavidin-HRP.

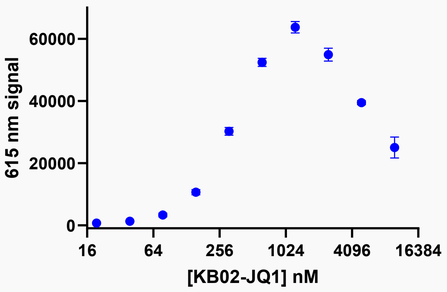


Figure 8. Small-molecule-mediated ternary complex formation with DCAF16/DDB1/DDA1 and BRD4.
Classic heterobifunctional hook effect observed. Second-generation AlphaLISA beads ("Omega" beads) were purchased from Biosignal2.

Conclusions

- Functional CRL reconstitution depends on Cullin neddylation, compatible E2 enzymes, accessory factors, and proper substrate format.
- Proximity and ubiquitylation assays provide distinct information; detection of an interaction does not necessarily predict substrate ubiquitylation.
- Tailored CRL systems enable direct functional evaluation of native and degrader-induced substrate recruitment. These studies are critical for both drug discovery and understanding fundamental aspects of ubiquitin signaling and CRL regulation.

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. Kagiou, C., *et al*. 2024, *Nature Communications* **2**. Zhang, C., *et al*. 2026, *JACS* **3.** Scott, D.C., *et al*. 2023, *Molecular Cell* **4.** Dai, X., *et al*. 2018, *Nature Medicine* **5.** Winter, G., *et al*. 2015, *Science*