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UBIQUITIN SIGNALLING

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Post-translational modification with ubiquitin (Ub) initiated by sequential actions of Ub-activating enzyme (E1), Ub-conjugating enzyme (E2) and Ub ligase (E3) regulates diverse cellular processes, including signal transduction, cell cycle progression, apoptosis and gene transcription. Deregulation in the Ub pathway is often associated with human pathogenesis, including cancer. Our group uses structural biology and biochemical approaches to study the enzymes in the Ub pathway to understand their regulation, mechanistic function and mutation-induced deregulation. We anticipate that the knowledge gained from our structural studies will assist in the development of selective therapeutic targets within the Ub pathway.

Ubiquitin conjugation cascade

Covalent attachment of Ub involves three key enzymes, namely E1, E2 and E3 (Figure 1). E1 adenylates Ub's C-terminus in the presence of Mg^{2+} and ATP, followed by formation of a covalent thioester intermediate with Ub. E1 then recruits an E2 and transfers the thioesterified Ub to the E2's catalytic cysteine, forming an E2~Ub thioester intermediate (~ indicates the thioester bond). E3 generally consists of an E2-binding module (HECT, RING, RBR or U-box domain) and a protein-protein interaction domain that can recruit the substrate directly or indirectly. With this configuration, E3 recruits E2~Ub and the substrate to promote Ub transfer from the E2 to a lysine side chain on the substrate. In humans, there are ~600 RING E3s, and we are interested in uncovering their regulation and function and exploring the Ub system for cancer therapeutics.

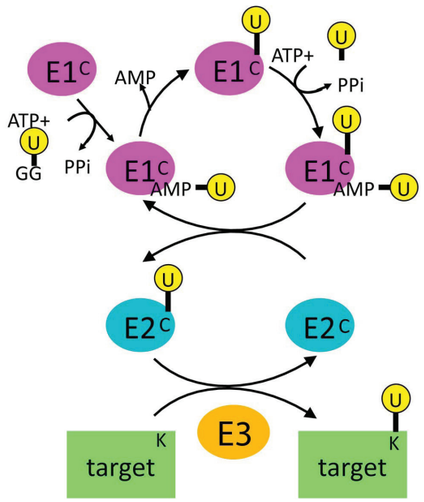


Figure 1
Enzymatic cascade for Ub modifications

Deregulation in CBL ubiquitin ligase

CBL proteins (CBLs) are RING E3s that negatively regulate receptor tyrosine kinases, tyrosine kinases and other proteins by promoting their ubiquitination and degradation by the proteasome or lysosome. Mutations in CBL have been observed in human patients with myeloproliferative diseases. Investigating the mechanism by which CBL mutants exert oncogenesis, we showed that CBL mutants inactivated E3 activity, thereby functioning as an adaptor to recruit other proteins such as CIN85 to elicit oncogenic signalling. Mechanistically, CBL mutants bound to receptor tyrosine kinases such as EGFR, which led to phosphorylation of CBL mutants' C-terminal tyrosines. Phosphorylated tyrosines induced conformational changes that enabled CBL mutant-CIN85 interaction. CBL mutants could not ubiquitinate CIN85, leading to deregulated CBL-CIN85 signalling which altered transcriptome landscape, that in turn upregulated PI3K-AKT signalling cascade to drive oncogenesis (Ahmed *et al.,* 2021, Oncogene). Our ongoing work is aiming to develop therapeutics targeting CBL mutant-EGFR interaction and thereby reducing the oncogenic property of CBL mutant.

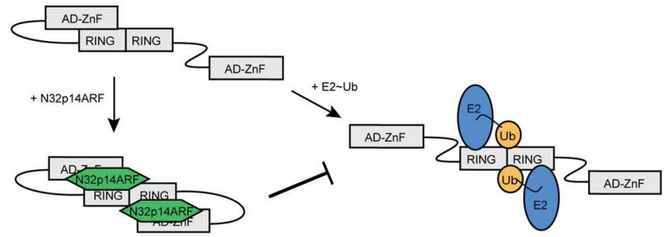
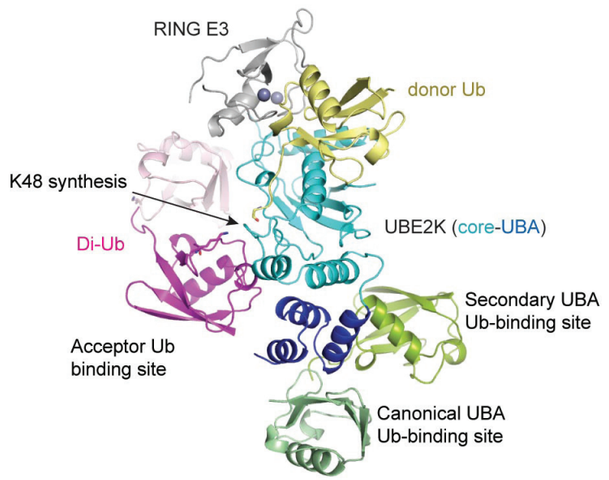


Figure 2
Regulation MDM2 E3 activity by p14ARF

MDM2 RING domain: regulation and targeting

MDM2 is a RING E3 that plays a critical role in the regulation of the p53 tumour suppressor protein by inhibiting p53's transcriptional activity and targeting it for proteasomal degradation. Approximately 50% of human cancers retain wild-type p53, but p53 expression is usually kept low due to amplification of MDM2 gene. Inhibition of MDM2-p53 interaction stabilises p53, resulting in elevated p53 activity that promotes cell cycle arrest and apoptosis in cancer cells. Small-molecule inhibitors targeting MDM2's N-terminal p53-binding domain are in clinical trials, but these compounds exhibit high on-target toxicities. We showed that inhibition of MDM2's E3 activity via mutagenesis led to p53 stabilisation but MDM2 mutants could still bind p53 and restrain its transcriptional activity. Upon stresses their interaction was abrogated leading to rapid p53 activation (Nomura *et al.,* 2017, Nature Structural and Molecular Biology). Expression of MDM2 E3-inactive mutant was tolerated in adult mice, despite high levels of p53. Upon γ-irradiation, p53 activity was rapidly activated in various tissues, but most tissues were able to dampen p53 activity and regained homeostasis, suggesting inhibition of MDM2 E3 activity might reduce on-target toxicities (Humpton *et al.,* 2021, Genes & Development). In an effort to target MDM2 E3 activity, we showed that MDM2 adopted an autoinhibited conformation where its acidic-zinc finger regions formed intramolecular interaction with the RING domain to perturb its E2~Ub binding affinity and E3 activity. p14ARF is a negative regulator of MDM2 and binds to MDM2's acidic region. We showed that binding of p14ARF to MDM2's acidic region strengthened MDM2's intramolecular interaction and massively inhibited its E3 activity (Kowalcyzk *et al.* 2022, Life Science Alliance). Our study provides the basis for p14ARF-mediated inhibition of MDM2 E3 activity (Figure 2) and reveals strategies for targeting MDM2 RING domain.

Figure 3
Structure of RNF38 RING domain bound to UBE2K-Ub/K48-Ub_2 complex



Mechanism of K48-linked polyUb chain synthesis

The K48-linked polyUb chain acts as a signal that targets protein substrates for proteasomal degradation. While the enzymes that assemble K48-linked polyUb chain are known, the mechanism of Ub chain synthesis remains elusive. We studied one of the E2 enzymes, UBE2K, that selectively catalyses K48-linked polyUb chain formation. To visualise this reaction, we chemically trapped UBE2K covalently linked to donor Ub and acceptor K48-linked di-Ub, where the C-terminus of donor Ub was linked to UBE2K's active site cysteine and K48 of the acceptor di-Ub was linked to an UBE2K active site residue. We then determined the crystal structure of this cross-linked UBE2K complex and a RING E3 (Figure 3). We performed various NMR analyses and mutagenesis coupled with biochemical assays to validate our structure and demonstrated that our structure approximated the transition state of the K48-linked Ub chain synthesis. Our structure revealed that UBE2K active site residues and the C-terminal Ub-associated (UBA) domain bound the acceptor Ub and oriented its K48 toward the UBE2K–Ub active site for catalysis. Importantly, the UBE2K active site residues imparted K48-linked specificity whereas the UBA domain functioned to stabilise the conformational flexibility of acceptor Ub. Unexpectedly, our structure unveiled multiple Ub binding surfaces on the UBA domain (canonical, secondary and acceptor; Figure 3). We showed that this multivalent Ub binding feature served to bring UBE2K to Ub-primed substrate (substrate modified with Ub). By localising UBE2K to Ub-primed substrates, where Ub concentration was enriched, weak acceptor Ub affinity could be overcome to accelerate Ub chain extension. Moreover, we showed that UBA domain exhibited a preference for K63-linked polyUb chain as the acceptor and thereby promoted branched K48-K63 polyUb chain formation. Our work explains the molecular basis for K48-linked Ub chain synthesis and how UBA domain promotes processive polyUb chain formation (Nakasone *et al.* 2022, Nature Chemical Biology).

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