

UBIQUITIN SIGNALLING



Group Leader
Danny Huang

Associate Scientist
Lori Buetow

Research Scientist
Feroj Syed

Scientific Officer
Michael McIlwraith

Graduate Students
Emily Dearlove
Shijie Wang
Alice Wicks'

'BBSRC CTP

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 Mg²⁺ 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.

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

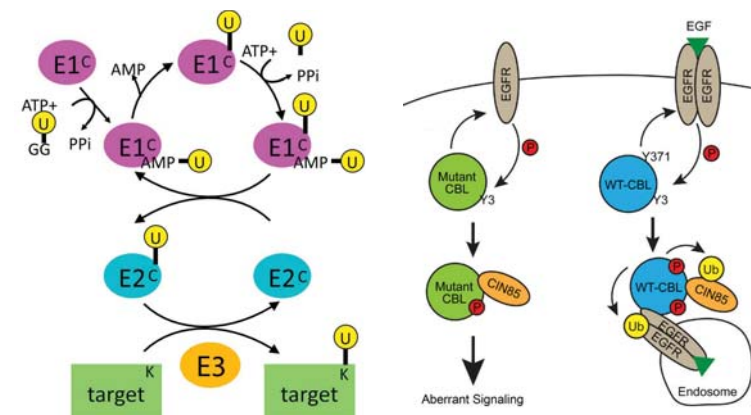
mutant-CIN85 interaction. CBL mutants could not ubiquitinate CIN85, leading to deregulated CBL-CIN85 signalling which altered transcriptome landscape, that in turn upregulated PI3K-AKT signalling cascade to drive oncogenesis (Ahmed *et al.*, 2021, *Oncogene*) (Figure 2). Over the past year, we have characterized an inhibitory molecule that binds CBL mutants and block its oncogenic property in cells and in a mouse xenograft model. Ongoing works are to explore the potential of this molecule in both WT and mutant CBL-driven cancers.

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

Figure 1. Enzymatic cascade for Ub modifications

Figure 2. Model showing mechanism of action of CBL mutant in driving oncogenesis



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 (Kowalczyk *et al.*, 2022, *Life Science Alliance*). Our study provides the basis for p14ARF-mediated inhibition of MDM2 E3 activity (Figure 3) and reveals strategies for targeting MDM2 RING domain. Currently, we are developing MDM2 RING inhibitors via protein design.

DELTEX ubiquitin ligases

We have characterised the DELTEX family of ubiquitin ligases. They harbour a conserved C-terminal RING domain followed by a DELTEX C-terminal domain (DTC). Our work revealed that the DTC domain contains an ADP-ribose/NAD⁺-binding pocket, enabling it to recruit

ADP-ribose-modified substrates in cells and catalyze their ubiquitination (Ahmed *et al.*, 2020, *Science Advances*). Poly-ADP-ribosylation is an early event in the DNA damage repair pathway, and we showed that DELTEX E3s are involved in this process. We are currently investigating the underlying mechanism further. Beyond protein substrate ubiquitination, we also demonstrated that DELTEX E3s can catalyze direct ubiquitin modification of ADP-ribose and NAD⁺ (Chatrin *et al.*, 2020, *Science Advances*), although the biological significance of this modification remains to be elucidated. Recently, we showed that DTX3L can bind single-stranded nucleic acids and catalyze ubiquitin modification at the 3'-OH group of ribose (Dearlove *et al.*, 2024, *eLife*). We are examining the functional relevance of this novel modification. Given the involvement of DELTEX E3s in DNA damage repair pathway, we hypothesized that these non-proteinaceous ubiquitination event may play a role in this process. Further characterization of these mechanisms could open avenues for therapeutic targeting in cancer.

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Figure 3. Regulation MDM2 E3 activity by p14ARF

