

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 functions 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 to explore the Ub system for cancer therapeutics.

Deregulation in CBL ubiquitin ligase

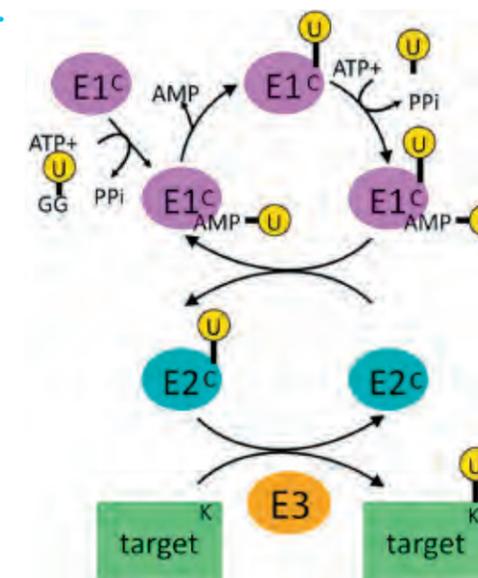
CBL proteins (CBLs) are RING E3s that negatively regulate RTKs, tyrosine kinases and other proteins by promoting their ubiquitination and degradation by the proteasome or lysosome. Mutations in CBL have been observed in human patients with myeloproliferative diseases. We investigated the mechanism by which CBL mutants exert oncogenesis and showed that CBL mutants inactivate E3 activity thereby functioning as adaptor to recruit other proteins such as CIN85 to elicit oncogenic signalling. Mechanistically, CBL mutants bind to receptor tyrosine kinases such as EGFR, which leads to phosphorylation of CBL mutants' C-terminal

tyrosines. Phosphorylated tyrosines induce conformational changes that enable CBL mutant-CIN85 interaction. CBL mutants cannot ubiquitinate CIN85, leading to deregulated CBL-CIN85 signalling which alters transcriptome landscape that in turn up-regulates PI3K-AKT signalling cascade to drive oncogenesis.

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 often due to amplification or high expression of MDM2. Inhibition of the 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 explored whether targeting the RING domain is a suitable strategy. We showed that MDM2 E3-inactive mutant cannot ubiquitinate and degrade p53. However, these mutants retained the ability to bind p53, thereby limiting p53's transcription activity in cells. Upon stresses, cells expressing E3-inactive MDM2 showed rapid p53 activation (Nomura *et al.* 2017 Nature Structural and Molecular Biology, 24, 578-587). In collaboration with Prof. Karen Vousden's group at the Francis Crick Institute, we showed that expression of MDM2 E3-inactive mutant is tolerated in adult mice. Despite high

Figure 1
Enzymatic cascade for Ub modifications



levels of p53, the MDM2 mutant was able to restrain p53 activity sufficiently for normal growth. Upon high dose of γ -irradiation, p53 activity was rapidly activated in various tissues, but most tissues were able to dampen p53 activity and regain homeostasis. These studies support the view that inhibitors that target MDM2 E3 activity could activate p53 in tumours with reduced on-target toxicities.

It remains unclear how p53 is rapidly activated upon DNA damage. MDM2 is phosphorylated near its C-terminal region, notably Ser429, which is adjacent to the RING domain after DNA damage. We showed that Ser429 phosphorylation enhances MDM2's E3 activity. A crystal structure of pSer429-MDM2 RING domain bound to E2~Ub revealed that pSer429 functions by stabilising Ub to keep E2~Ub in the active conformation to enhance the activity

(Figure 2A). Upon DNA damage, Ser429 phosphorylation enhanced MDM2 autoubiquitination and degradation in cells explaining the rapid p53 activation. We also described a strategy for preparation of MDM2 RING domain for structural analyses to enable rapid development MDM2 RING inhibitor.

Insights into Deltex ubiquitin ligases

Deltex (DTX) family E3s share a highly conserved C-terminal RING domain followed by a Deltex C-terminal domain (DTC). DTXs have been linked to developmental processes involving Notch signalling and histone ubiquitination during DNA damage repair. However, their functions remain largely unknown. We discovered that DTX E3s harbour dual activities and catalyse both ubiquitination of ADP-ribosylated substrate and ADP-ribosylation of Ub. We showed that DTX's C-terminal DTC domain harbour a conserved pocket that binds both ADP-ribose (ADPr) and NAD⁺ (Figure 2B). The DTC domains also bind poly-ADP-ribosylated substrates in cells. The proximity of RING and DTC domain enables the DTX to catalyse the ubiquitination of ADP-ribosylated substrates. Interestingly, when DTC domain is bound to NAD⁺, it catalyses Ub ADP-ribosylation. Mechanistically, the flexible linker connecting the RING and DTC domain enables the juxtaposition of the RING domain bound E2~Ub and the DTC-bound NAD⁺; the reaction proceeds when Ub is released from E2, leading to ADPr modification at the C-terminal Gly76 of Ub. ADPr-modified Ub is a new post-translational code with unknown function, and we are investigating this exciting crosstalk. Moreover, we have shown that DTXs display different interactomes and are investigating their cellular functions.

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Figure 2
Structure and mechanism of RING E3s
A) Crystal structure of pSer429-MDM2 RING homodimer bound to E2~Ub.
B) Crystal structures of DTX RING-DTC domain bound to ADPr (left) and NAD⁺ (right).

