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UBIQUITIN SIGNALLING

Group Leader
Danny Huang

Associate Scientist
Lori Buetow

Research Scientists
Mads Gabrielsen[1]
Karolina Majorek[1]
Mark Nakasone[1]
Feroj Syed

Scientific Officer
Gary Sibbet

Graduate Students
Dominika Kowalczyk
Helge Magnussen[1]
Chatrin Chatrin

[1]ERC

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 X-ray crystallography 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 (Fig. 1). E1 initiates the cascade by adenylating Ub's C-terminus in the presence of Mg^{2+} and ATP, followed by the 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 plays a pivotal role in determining substrate fate. In general, E3 consists of an E2-binding module (HECT, RING 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, the Ub pathway consists of two E1s, ~30–40 E2s and ~600 E3s that collectively ubiquitinate thousands of different substrates. Our group is interested in understanding the regulation and mechanistic functions of RING E3s, with a particular focus on RING E3s that have been linked to cancer.

Ligase-independent function of MDM2 in limiting p53 activity

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. MDM2 contains a C-terminal RING domain, which dimerises with itself or with an inactive RING domain from MDMX to form active MDM2 homodimer or MDM2-MDMX heterodimer, respectively. Mouse model studies have shown that both complexes have non-redundant roles in the inhibition of p53 activity, as loss of Mdm2 or MdmX leads to embryonic lethality at different developmental stages. However, it remains unclear how both complexes regulate p53, as it is difficult to separate their activities in cells. MDM2 inhibits p53 activity by binding to p53 via its N-terminal domain and other regions and recruits an E2~Ub conjugate via its RING domain to ubiquitinate p53. Small-molecule inhibitors targeting MDM2's N-terminal p53-binding domain have been developed, but these compounds exhibit high toxicity due to high levels of p53 activity, thereby limiting their efficacy. Here we investigated the effects of inhibition of the RING domain on p53 transcriptional activity.

We determined a 2.4 Å crystal structure of MDM2-MDMX RING dimer bound to an E2 UbcH5B covalently linked to Ub. The structure reveals the mechanism of E2~Ub activation by the MDM2-MDMX heterodimer and provides a rationale for how the MDM2 homodimer binds and activates the E2~Ub complex. Guided by the crystal structure, we designed MDM2 mutants that prevent E2~Ub binding without altering the RING domain structure. These mutants lost MDM2's E3 activity and were unable to ubiquitinate and degrade p53. However, they retained the ability to bind p53, thereby limiting p53's transcription activity. Cells expressing these mutants retained basal p53 levels and therefore responded more quickly to cellular stress than cells expressing wild-type MDM2. Our work reveals a ligase-independent role of MDM2 in p53 regulation and suggests that targeting the MDM2 E3 ligase activity could widen the therapeutic window of p53 activation in tumours, since rapid p53 induction can be achieved while basal p53 control by MDM2 is maintained.

Figure 1
Enzymatic cascade for Ub modifications

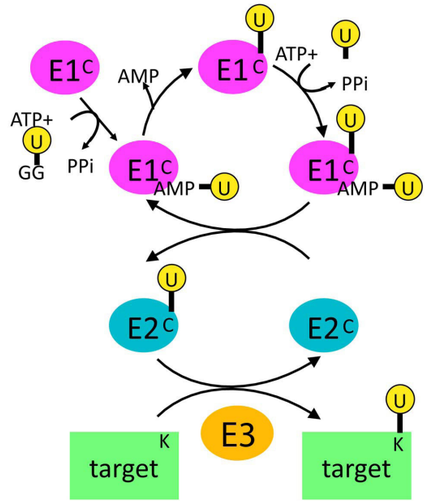
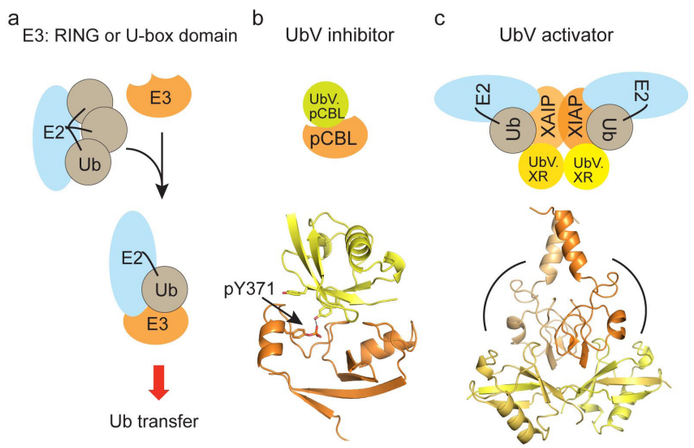


Figure 2
Targeting the RING domains
(a) Activation of the E2~Ub complex by the RING or U-box domain. The RING or U-box domain binds the E2~Ub complex and promotes the formation of the closed E2~Ub complex to facilitate Ub transfer. **(b)** UbV.pCBL functions as an inhibitor. It binds the E2~Ub binding surface of the pCBL RING domain and competes against E2~Ub binding. The crystal structure of the pCBL RING domain bound to UbV.pCBL reveals that phosphoTyr371 interacts with Tyr68 from UbV.pCBL. **(c)** UbV.XR functions as an activator. It dimerises and binds to a surface on the XIAP RING domain dimer that is remote from the E2~Ub binding site. UbV.XR contacts the closed E2~Ub conformation and further stabilises it to activate the ligase activity. The crystal structure of the XIAP RING domain dimer bound to UbV.XR is shown with the E2~Ub binding site in each RING domain indicated by an arc.



Selective targeting of the catalytic domain of RING E3s

The catalytic domain of RING E3s, commonly known as the RING domain, contains ~75–100 amino acid residues that form two loops stabilised by two Zn^{2+} ions. The RING domain promotes Ub transfer by binding and stabilising the E2~Ub conjugate in a closed conformation to facilitate catalysis (Fig. 2a). Due to the small surface area, targeting the RING domain remains a major challenge. Development of a general platform for targeting the RING domain would enable us to address the biological functions of these enzymes and to investigate whether modulation of the ubiquitin ligase activity could be a suitable approach for targeting RING E3s.

In collaboration with Professor Sachev Sidhu's lab at the University of Toronto, we have utilised a phage-displayed ubiquitin variant (UbV) library to screen for UbV(s) that bind selectively to the RING or U-box domain. The UbV library contains native Ub sequence that was randomised to generate billions of ubiquitin variant sequences. We identified three UbVs (UbV.E4B, UbV.pCBL and UbV.XR) that bind selectively to the RING or U-box domain of monomeric UBE4B, phosphorylated active CBL, and dimeric XIAP, respectively. We showed that UbV.E4B and UbV.pCBL function as inhibitors that bind selectively to the E2~Ub binding surface on the U-box domain of UBE4B and the RING domain of pCBL, respectively, thereby blocking E2~Ub binding. Interestingly, UbV.pCBL was selective only against Tyr371-phosphorylated CBL; the binding specificity was revealed in the crystal structure (Fig. 2b). Furthermore, cell-based analyses showed that UbV.E4B inhibits UBE4B-mediated p53 ubiquitination and UbV.pCBL inhibits CBL-catalysed EGFR ubiquitination in an EGF-dependent manner, a condition that leads to Tyr371 phosphorylation of CBL. Inhibition of EGFR ubiquitination resulted in EGFR stabilisation, decreased EGFR accumulation in early endosomes, and prolonged downstream signalling events.

In contrast to UbV.E4B and UbV.pCBL, UbV.XR binds XIAP dimeric RING domain and activates the ligase activity. The crystal structure of XIAP-UbV.XR showed that UbV.XR binds to a region in XIAP dimeric RING domain that is remote from the E2~Ub binding site (Fig. 2c). Structural modelling and biochemical analyses revealed that UbV.XR contacts the E2~Ub complex to assist stabilisation of the E2~Ub complex in the closed active conformation, thereby enhancing the ligase activity. When UbV.XR was introduced into HEK293T cells, it bound XIAP and enhanced SMAC ubiquitination upon induction of apoptosis. Collectively, our work demonstrates the versatility of the UbV technology in the identification of inhibitors and activators of RING/U-box E3s.

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