

mutant ER $\alpha$  LBD structures may explain instability in the absence of ligand or with specific ligands<sup>1,2,7,9</sup>. Nettles *et al.*<sup>5</sup> address this instability through use of a specific point mutation, Y537S, which stabilizes H12 in an agonist conformation and is shown to overcome misfolding of the apo-ER $\alpha$  LBD. One might predict that stabilizing H12 on top of the ligand binding cavity with the Y537S mutation would prevent efficient access of ligands to the binding pocket. Unexpectedly, analysis of apo Y537S ER $\alpha$  crystals suggests that ligand entry can take place through a previously undescribed channel created by a switch in the position of His524, which normally closes this channel in the presence of ligand. Accordingly, mutant Y537S was amenable to the rapid generation of more than ten structures via cocrystallization and soaking strategies. Since this channel appears conserved in all steroid receptors, use of mutations stabilizing H12 in the agonist conformation may be generally applicable to structural studies of this class of nuclear receptors.

Importantly, the mutation introduced did not affect the interaction of the receptor with ligands, and structures obtained with the mutant receptor could be superimposed to those previously generated with wild-type ER $\alpha$ . However, the restriction imposed on the positioning of H12 may limit the application of this strategy with antagonists having bulky side chains. Instead, a different mutation (L536S)

was designed to stabilize H12 in the antagonist conformation seen in the presence of tamoxifen and raloxifene (Fig. 1)<sup>1,2</sup>, and this mutation was shown to be compatible with binding of raloxifene in a cocrystallization approach. It will be of interest to determine whether the L536S mutation allows apo receptor crystallization and soaking with a complementary range of ligands. Use of different stabilized structures together with monitoring of the antagonist/agonist properties of ligands in mutant versus wild-type receptors may also provide information on H12 dynamics in the presence of these ligands. However, whether some ligands such as full antiestrogens are compatible with H12-stabilizing mutations remains at present an open question, as H12 was found to be displaced and unstructured with this type of ligand<sup>7</sup>.

Nettles *et al.*<sup>5</sup> illustrate the utility of their approach by solving the structure of Y537S ER $\alpha$  with several NF $\kappa$ B-selective ligands (Fig. 1), revealing the basis for their destabilizing effect on the ER $\alpha$  LBD conformation. These ligands retain the capacity of natural estrogens to suppress NF $\kappa$ B activity in an ER $\alpha$ -dependent manner, but they result in partial or very poor levels of ER $\alpha$  transcriptional activity. Efficient suppression of ER $\alpha$  transcriptional activation properties correlates with alterations in the conformation of helix 11, interfering with stabilization of H12 in the agonist conformation.

An elegant demonstration of the functional importance of these structural effects is provided by replacement of ethyl by methyl groups in one compound, which shifts H11 closer to the agonist conformation, thereby yielding increased ER $\alpha$  transactivation. Thus, while the mechanisms of NF $\kappa$ B transrepression remain imperfectly understood, they differ from those underlying transcriptional activation by a lack of sensitivity to H11-H12 conformation.

This new approach to crystallization of steroid receptors should greatly facilitate structure-activity relationship studies and rational design of steroid analogs targeting specific receptors and also potentially specific receptor-interacting protein complexes. There is no doubt that this work will thus considerably accelerate drug discovery, and stimulate research into the mechanisms of action of steroid receptors through both genomic and alternative pathways.

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## OTU takes the chains OUT

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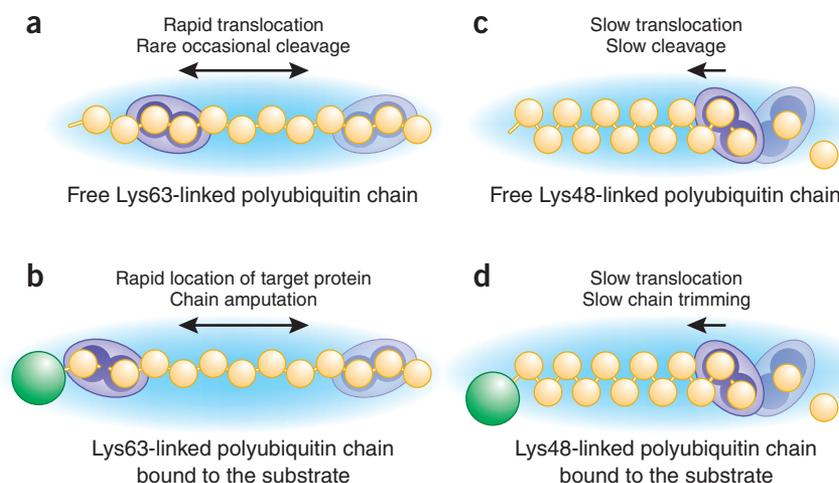
**A20 protein, a regulator of inflammation and cell survival, modulates cellular signaling via two apparently opposite enzyme activities. Recent studies elucidate the unusual structural organization of the A20 protease domain and provide new mechanistic insights into its biological function.**

Many critical biological processes are regulated by ubiquitination—the covalent modification of a target protein with the small protein ubiquitin. Often a long polyubiquitin chain is formed by enzymes called ubiquitin ligases. When the ubiquitins are linked to each other through the lysine found at

position 48 (Lys48 chains), the target protein is directed to the proteasome for degradation. If lysine 63 is used instead (Lys63 chains), it can serve as a signal for the target to assemble with other proteins. Like most regulatory modifications, ubiquitination is a reversible process: the attached ubiquitin can be removed by specific proteases called deubiquitinating enzymes (DUBs). Most of these enzymes belong to the class of cysteine proteases sharing a common papain-like fold and a similar catalytic mechanism. However, Lin *et al.*<sup>1</sup> present structural and biochemical evidence that DUB A20 may function differently, using attenuated protease activity as a way to increase the specificity of cleavage.

A20 was discovered as a protein that downregulates the activation of transcription factor NF $\kappa$ B. This factor has a pivotal role in initiating inflammation and raising an effective immune response. The activation of NF $\kappa$ B involves a cascade of biochemical reactions initiated by inflammatory signals at the cellular surface and transduced to the nucleus. For efficient transduction and termination of this signal, some intracellular proteins (notably TRAFs and RIP) must be modified with Lys63 polyubiquitin chains. A20 inhibits NF $\kappa$ B signaling by two mechanisms<sup>2,3</sup>. First, its N-terminal protease domain disassembles the Lys63 polyubiquitin chains on TRAFs and RIP. Second, the

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**Figure 1** Model of A20 function showing the low catalytic activity of the OTU domain, its high affinity toward polyubiquitin chains and differential chain cleavage. A key feature of this model is that after initial binding of the OTU domain to the polyubiquitin chain, subsequent events are determined by lateral movement of the protein along the chain and eventual cleavage. **(a)** In the case of free polyubiquitin chains, if the OTU translocation is rapid enough (Lys63-linked chain), the cleavage would happen very rarely because the attenuated active site does not have time for the structural reorganization required for catalytic events. **(b)** When the same chain is attached to the substrate, the OTU would stop sliding and remain tightly bound upon encountering the obstacle at the junction between the substrate and the chain. Then the amputation would occur, releasing the whole chain in one catalytic event. **(c,d)** On the other hand, the slow rate of OTU translocation on the chain would result in low-efficiency trimming either in the absence **(c)** or presence **(d)** of the substrate (Lys48-linked chain).

A20 C-terminal zinc fingers act as a ubiquitin ligase to build up Lys48-linked polyubiquitin chains on RIP proteins to induce their degradation.

Though it is not unusual to find ligases and DUBs physically associated, the striking combination of two apparently opposite enzyme activities in one protein suggests the unique organization and mechanism of the A20 protein. The A20 protease domain belongs to the ovarian tumor (OTU) superfamily of cysteine proteases<sup>4</sup> that have DUB activity. Structurally and mechanistically, however, the OTU domain remains relatively uncharacterized. The new study by Lin *et al.*<sup>1</sup> and an independent report from Komander *et al.*<sup>5</sup> show that the structure of the N-terminal OTU domain of A20 is significantly different from that of other cysteine proteases. Surprisingly, the OTU active site has elements that more closely resemble serine proteases; the oxyanion hole is formed by main chain amides in a loop that immediately precedes the active site cysteine rather than a side chain amide, as is characteristic of other cysteine proteases<sup>1,5–7</sup>. The loop is shifted toward catalytic residues, generating

a much more spatially restricted active site cleft than in any known cysteine protease. Lin *et al.*<sup>1</sup> suggest that this organization of the active site may underlie the apparently low catalytic activity and substrate selectivity of the A20 OTU domain.

From their analysis, Lin *et al.*<sup>1</sup> improve our understanding of the specificity of A20 for cellular substrates. They show that consistent with its physiological function, A20 has negligible affinity toward single ubiquitin-based substrates and inhibitors, preferring instead polyubiquitin chains. Comparison of the cleavage by A20 of free polyubiquitin chains led both research groups to the striking observation that, in contrast to the observed specificity of A20 for Lys63 linkage *in vivo*, Lys63 chains are much poorer substrates than Lys48 chains. The apparent paradox is explained in a series of elegant biochemical experiments in which Lin *et al.*<sup>1</sup> show that A20 effectively deubiquitinates Lys63-linked polyubiquitinated TRAF6 with concomitant appearance of free chains. This suggests that A20 effectively amputates Lys63-linked chains attached to the substrate without cleaving them *per se* by recognizing

and cleaving at the junction between the substrate and the Lys63-linked chain.

Based on these data one might speculate that A20 binds to and slides along the polyubiquitin chain via one-dimensional diffusion or a similar ‘hopping’ mechanism. Such a strategy is used by some DNA repair enzymes to locate specific sites among a vast excess of nonspecific DNA<sup>8</sup>. According to the model (Fig. 1), the difference in polyubiquitin cleavage may be explained by both efficiency of A20 sliding and its specificity toward particular chain linkage. Thus the higher apparent cleavage of free Lys48-linked chains by A20 may reflect the lower rate of OTU translocation compared with that on free Lys63-linked chains. With polyubiquitinated substrates A20 will rapidly find and pause at the junction between the substrate and the Lys63-linked chain, thus having enough time to release the whole chain in one catalytic event. In contrast, the Lys48-linked polyubiquitin substrate assembled by the ligase domain of A20 would only slowly be trimmed, allowing assembly of the intact degradation signal. A comparison of the cleavage of physiological A20 substrate RIP1, which can be modified with both Lys48- and Lys63-linked polyubiquitin chains, could test this hypothesis.

The amputation of whole polyubiquitin chains represents a rare mechanism used by DUBs, most notably by the RPN11 subunit of the proteasome<sup>9</sup>. This strategy may provide a time advantage in dynamic biological processes where the primary goal is a prompt removal of a specific polyubiquitin tag rather than chain disassembly. Such a mechanism could also be involved in the correction of heterogeneous and forked chains to prevent accumulation of nondegradable polyubiquitin conjugates<sup>10</sup>. Further studies should reveal whether other OTU proteases share the same mechanism of substrate cleavage.

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