CHAPTER 6

# MECHANISM, SPECIFICITY AND STRUCTURE OF THE DEUBIQUITINASES

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**Abstract:** Removal of ubiquitin from modified proteins is an important process to regulate the ubiquitin system. Roughly 100 dedicated enzymes for this purpose, the deubiquitinases, exist in human cells and are intricately involved in a wide variety of cellular processes, although many enzymes remain unstudied to date. The deubiquitinases consist of five enzyme families that contain USP, OTU, UCH, Josephin, or JAMM/MPN+ domains providing catalytic activity. We now understand the catalytic mechanisms of all deubiquitinase families from structural work and more importantly, have obtained insight into an unanticipated variety of ways to exercise specificity. It emerges that deubiquitinases exploit the entire complexity of the ubiquitin system by recognizing their substrates, particular ubiquitin chain linkages and even the position within a ubiquitin chain. This chapter describes the mechanisms of deubiquitination and the different layers of deubiquitinase specificity. The individual deubiquitinase families are discussed with a focus on structure, regulation and specificity features for selected enzymes.

## INTRODUCTION

Protein ubiquitination is emerging as one of the most important regulatory posttranslational modifications. Most prominent and well-studied are its roles in protein degradation,[1] however, recent years have seen an explosion of data on nonproteolytic roles of ubiquitination in cell signalling processes, intracellular trafficking and the DNA damage response.[2] The versatility to modulate such diverse processes is achieved by the ability of ubiquitin to form at least eight different types of polymers (reviewed in refs. 3,4). In such ubiquitin chains, isopeptide bonds are formed between the ubiquitin C-terminus and one

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of seven lysine residues of a second ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63). Alternatively, also the N-terminal amino group can be used for ubiquitin linkages to generate linear ubiquitin chains.[5] The linkage type of the ubiquitin chain determines whether a ubiquitination event will trigger proteasomal degradation (mediated by Lys48- and Lys11-linked chains and possibly other chain types) or signalling processes such as protein kinase activation, or DNA repair pathways (mediated by Lys63-linked and linear chains).[4]

Like other posttranslational modifications, ubiquitination is reversible. The human genome encodes ~98 deubiquitinating enzymes, also known as deubiquitinases or DUBs, which provide different functionalities and specificities to carefully regulate ubiquitination events. These enzymes cluster in five structurally unrelated families:[6,7] the ubiquitin specific proteases (USP, 56 individual members in humans plus 11 additional genes from the USP17 multigene family),[8] the Ovarian Tumor (OTU) DUBs (15 members), the Ubiquitin C-terminal hydrolases (UCH, 4 members), the Josephin domain DUBs (4 members) and the JAB1/MPN/MOV34 (JAMM/MPN+) DUBs (8 members).[6,7]

An important role of DUBs is the maintenance of a free ubiquitin pool in cells. Ubiquitin genes produce polyubiquitin precursor proteins and specialized DUBs such as USP5/IsoT are required to process these precursors into monoubiquitin.[9] Ubiquitin has a half-life of several days in cells, which is achieved by recycling of ubiquitin from degraded substrates. The proteasome itself harbors three DUBs (USP14, UCHL5 and POH1) that hydrolyze the chains prior to degrading of the substrate, hence recycling ubiquitin for further use.[10] These roles of DUBs in maintaining a stable pool of monoubiquitin are performed by a handful of dedicated enzymes.

The majority of DUBs however directly regulate protein ubiquitination events. Most commonly, ubiquitination will lead to protein degradation and hence deubiquitination has a stabilizing effect, actively increasing protein levels in cells. Deubiquitination can also inhibit cellular signalling cascades that are activated by nondegradative chains types. As protein homeostasis as well as cell signalling often requires tight temporal and spatial regulation, the DUBs affecting these pathways are also regulated in many different ways. Furthermore, DUBs have maintained remarkable specificity, with regard to the selection of substrates, their preference for particular chain types and even their positioning on a ubiquitin chain.

De-regulation of deubiquitination can lead to imbalances of protein levels and hence to disease. For example, the degradation of the oncogene c-myc is mediated by USP28, which retains MYC in the nucleus and prevents it from entering the nucleolus, where it is degraded.[11] Proliferation of some cancer cell lines depends on high MYC levels and knock-down of USP28 inhibits growth of these cell lines, suggesting an oncogenic role of USP28.[11] However, USP28 also stabilizes several important mediators of the DNA damage response, including Chk2 and 53BP1, after DNA damage has occurred.[12] Hence, loss of USP28 attenuates the cellular response to DNA damage, rendering USP28 a likely tumor suppressor candidate. A similarly complex example is the regulation of the p53 tumor suppressor by the deubiquitinase USP7. USP7 is thought to directly stabilize p53 levels, but in addition, USP7 also stabilizes the levels of the p53-destabilizing E3 ubiquitin ligase, MDM2 in cells.[13,14] These two examples illustrate the importance of DUBs in regulating protein stability.

Several cell-signalling DUBs have further well-established links to cancer. Familial cylindromatosis, a rare benign skin cancer affecting hair follicles and sweat glands of skin and neck, has its genetic cause in truncation of the *cyld* tumor suppressor gene.[15]

Truncation ablates the function of the USP deubiquitinase domain of CYLD.[16-19] This domain has specificity for Lys63-linked and linear polyubiquitin chains[19,20] and has been implicated in regulation of nondegradative signalling pathways leading to the activation of the NF-κB transcription factor.[21] CYLD also has roles in numerous other Lys63-dependent processes and may serve as a general housekeeping enzyme regulating Lys63 ubiquitin linkages.[22] Other DUBs affecting primarily nondegradative ubiquitination events are the NF-κB regulator and tumor suppressor A20,[23,24] the TRABID enzyme involved in the Wnt/β-catenin pathway[25] and OTUD5 involved in the interferon response factor (IRF)-3 signalling pathway.[26]

Hence, DUBs have established roles in cancer, but also in inflammation and immune responses and in neurological disorders. This has led to an increasing interest to target these enzymes pharmacologically, for which a detailed mechanistic and structural insight is essential. This chapter provides an overview of the structural features of the deubiquitinases and discusses their mechanism and common concepts of specificity and regulation. For further information, readers are referred to recent reviews on the topic.[7,27,28]

## MECHANISMS OF DEUBIQUITINATION

DUBs are proteases that hydrolyze the isopeptide bond between the ubiquitin C-terminus and the Lys ε-amino group. Four of the five human DUB families (USP, OTU, UCH, Josephin) are Cys proteases while the JAMM/MPN+ DUBs are zinc dependent metalloproteases.

### Mechanism of Cys-Dependent DUBs

The Cys-dependent deubiquitinase families comprise a catalytic diad or triad and their mechanism is similar to that of the Cys protease papain.[29,30] A catalytic Cys performs a nucleophilic attack on the isopeptide linkage of a ubiquitinated Lys residue. This is facilitated by a nearby His side chain that lowers the pKa of the Cys. A third residue, usually Asp or Asn, aligns and polarizes the catalytic His. This is not always essential and some enzymes lack the third residue and polarize the His by other means. This mechanism has two additional features. A negatively charged transient reaction intermediate is stabilized by an oxyanion hole formed nearby by hydrogen-donating residues. A more stable acyl-intermediate is formed when the carboxyl-group is covalently bound to the enzyme, after the amino group has been hydrolyzed. The reaction cycle is completed by water-mediated hydrolysis of the acyl-Cys intermediate.

The mechanism of Cys-based deubiquitinases has been exploited by the generation of modified ubiquitin-derived probes that have reactive C-termini.[31,32] In the simplest molecule, ubiquitin aldehyde, the C-terminal carboxyl group of Gly76 is exchanged to an aldehyde group, which after binding to the catalytic Cys, is not hydrolyzed by water. This molecule acts as a potent and specific inhibitor of Cys-dependent deubiquitinases.[33] These ubiquitin probes have been improved since[34] and several probes are commercially available, including ubiquitin vinyl-sulfone (Ub-VS) and ubiquitin vinyl methyl ester (Ub-VME). Ubiquitin probes were instrumental in identifying novel deubiquitinases in cells[31] and to obtain the first ubiquitin-DUB complexes for structural characterisation.[35-37] However, different DUBs display different affinities for individual probes[38] and some enzymes cannot be modified by these reagents.

### Mechanism of Metalloprotease DUBs

JAMM/MPN+ family deubiquitinases are zinc-dependent metalloproteases. Within their catalytic site, invariant His, Asp and Ser side chains coordinate two zinc ions.[39] The structure of the first JAMM/MPN+ domain revealed similarities to cytidine deaminase, suggesting that these families were evolutionarily related.[40] The catalytic mechanism was proposed to be similar between these two hydrolytic enzymes. The zinc ion in the catalytic site activates a water molecule to form a hydroxide ion, which is able to attack the carboxyl carbon in the isopeptide link. The transient tetrahedral intermediate collapses with elimination of the ε-amino group and replacement of the amine with a hydroxyl group from the activated water molecule. A nearby invariant Glu residue acts both as a proton acceptor and donor in this catalytic cycle.[40] These predictions were recently supported by crystal structures of the AMSH-LP JAMM/MPN+ domain in isolation and bound to diubiquitin (see below).[41]

## CONSIDERATIONS FOR DEUBIQUITINASE SPECIFICITY

The 98 human DUBs are a diverse superfamily of enzymes. As will be discussed in detail below, the catalytic domains of the five DUB families share no sequence similarity and have distinct structural folds. Most DUBs however hydrolyze ubiquitin chains into monoubiquitin. Hence they can bind to two ubiquitin moieties, placing the isopeptide bond to be cleaved across their active site. In this arrangement, the 'distal' ubiquitin molecule presents its C-terminal Gly to the catalytic centre, while the 'proximal' ubiquitin is bound through its modified Lys. All DUBs analyzed to date bind ubiquitin through a significant distal binding site, while the proximal ubiquitin binding site is less extensive. The catalytic centre, bound to the flexible linker between ubiquitin moieties, rigidifies the linker region by tight interactions. While these general principles hold true for most DUBs, subtle differences in ubiquitin binding can change enzymatic properties significantly and contribute to DUB specificity.

It is important to comprehend the complexity of the ubiquitin system in order to discuss DUB specificity. In contrast to other modifications such as phosphorylation or acetylation, where a single modifying group is attached, ubiquitination is further organized by its polymeric nature. Ubiquitin chains are the principal outcome of ubiquitination and have different structural and topological features. By dealing with ubiquitin chains, DUBs face many additional layers where decisions regarding specificity have to be made. It is not yet clear whether all the ways to exercise specificity are employed in vivo, yet many observations suggest that DUBs exploit the system to its full potential. The following section outlines the emerging concepts in DUB specificity.

### Ubiquitin versus Ubiquitin-Like Protein Cleavage

Ubiquitin is one of 17 small ubiquitin-like (UBL) modifiers in humans which all contain the characteristic ubiquitin fold.[42] Several UBLs, including SUMO, Nedd8, ISG15, FAT10 and ATG12 modify proteins using a similar mechanism compared to ubiquitin.[42,43] The result is a topologically similar modification (SUMO, Nedd8 and Atg12 are roughly the same size and shape as ubiquitin, while ISG15 and FAT10 resemble diubiquitin) yet

DUBs are able to distinguish between ubiquitin and UBLs. The key to this selectivity lies partly in the C-terminal four residues preceeding the Gly-Gly motif. SUMO, Atg12 and FAT10 share no sequence similarity with ubiquitin within these residues. However, Nedd8 has a similar sequence and ISG15 has an identical sequence compared to ubiquitin. It is therefore not surprising that both Nedd8 and ISG15 can also be hydrolyzed by some cross-reactive DUBs (see below for examples).

## Isopeptide versus Peptide Bond Cleavage

Not all ubiquitin chains are linked to Lys residues via isopeptide bonds, but chains can also be linked through the α-amino group of the N-terminus (linear ubiquitin chains).[5] This chain type has nonproteolytic roles in NF-κB signaling[44] and linear chains are also the source of monoubiquitin in cells as ubiquitin is translated from linear polygenes. This requires DUBs to deal with this particular chain type and peptide bonds. Due to structural differences between the isopeptide (linked through an elongated, flexible side chain) and the peptide bond (bulky side chain of Met1, Ramachandran restraints), cleavage of linear chains requires a more spacious active site environment.[20] Recent data shows that USP enzymes can cleave linear chains, albeit with lower activity. Most other DUB families do not hydrolyze this chain type, although enzymes acting on linear chains may exist within these families.[20] Cleavage of peptide bonds by USPs may also allow them to hydrolyze non-ubiquitin sequences and was suggested to be used in the observed USP1 autoproteolysis within its USP domain.[45]

## Linkage Specificity within a Ubiquitin Chain

The most striking layer of DUB specificity is the ability of many enzymes to select between different ubiquitin chain linkages.[20] Importantly, chain linkage specificity is not determined by DUB family. This is in contrast to e.g., phosphatases that utilize different enzyme families for removal of phosphates from Tyr, or Ser/Thr residues.[46] For example, OTU and USP family enzymes have evolved Lys48- and Lys63-specific members.[20] The JAMM/MPN+ family of DUBs may have intrinsic specificity for Lys63-linked chains (see below).

Currently, however, only three ubiquitin chain types (Lys63-, Lys48-linked and linear) are available for in vitro studies of DUB specificity. Hence the overall picture remains incomplete and requires development of new and better reagents and assays. As highly specific DUBs exist, it is possible that even new DUB families may be discovered once proper reagents are available.

## Exo- vs. Endo Activity within a Ubiquitin Chain

Polymers of ubiquitin can be cleaved from the end (exo) or within a chain (endo) and both mechanisms have been described.[19,47] This mechanistic difference has profound consequences. An endo-DUB would be able to remove entire chains from substrates, reversing polyubiquitination most efficiently. It would however result in free chains and further DUB action (likely by distinct enzymes) is required to recycle monoubiquitin from the released chains. In contrast, exo-DUB activity seems inefficient if chains are long; such activity would be required though for recycling, e.g., proteasome-bound, DUBs.

## Chain Cleavage versus Substrate Deubiquitination

Ubiquitination can often be divided into two independent steps, chain initiation and chain elongation. One mechanistic reason for this is that the sequence context of the 'first' ubiquitin on a substrate Lys is distinct from the (always equivalent) ubiquitin sequence used for elongating the chain. DUBs face the same problem. Some DUBs may only target ubiquitin-ubiquitin linkages, but their action might not remove the proximal ubiquitin, leaving the substrate monoubiquitinated. In fact, it is often not clear what the physiological end product of a deubiquitination reaction is. Ubiquitin chain editing,[48] i.e., the switch from one chain type (e.g., a 'signalling' Lys63-linked chain) to another type (e.g., 'degrading' Lys48-linked chain) may benefit from substrates not fully deubiquitinated. In such scenario, DUB action on a substrate leaves a platform, i.e., monoubiquitin, for subsequent ubiquitination with a different chain type. Enzymes that combine DUB and E3 ligase activity have been described[24,48] and many DUBs interact with E3 ligases.[49]

## Sequence Specific Deubiquitination

There may be DUBs that act on monoubiquitinated targets, e.g., those left by prior chain deubiquitination (see above). These DUBs may specifically recognize a ubiquitinated sequence context in target proteins and hence hydrolyze monoubiquitin, or even entire ubiquitin chains en bloc. This would allow for a great level of specificity, yet such sequence specific DUBs have not been formally described yet. However, nonspecific DUBs such as USP family members, may be able to accommodate a wider range of sequences in their proximal binding site and hence may completely deubiquitinate substrates.

## Substrate Recognition and Specificity

In order to function within a particular pathway, DUBs need to select their substrate proteins. Many DUBs contain additional protein interaction domains to facilitate direct substrate interaction, yet also indirect means, e.g., by localizing DUBs to specific places in the cell may aid such selectivity.[7] Localisation of a DUB via protein interaction domains may affect other layers of specificity, such as linkage preference. Formation of a DUB-substrate complex would significantly increase the local concentration of particular ubiquitin linkages, potentially overriding the intrinsic linkage preference of the DUB.

## THE FIVE HUMAN DUB FAMILIES

A surge of data in the last years has revealed many aspects of DUB biology and in particular structural studies by X-ray crystallography and NMR have yielded important insights in DUB activity, specificity and regulation. In the following section, the five human DUB families are discussed individually and recurrent mechanisms of regulation and specificity are outlined.

## USP Domain DUBs

USP family DUBs comprise the largest and most diverse family of deubiquitinases in mammalian cells with 56 distinct members. Another 12 USP17 (also known as DUB3)-like USP genes exist. USP domain DUBs are usually large proteins (between 350 and 3400 amino acids (Aa), average size ~1000 Aa) with a core catalytic domain of ~350 Aa. Outside of their catalytic core, USP enzymes comprise numerous other domains, including protein interaction domains that facilitate substrate binding, or domains determining subcellular localization. Only USP19, USP30 and USP48 contain predicted transmembrane regions. USP19 is anchored at the endoplasmic reticulum,[50] while USP30 is localized in the outer membrane of mitochondria.[51] In addition, ubiquitin binding domains (UBDs) such as zinc-finger ubiquitin specific protease (ZnF UBP), ubiquitin interacting motifs (UIM) and ubiquitin associated (UBA) domains are found in several enzymes.[6,7] Finally, ubiquitin-like (UBL) domains are found in at least 18 USP domain DUBs.[52] The presence of UBL domains might suggest a common autoregulatory mechanism that remains unstudied to date.

The USP domain itself consist of three sub-domains, Palm, Thumb and Fingers, resembling a right hand[35] (Fig. 1A). The catalytic centre lies at the interface between Palm and Thumb, while the Fingers domain grip the distal ubiquitin. Dramatic conformational changes are present in USP domains upon ubiquitin binding.[35,47] In USP7, the catalytic Cys shifts upon ubiquitin binding from a catalytically unproductive position to an active position where it interacts with the catalytic His residue[35] (Fig. 1B). In contrast, the catalytic machineries of USP14 and USP8 are properly aligned for catalysis in absence of ubiquitin, however ubiquitin-binding surface loops block the ubiquitin binding site[47,53] and these loops undergo conformational changes upon ubiquitin binding in USP14.[47] Furthermore, in USP8, which has so far only been crystallized without ubiquitin, the Fingers domain is tightened inward, additionally blocking the ubiquitin binding site (Fig. 1C).[53] Inactive conformations are not a global feature of USPs, as the CYLD USP domain was poised for catalysis and did not show a blocked active site cleft (Fig. 1D).[54]

Most of the analyzed USP family enzymes are nonspecific and will cleave any chain type,[20] yet some members show distinct specificities. USP14 preferentially cleaves Lys48-linked ubiquitin chains,[47] while CYLD specifically hydrolyzes Lys63-linked and linear chains.[20] The structures of USP14 and CYLD have given insights into their mechanism of action and specificity. The structure of the Lys63-specific enzyme CYLD has revealed that the proximal ubiquitin binding site and in particular an extended loop in this region, contribute to the observed linkage specificity (Fig. 1D).[19] USP domains can have endo- and exo-activity against polyubiquitin chains. The Fingers-subdomain of USP7 and USP14 wraps around the distal ubiquitin, restricting access to Lys48 and Lys63 (Fig. 1A). This allows these USPs to bind to the distal end of a chain only and consistently, USP14 acts primarily as an exo-DUB.[47] In contrast, CYLD lacks the Fingers subdomain, allowing Lys63 (and linear) chains to continue from the distal ubiquitin (Fig. 1D). Hence CYLD can interact with a ubiquitin chain at any point including at internal positions and has endo-activity.[19] Several USP domains are cross-reactive with other UBL modifiers.[55,56] These enzymes include USP18 and USP13 that interact with ISG15 suicide probes (ISG15-vinyl sulfone, similar to UbVS, see above) better than with ubiquitin probes and several other USP domains that bind to both ubiquitin and ISG15 probes.[55] Equivalent studies are important for other UBL modifiers with more elusive roles.
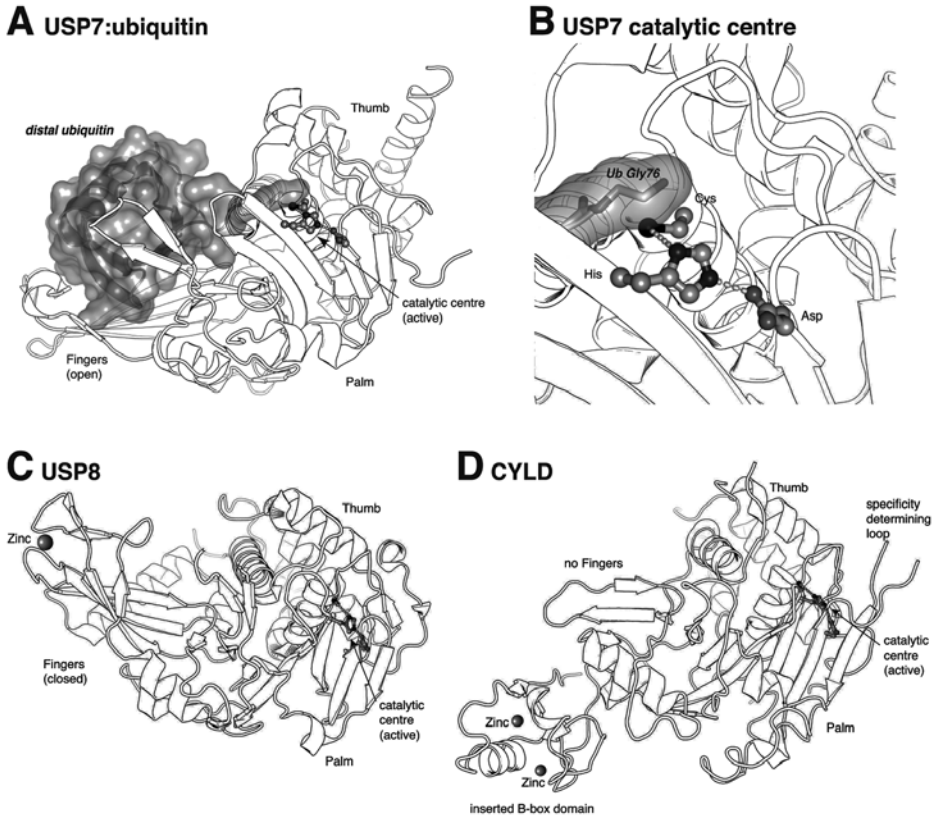
**Figure 1.** Structures of USP domain deubiquitinases. A) Structure of USP7 (also known as HAUSP) bound to ubiquitin (pdb-id 1nbf).[35] The USP domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. Hydrogen bonds are indicated by dotted lines. The Fingers, Palm and Thumb domains are indicated. B) Close-up view of the active site of USP7 bound to ubiquitin. The catalytic triad residues and their interactions are shown. C) Structure of USP8 (pdb-id 2gfo).[53] In the absence of ubiquitin, the Fingers subdomain is closer to the Thumb/Palm preventing ubiquitin binding. The Fingers subdomains of 45 out of 56 USP domain DUBs including USP8 comprise a functional zinc-binding site (zinc indicated as a grey sphere).[57] D) Structure of CYLD (pdb-id 2vhf).[19] CYLD does not contain a Fingers subdomain, allowing it to act as an endo-deubiquitinase against Lys63-linked and linear chains. A specificity determining loop near the active site disfavors Lys48-chain binding. The CYLD USP domains contains a zinc-binding B-box domain inserted in its sequence.

An intriguing structural feature of USP domains is their disrupted catalytic domain. The catalytic core of USP domains comprises ~350 residues, yet more than half of the human USPs have catalytic domains of much larger sizes (400-850 Aa) annotated.[57] This is an artefact from the bioinformatic annotation, which defines USP domains as the region between the N-terminal Cys-box and C-terminal His- and Asp-boxes that contain the residues of the catalytic triad. More detailed analysis shows that the USP domain core can be subdivided into six conserved sequence boxes, spanning ~350-400 residues, in all human USP domains.[57] The five boundaries between boxes are points where large insertions occur. These inserted sequences contain additional independently

folded domains, including protein interaction domains (e.g., B-box in CYLD (Fig. 1D)[19] and MYND domain in USP19)[50] and ubiquitin binding domains (e.g., UBA domains in USP5,[58] or UIM motifs in USP37).[57] Seven USPs contain ubiquitin-like folds as an insertion.[52,57] Although not yet backed up by structural work, the UBL insertions are likely positioned near the distal ubiquitin binding site, where they may directly alter USP function.[52] Structures of USP domains containing an insertion will likely yield interesting insights regarding regulation of these enzymes.

Further regulation of USP domain DUBs is provided by interacting proteins, and more than 770 DUB interacting proteins have recently been revealed.[49] Many USP family members interact with WD40 repeat containing proteins. The WD40 protein UAF1 (USP1 associated factor, also known as WDR48) was shown previously to interact with USP1, USP12 and USP46 and more importantly to allosterically activate these USP enzymes.[59,60] Another commonly observed interaction exists between DUBs (not only USP domains, but also other classes) and E3 ubiquitin ligases.[7,49] DUB activity may prevent autoubiquitination, a common feature of E3 ligases, or alternatively, E3 ligases might down-regulate DUBs. This yet again illustrates intricate interplay between ubiquitination and deubiquitination.

To date, most USP domain containing enzymes remain poorly characterized and virtually no literature exists for more than 25% of the USP proteins. This is likely to change with new genome wide screens, which have proven highly successful in identifying new DUB functions (see ref. 61 for an example). Still, biochemical characterisation is important to understand more about this enzyme family.

## OTU Domain DUBs

Human cells contain 15 OTU domain DUBs, only half of which have been studied to date. Several OTU enzymes are involved in cell signalling processes, regulating NF-κB signalling (A20, Cezanne1/2),[24,62] Wnt signalling (TRABID)[25] and IRF3 signalling (OTUD5, also known as DUBA).[26] Other OTU members have more elusive roles. OTU family proteins range in size from 230 Aa to 1222 Aa and like USP domains, often contain additional domains with links to the ubiquitin system, including UIM and UBA domains and UBL folds.[7]

The structure of the OTU domain does not resemble that of USP domains, yet the catalytic residues of the active enzymes superpose well (Fig. 2A,B).[54] The OTU domain core comprises ~150-200 residues,[63] however, a subclass of enzymes, (A20, Cezanne1/2, TRABID, VCIP135) contain an extended catalytic core of ~360 residues (Fig. 2D[54] and D.K., unpublished). Like some USP domains, the distal binding site of OTU domains undergoes a disorder-to-order transition upon ubiquitin binding.[37] At least in one case (OTUB1),[38] the active site is in an unproductive configuration and requires conformational changes prior to activation (Fig. 2C). The catalytically inactive resting state found in many DUBs, not only OTU members, may protect the catalytic Cys residue from oxidative stress. A low pKa Cys residue in the active site would be attacked by reactive oxygen species (ROS) and it has been suggested that high levels of ROS affect the function of the OTU DUB Cezanne.[64] ROS may also regulate other deubiquitinase classes.

OTU family enzymes display marked chain linkage specificity. TRABID and DUBA are Lys63-specific,[20,26] while OTUB1 is Lys48-specific.[38] The A20 OTU domain is Lys48-specific in vitro,[54,65] yet the substrates of A20 are modified with Lys63-linked chains. A20 was shown to act on Lys63-polyubiquitinated substrates
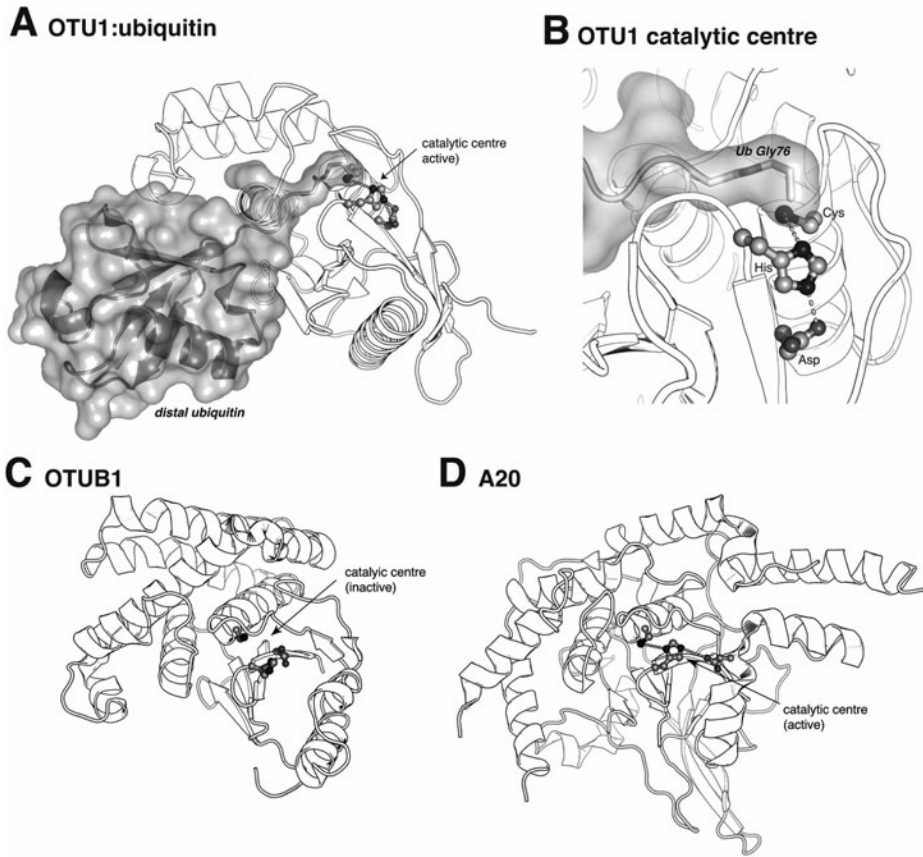
**Figure 2.** Structures of OTU domain deubiquitinases. A) Structure of OTU1 bound to ubiquitin (pdb-id 3by4).[37] The OTU domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. Hydrogen bonds are indicated by dotted lines. B) Close-up view of the active site of OTU1 bound to ubiquitin. The catalytic triad residues and their interactions are shown. C) Structure of OTUB1 (pdb-id 2zfy).[38] The Otubains (OTUB1 and OTUB2) contain several additional helices. D) Structure of A20 (pdb-id 2vfj).[54] The A20 catalytic domain is ~150 residues longer and contains additional structural elements.

such as TRAF6, releasing whole chains from the proteins, potentially by cleaving the proximal ubiquitin.[65] Most OTU domains do not cleave linear chains efficiently and hence may be strict isopeptidases,[20] however, OTUB1 was suggested to cleave both ubiquitin and Nedd8 conjugates.[38]

## UCH Domain DUBs

The UCH family of deubiquitinases contains four members, two of which consist of only a catalytic domain (UCHL1 and UCHL3, ~200 Aa).[6,7] UCHL1 and UCHL3 have roles in brain function[66-68] and the Ile93Met point mutant of UCHL1 is associated with familial Parkinson's disease.[69] A third member, UCHL5 (also known as UCH37) contains

a 100 Aa extension which is essential to bind to the proteasome subunit Rpn13.[70-72] Proteasome-bound UCHL5 is one of three DUBs that recycle ubiquitin chains from proteasome substrates.[10] The fourth human UCH enzyme, BAP1 (BRCA1 associated protein-1), contains a C-terminal extension of >500 Aa. BAP1 is a tumor suppressor and interacts with the BRCA1/BARD1 E3 ubiquitin ligase involved in DNA repair, yet its roles in the DNA damage response are debated.[73-75] Recent data shows that BAP1 also interacts with the cell cycle regulator host cell factor-1 (HCF1).[76,77] Human NCI-H226 squamous lung carcinoma cells harbor a deletion of BAP1 and overexpression of BAP1 in this cell line blocks their proliferation and tumor growth in mice.[77]

Structures of UCH domain reiterate common principles of DUB regulation and specificity. The catalytic residues in ubiquitin-free UCHL1 are in a nonproductive conformation[78] and need to undergo a conformational change upon binding to ubiquitin. In the active ubiquitin bound conformation of UCHL3 (Fig. 3A) or Yuh1 (the single yeast UCH enzyme), the catalytic triad residues superpose well with other DUB classes and several loops are remodelled upon ubiquitin binding (Fig. 3B).[36,79] The most striking
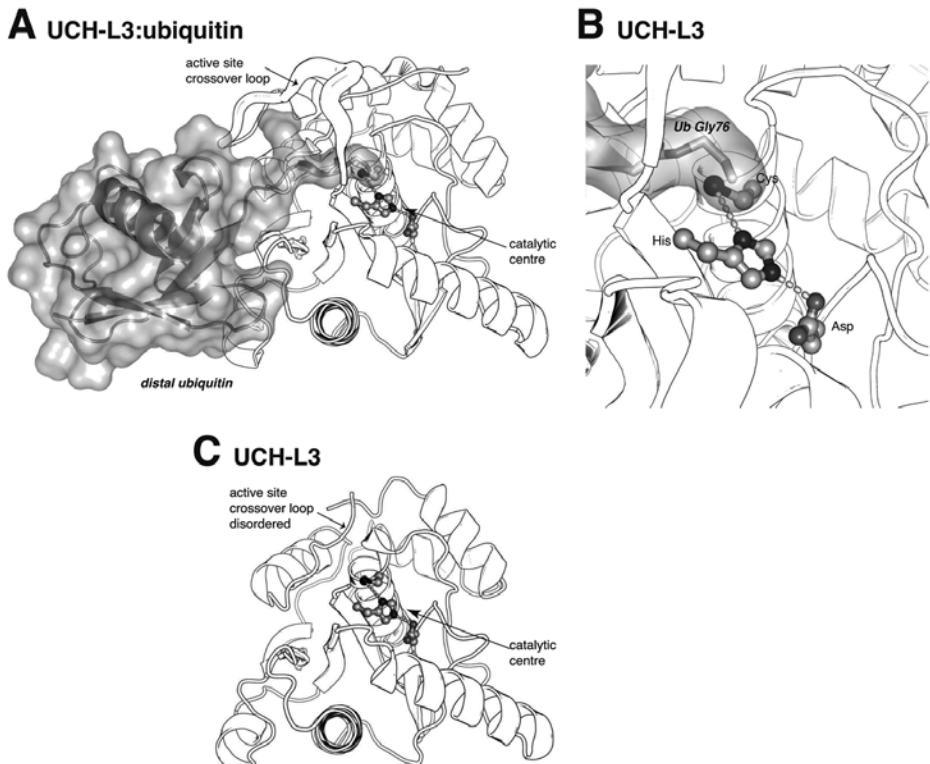


**Figure 3.** Structure of a UCH domain deubiquitinase. A) Structure of UCHL3 bound to ubiquitin (pdb-id 1xd3).[79] The UCH domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. Hydrogen bonds are indicated by dotted lines. The active site crossover loop forming across the ubiquitin C-terminus at the active site is indicated. B) Close-up view of the active site of UCHL3 bound to ubiquitin. The catalytic triad residues and their interactions are shown. C) Structure of UCHL3 in the apo form without ubiquitin (pdb-id 1uch).[30] The active site crossover loop is disordered.

feature of UCH enzymes is a large surface loop, the active site crossover loop, which forms upon ubiquitin binding (Fig. 3A,C).[36,79] The ubiquitin C-terminus has to thread through this loop in order to reach the active site. This poses a significant steric constraint and does not allow binding of folded ubiquitinated proteins of more than approximately 10 Å in diameter. This structural feature excludes ubiquitin chains, which would be too big to enter through the crossover loop. Indeed, UCH enzymes have negligible activity against ubiquitin polymers of any linkage type in vitro.[20,80] Only significant extension of the crossover loop allows polyubiquitin cleavage.[80] Hence, UCH enzymes with their restricted accessibility to the active site, can act on ubiquitination sites in unfolded regions of proteins (and maybe perform chain amputation) and on ubiquitin-peptide conjugates which may be a by-product of proteasomal degradation. Interestingly, proteasome-bound UCHL5 can act against polyubiquitin chains, despite a predicted analogous active-site crossover loop.[81] Hence, either proteasome interaction induces a conformational change in UCHL5 to remodel the obstructing loop, or the proteasome unfolds ubiquitin polymers significantly so they can enter through the cross-over loop. UCHL3 but not UCHL1 is inhibited by diubiquitin[82] and UCHL5 also does not hydrolyze diubiquitin efficiently.[70] The molecular basis for this inhibition is not clear at the moment.

## Josephin Domain DUBs

Four human DUBs contain a catalytic Josephin domain, which was identified by bioinformatics[83] and subsequently validated to be catalytically active.[84] The most prominent member of Josephin DUBs is Ataxin-3. Ataxin-3 is the protein mutated in Machado-Joseph disease (MJD, SCA3), the most common form of spinocerebellar ataxias.[85] Ataxin-3 contains a stretch of Gln residues (polyQ), which is significantly extended in the disease state as the consequence of amplification of an unstable CAG triplet repeat. The resulting polyQ stretch leads to protein aggregation in the form of intracellular inclusion bodies.[86]

Josephin domains have been studied by nuclear magnetic resonance (NMR) techniques and currently several inactive structures are available, where the catalytic triad is in nonproductive conformations (Fig. 4).[87-90] The key feature of Josephin domains is a large helical lever that restricts access to the active site in absence of ubiquitin (Fig. 4A, C, D).[87,88] NMR-based docking analyzes of diubiquitin onto Ataxin-3 suggest that ubiquitin binding stabilizes an active conformation of Ataxin-3.[90] Interestingly, Ataxin-3 catalytic activity is activated by ubiquitination of the Josephin domain itself by an unknown E3 ligase.[91] It is tempting to speculate that ubiquitination stabilizes the helical lever in an open conformation.

Ataxin-3 contains three UIM motifs in its C-terminal part.[92] The two Josephin-proximal UIMs were recently shown to preferentially interact with Lys48-linked ubiquitin chains,[93] however, Ataxin-3 was also suggested to edit Lys63-linkages in mixed linkage chains.[94] The substrates of Ataxin-3 and the roles of the remaining Josephin domain proteins are currently unclear.

## JAMM/MPN+ Domain DUBs

Eight human DUBs contain a JAMM/MPN+ metalloprotease domain and these proteins often operate as part of multi-subunit protein complexes. A JAMM/MPN+ DUB in the proteasome, POH1, contributes to recycling ubiquitin chains,[10] while AMSH and
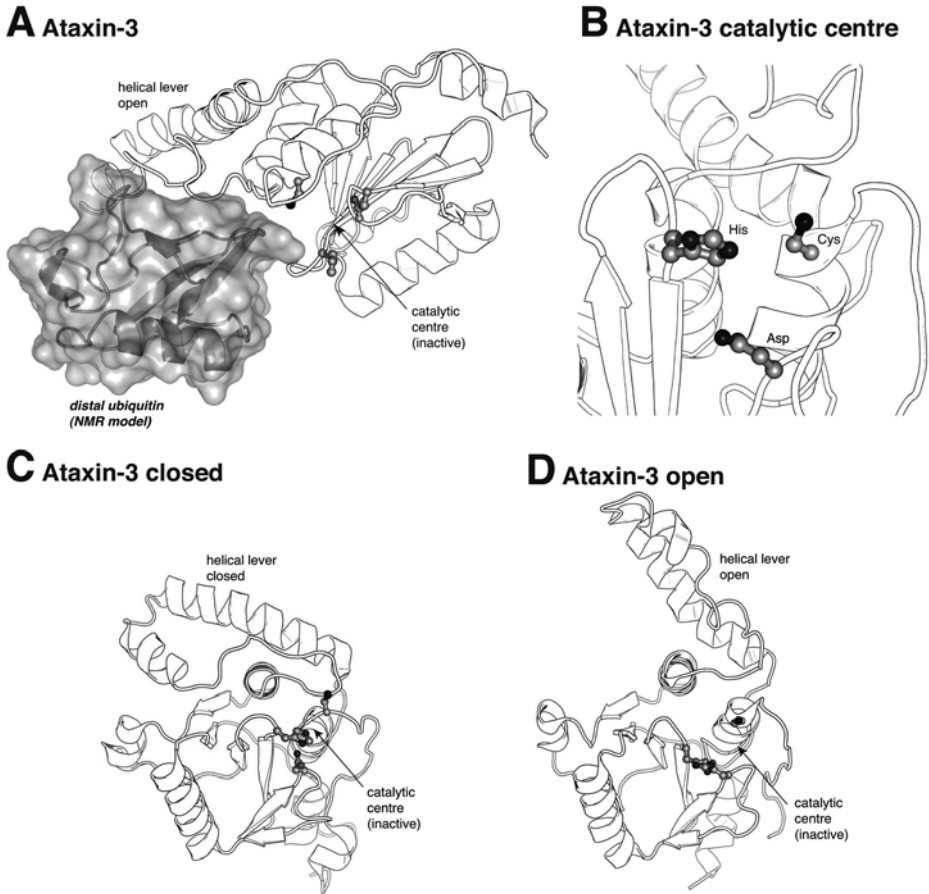
**Figure 4.** Structure of a Josephin domain deubiquitinase. A) Structure of Ataxin-3 bound to ubiquitin (pdb-id 1jri).[90] The Josephin domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey colors. Ubiquitin is shown under a grey semitransparent surface. The helical lever regulating access to the active site is labelled. B) Close-up view of the active site of Ataxin-3. The invariant catalytic residues have been verified by mutagenesis, but are in an unproductive conformation in all structures. C,D) Structure of Ataxin-3 in absence of ubiquitin (pdb-id 2aga,)[87] and in presence of diubiquitin (2jri).[90] Several NMR models have indicated high flexibility of the helical lever that moves between closed (C) and open (D) conformations. Diubiquitin has been omitted from D for clarity.

AMSH-LP are associated with the ESCRT machinery and are involved in membrane receptor trafficking.[95] BRCC3 has been found in two DNA repair complexes, the BRISC complex and the BRCA1 A complex.[96-99] CSN5 is a component of the COP9 signalosome and acts as deneddylating enzyme to remove the activating Nedd8 modification from Cullin E3 ligases.[100] MYSM1 is part of a histone deubiquitinase complex.[101] PRPF8, a splicing factor, contains an impaired metal binding site and hence may have lost DUB activity.[102] The remaining enzyme, MPND has not been studied to date.

Most JAMM/MPN+ DUBs cleave Lys63 ubiquitin chains and some (AMSH, AMSH-LP, BRCC3) with exquisite specificity.[96,103] The molecular basis for this linkage
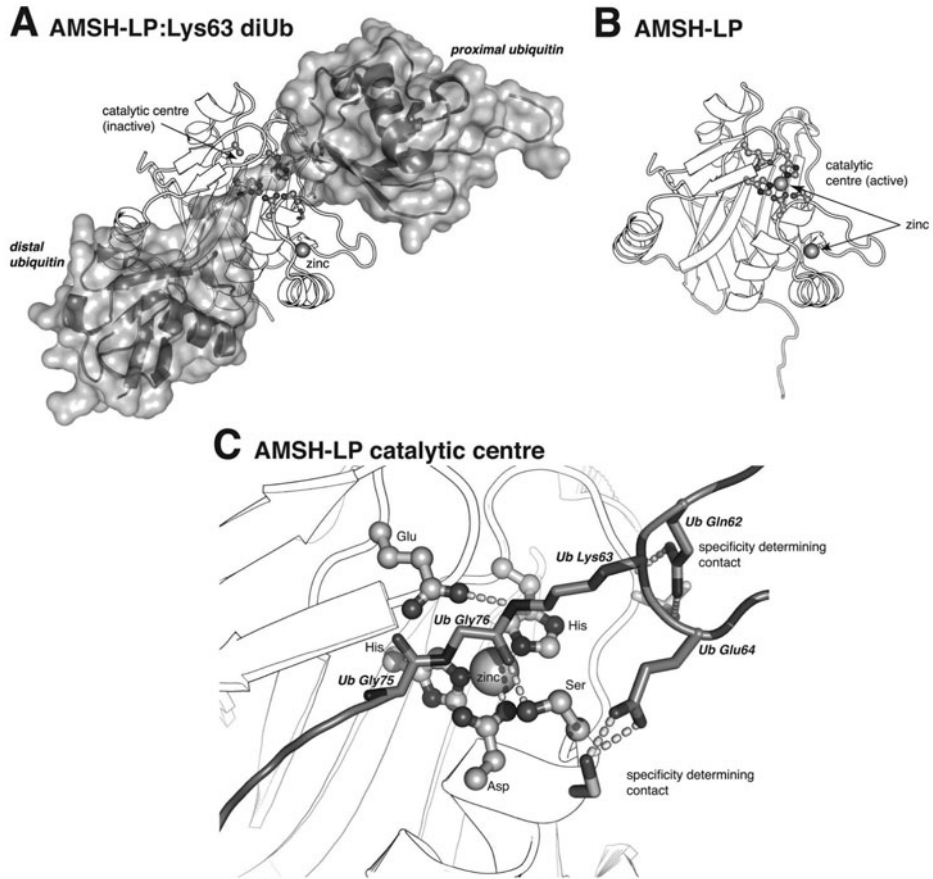
**Figure 5.** Structure of a JAMM/MPN+ domain deubiquitinase. A) Structure of AMSH-LP bound to Lys63-linked diubiquitin (pdb-id 2znv).[41] The JAMM/MPN+ domain (white) is shown in cartoon representation and the catalytic centre residues are shown as stick models in grey and zinc ions as grey spheres. The Lys63-linked diubiquitin is shown under a semitransparent surface and binds across the active site. The complex was obtained by disrupting the primary zinc binding site and mutation of the catalytic Glu residue. B) Structure of the AMSH-LP JAMM/MPN+ domain without ubiquitin (pdb-id 2znv).[41] The enzyme is in an active configuration with two zinc ions. C) Catalytic centre of the AMSH-LP enzyme. The active zinc-bound form is superposed onto the ubiquitin complex. The catalytic residues and their interactions are shown. Also the Lys63-adjacent residues Gln62 and Glu64 are shown in the proximal ubiquitin, which make specificity-determining contacts to the AMSH-LP protein.

specificity was revealed in the crystal structure of AMSH-LP bound to Lys63-linked diubiquitin (Fig. 5).[41] Apart from representing the first DUB structure with a substrate chain bound across the active site, this structure also gave important insights into Lys63 specificity of DUBs. Lys63-linked polyubiquitin chains show an extended conformation[20] and AMSH-LP exploits this, by stretching the Lys63-linkage maximally (Fig. 5A,C).[41] The linker residues are contacted by the protein and furthermore, the sequence context of the Lys63 residue, Gln62 and Glu64, are specifically contacted

by the AMSH-LP JAMM/MPN+ core (Fig. 5C).[41] Hence similarly to CYLD,[19] the proximal ubiquitin containing the Lys residue of the linkage plays an important part in determining the linkage specificity of the DUB. The molecular details for Nedd8 cleavage by the CSN5 JAMM/MPN+ domain, or for the activity of POH1 in the proteasome are less clear.

## CONCLUSION AND FUTURE PERSPECTIVES

Protein deubiquitination is being recognized as a key instrument to understand the complex ubiquitin system. The systematic analysis of DUB involvement in biological processes, facilitated by powerful siRNA screening methods[16,26,61] and by the recent comprehensive analysis of DUB interacting proteins,[49] allowed deep insights into ubiquitin mediated regulatory cascades. The prevalent idea that ubiquitination is primarily a degradation signal has been challenged by identification of DUBs such as TRABID[25] and DUBA,[26] which are specific for nondegradative Lys63-chains. This chain type was not known to be involved in the pathways regulated by these DUBs (Wnt- and IRF signalling, respectively) opening new avenues for understanding of, but also for interfering with, these pathways.

Chain linkage specificity will be a hot topic in the years to come, as the abundance of atypical chain types has just been realized through powerful developments in proteomics.[104,105] However, in order to gain further insight, novel tools have to be developed. Most importantly, chain synthesis of the remaining chain types has to be achieved. DUBs will undoubtedly play a major role to unravel the roles of novel ubiquitin modification and furthermore, the specific members have great potential to become important tools in ubiquitin research.

Despite much progress to understand the deubiquitinases at a structural level, more work lies ahead. The key to understanding DUB specificity is to obtain further structures of DUBs bound to ubiquitin polymers of different linkages. Also the recent identifications of allosteric DUB activators require further studies. Most DUBs are poor enzymes and hydrolyze ubiquitin polymers with slow kinetics. The reasons for this may be non-ideal substrates, or general allosteric mechanisms regulating DUB activity that have not been uncovered. With more DUB structures available, the subtle differences will become apparent.

Numerous DUBs have tight links with human disease. As proteases were in the focus of pharmaceutical intervention for a long time, it is surprising that there has been relatively little progress on the development of DUB inhibitors (for a recent review see ref. 32). The potential of DUBs as drug targets is being realized, but requires careful biochemical and genetic analysis, as well as better assay technologies.[106] This area of research promises to yield exciting and interesting insights in the years to come.

## ACKNOWLEDGEMENTS

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