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"Deubiquitylating enzymes and drug discovery: emerging opportunities"

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Abstract

More than a decade after a Nobel Prize was awarded for discovery of the ubiquitin-proteasome system and clinical approval of proteasome and ubiquitin E3-ligase inhibitors, first-generation deubiquitylating enzyme (DUB) inhibitors are now approaching clinical trials. However, although our knowledge of the physiological and pathophysiological roles of DUBs has evolved tremendously, the clinical development of selective DUB inhibitors has been challenging. Here, we discuss these issues and highlight recent advances in our understanding of DUB enzymology and biology, as well as technological improvements, that have contributed to the current interest in DUBs as therapeutic targets in diseases ranging from oncology to neurodegeneration.

Introduction

The sequential enzymatic processes that covalently attach ubiquitin, a 76-residue polypeptide, to target proteins – a process known as ubiquitylation - are now well understood (Figure 1a)[1]. In some cases, a single ubiquitin is attached to the target protein, while in others, multiple mono-ubiquitin adducts are conjugated to different residues of the target. In many instances, various types of ubiquitin chains are produced, wherein one ubiquitin moiety is attached to a free amino group of another. This leads to linear ubiquitin chains and chains involving internal ubiquitin lysine residues K6, K11, K27, K29, K33, K48, K63, as well as mixed ubiquitin chains containing different linkages, or linkages between ubiquitin and ubiquitin-like proteins (Ubls) that include SUMO (small ubiquitin-like modifier) and NEDD8 (neuronal-precursor-cell-expressed developmentally downregulated protein-8).

These different types of ubiquitin/Ubl modifications, sometimes referred to as "the ubiquitin code", have specific and diverse effects on protein and cell physiology. For example, such modifications can target proteins that are damaged, improperly folded, or have intrinsically short half-lives for degradation via the ubiquitin-proteasome system (UPS)[2]. Here, appropriately polyubiquitylated proteins are recognized and degraded by the 26S macromolecular proteasome complex[3] via mechanisms that have been extensively reviewed elsewhere[4,5]. In other instances, ubiquitylation regulates protein interactions, localisation and enzymatic activities, thereby affecting cellular processes including transcription, DNA-damage signalling and DNA repair, cell cycle progression, endocytosis, apoptosis and various others[6-9]. Such control mechanisms often involve ubiquitin-binding proteins, many of which exist in eukaryotic cells[10]. The recent demonstration of post-translational modification of ubiquitin itself provides an additional layer of regulation that impacts on various cellular processes[11].

Like other posttranslational modifications, ubiquitylation is reversible, with peptidases termed deubiquitylating enzymes (DUBs) cleaving ubiquitin from substrate proteins, editing ubiquitin chains and processing ubiquitin precursors[12]. Some DUBs and related enzymes are involved in editing or processing Ubls and their conjugates[13]; prime examples of these being the SENP (sentrin/SUMO-specific protease) proteins that process SUMO precursors and SUMO-conjugates[14]. DUBs are classified into six families based on sequence and domain conservation (Figure 1b): USPs (ubiquitin specific proteases), UCHs (ubiquitin COOH- terminal hydrolases), MJDs (Machado-Josephin-domain containing proteases), OTUs (ovarian tumour proteases), MINDY (motif interacting with ubiquitin-containing

novel DUB family) and JAMMs (JAB1/MPN/MOV34 family). SENPs and the first five DUB families are cysteine peptidases, while JAMMs are zinc metallo–peptidases.

Ubiquitylation and related processes control myriad aspects of human cell biology and physiology, and defects in such processes contribute to many diseases. Accordingly, DUB deregulation contributes to various sporadic and genetic disorders. Notable examples include: the UCH family member BAP1, mutated in melanoma, mesothelioma and renal-cell carcinoma[15]; USP6, translocated in aneurysmal bone cysts[16]; USP7, mutated in neurological disorders[17]; USP8 whose mutations cause Cushing's disease (CD)[18,19]; USP9X, whose mutations produce developmental disorders[20] and whose expression is dysregulated in cancer[21]; USP15, amplified in certain glioblastoma, breast and ovarian cancers[22]; and CYLD, commonly mutated in cylindromatosis[23]. Deregulation of MJD family DUBs has also been linked to diseases associated with polyglutamine amplification. For example, expansion of DNA "CAG" trinucleotide repeats in ATAXIN-3 (ATXN3) causes Machado-Joseph disease[24]. Furthermore, mutations in the JAMM family member AMSH (STAMBP) cause microcephaly-capillary malformation syndrome[25].

There has been growing interest in exploiting components of the ubiquitylation machinery as therapeutic targets[26]. While there has been strong progress in developing small-molecule inhibitors of ubiquitin/Ubl E1 enzymes[27] , the highly pleiotropic nature of E1s means that such drugs will likely be confined to acute settings, such as in the treatment of aggressive cancers. Given their greater numbers and diversity, E2s, E3s and DUBs offer the potential for developing drugs with more specific effects. In particular, being a group of diverse enzymes with well-defined catalytic clefts, DUBs are intrinsically attractive as potential drug targets[26]. However, as we discuss further below, until recently the development of selective DUB inhibitors has been limited by insufficient understanding of DUB biology, difficulties in establishing robust biochemical assays suitable for compound screening, limitations in cellular and *in vivo* models to assess DUB activity or inhibition, and the pleiotropic nature of various small-molecule DUB inhibitors. With many of these issues now being largely overcome, the rate of progress of DUB drug discovery has quickened over the past few years, with various selective compounds being described and characterized by both academic groups and companies.

In this review, we discuss how DUBs and their deregulation impact on human disease, particularly cancer, neurodegeneration and inflammation (Table 1), and highlight the therapeutic potential for pharmacological modulation of DUB activities. Recent advances in assay development and screening

technologies, which are enabling researchers and drug developers to overcome recurrent challenges in the clinical translation of DUB inhibitors, are also discussed.

DUBs in oncology

Accumulating evidence implicates DUBs in tumorigenesis at multiple levels (Figure 2). First, DUBs such as BAP1, UCHL1 and CYLD have been described as displaying intrinsic oncogenic or tumour suppressor activities[28]. Second, some DUBs such as USP22 are connected to controlling key epigenetic changes that promote tumour development[29]. Third, through their deubiquitylating activities, various DUBs, such as USP7 and USP28 have been reported to regulate the levels and/or activities of various oncogene or tumour suppressor proteins[30,31]. Fourth, DUBs modulate other therapeutically relevant cellular components and processes, such as the ubiquitin proteasome system (e.g. USP14 and UCHL5)[32], stem-cell renewal (e.g. USP16 or USP22)[29,33], DNA-damage responses and repair (e.g. USP1, USP11)[9], immuno-oncology (e.g. USP7)[34], or receptor tyrosine kinases (e.g. USP8, USP9X)[35,36]. Consequently, and as described in more detail below, various DUBs are emerging as attractive targets for the development of novel cancer therapies.

Proteasomal DUBs

The successful targeting of the proteasome for cancer therapy is underlined by the clinical success of Bortezomib, a broadly acting proteasome inhibitor, in refractory multiple myeloma[37] or mantle cell myeloma[38]. However, three DUBs associated with proteasome functions, POH1, USP14 and UCHL5 (UCH37), may represent more specific anticancer targets. To facilitate the degradation of proteasome-targeted substrates, these specialised DUBs remove ubiquitin moieties that would otherwise impede entry into the 20S proteasome catalytic core[39].

The JAMM metallo-protease POH1 has been highlighted as a potential therapeutic target through studies showing that its levels inversely correlate with survival of multiple myeloma patients and that its depletion impairs proliferation of multiple myeloma cells[40]. In addition, nuclear POH1 is elevated in hepatocellular carcinomas and correlates with E2F1 overexpression and tumour growth[41]. POH1 has also been reported to regulate the ubiquitylation and stability of the oncogene, receptor tyrosine kinase ERBB2[42]. Furthermore, as POH1 has been connected to promoting cellular responses to DNA double-strand breaks, particularly by the process of homologous recombination, POH1 inhibition could potentially sensitise cancer cells to DNA-damaging agents and/or preferentially kill cancer cells that rely strongly on homologous recombination[43].

Another potential anticancer therapeutic target is USP14, which is primarily associated with the proteasome 19S regulatory particle, where it potentiates ubiquitin recycling[44]. USP14 is not constitutively active but reversibly associates with the 19S RPN1 subunit, which enhances its activity[45]. USP14 inhibits proteasomal degradation of ubiquitin-protein conjugates by trimming ubiquitin chains on protein substrates prior to their degradation[46]. USP14 expression is upregulated in non-small cell lung cancer, especially in adenocarcinoma[47], and its levels are reportedly elevated in ovarian cancer samples[48]. In line with this, USP14 is connected with several important signalling pathways, for example as a substrate of AKT that mediates intracellular signalling for growth factors[49] and a modulator of dishevelled, a key positive regulator of Wnt signalling[50].

Like USP14, the DUB UCHL5 reversibly interacts with the proteasome[51], binding to the RPN13/ADMR1 receptor[52] in a manner that enhances UCHL5 isopeptidase activity[51,53]. A key function of UCHL5 is to remove distal ubiquitin moieties from polyubiquitylated proteins, thereby liberating proteins from destruction[54], or facilitating destruction of certain substrates, as described for inducible nitric oxide synthase and IκB-α[55]. It therefore appears that, like USP14, UCHL5 suppresses the destruction of certain proteins, while promoting degradation of others. Notably, RNA interference studies showed that depletion of either USP14 or UCHL5 alone had no detectable effect on cell growth, proteasome structure or proteolytic capacity, but did accelerate cellular protein degradation[53]. By contrast, depletion of both DUBs decreased protein degradation, suggesting that they have overlapping functions. UCHL5 is over-expressed in epithelial ovarian cancer, which is associated with advanced tumour progression and poor clinical outcome[56]. UCHL5 is also over-expressed in hepatocellular carcinoma, and was shown to promote cell migration and invasion[57].

These proteasome-associated DUBs represent attractive drug targets, as their inhibition might have substantial effects on cancer-cell physiology but with fewer toxicities than are seen with drugs targeting core proteasome catalytic function[58]. Indeed, VLX1570 (Table 2), the most advanced reported DUB inhibitor, which was recently in Phase I trials (now suspended) for treatment of multiple myeloma and solid tumours[59], has been described to target USP14 and UCHL5[60]. VLX1570 is a ring-expanded version of the compound b-AP15 (VLX1500) identified from cell-based screens looking for compounds inducing p53-independent apoptosis. Cells treated with b-AP15 accumulate polyubiquitin chains[61], and it has been claimed that b-AP15 targets USP14 and possibly also UCHL5[60]. This compound was reported to be reversible and reasonably selective against other DUBs[60] in a cell-based activity probe assay, with an IC_{50} of ~2 µM against purified 19S proteasome DUB activities. b-AP15 has strong activity when tested in various *in vivo* solid tumour models[59], including multiple myeloma[62], but it

remains to be seen whether VLX1570 selectivity will be sufficient to deliver on its promise as a next-generation proteasome inhibitor. Cleave Biosciences has also published a series of patent applications describing compounds that inhibit JAMM proteases, providing potential angles for developing selective POH1 inhibitors (Table 2)[63-65].

DUBs linked to DNA repair

One hallmark of cancer is the down-regulation, loss or deregulation of certain DNA repair and DNA-damage response (DDR) pathways and/or strong reliance on such pathways[66,67]. DNA repair and DDR mechanisms are regulated by post-translational modifications, such as ubiquitylation, with many DUBs strongly linked to such processes[9,68].

One example of this is USP1, a DUB identified as a regulator of FANCD2 ubiquitylation, a key protein involved in the Fanconi anemia (FA) pathway of DNA crosslink repair[69,70]. USP1 influences accumulation of the FA core complex at DNA-damage sites and deubiquitylates FANCD2/FANCI in a cell-cycle dependent manner[69]. USP1 also removes mono-ubiquitin from PCNA, a DNA-replication component that also functions in DNA repair by translation synthesis[71]. Other USP1 activities include functioning in a feedback loop to limit DDR CHK1 protein kinase activity[72] and regulating cellular differentiation in osteosarcoma cells by deubiquitylating and hence affecting the stability of ID (inhibitors of DNA binding) proteins[73]. *In vitro*, USP1 activity is greatly stimulated by UAF1 (WDR48), enhancing USP1 catalytic turnover (k_{cat}) but not affinity (K_m) for mono-ubiquitylated substrates[74]. Selective USP1 inhibitors with sub-micromolar potency have been identified[75], with one, pimozide, shown to re-sensitise platinum-resistant non-small lung cancer cells and promote FANCD2 and PCNA mono-ubiquitylation[75]. However, while these studies indicated on-target effects, DUB selectivity profiling suggested that pimozide might be less selective than initially described[76]. Optimisation of certain USP1 screening hits has generated additional molecules[77], most notably a selective pyrimidine-core compound, ML323 (Table 2). This molecule allosterically blocks complex formation between UAF1 and USP1[78], potentiates cisplatin cytotoxicity and increases PCNA and FANCD2 mono-ubiquitylation in cells[77]. So far, however, little progress has been made in advancing selective USP1 inhibitors into clinical development.

Another DUB linked to DNA repair is USP11, which was initially described to complex with the DDR tumour suppressor BRCA2 to promote the DNA double-strand break repair pathway of homologous recombination[79]. Depletion of USP11 has been shown to sensitize cells to AZD2281/olaparib, which inhibits the DDR enzyme PARP[80]. Recently, an interaction between BRCA1 and PALB2 – which

functionally cooperate with BRCA2 in DNA repair – was shown to be under ubiquitin control, with PALB2 ubiquitylation suppressing its interaction with BRCA1 in a manner counteracted by USP11[81]. The only currently reported USP11 inhibitor is the topoisomerase inhibitor mitoxantrone (Table 2)[82]. While the authors reported low nanomolar potency in a pancreatic ductal adenocarcinoma cell survival model, no further development of this compound has been reported. Given the apparent amenability of USP11 to small-molecule inhibition, it is notable that USP4, a DUB closely related to USP11, was recently shown to play important roles in the DDR via promoting early stages of homologous recombination[83].

USP9X[21], which maintains DNA replication-fork stability and DNA-damage checkpoint responses by regulating the protein CLASPIN during S-phase[84], may represent another potential therapeutic target. USP9X has been shown to affect radiosensitivity in glioblastoma cells by MCL1-dependent and -independent mechanisms[85]. The best-described USP9X inhibitor is WP1130 (Table 2), identified in a screen for JAK2 inhibitors, which was shown to inhibit USP9X as well as other DUBs (USP5, USP14 and UCHL5)[86,87]. The covalent mechanism-of-action of this compound was shown via mass spectrometry to be reversible[76].

Regulation of oncogenes and tumour suppressors

Various DUBs have been reported to have connections to tumour suppressor or oncogenic functions, and may therefore represent potential therapeutic targets[88].

p53 regulation: Several DUBs have been linked to regulation of the tumour suppressor protein p53, which plays pivotal roles in cellular stress responses and is lost or mutated in many cancers[89]. Human HDM2 is a RING-type ubiquitin E3 ligase and key negative regulator of p53, via its ability to ubiquitylate p53 and target it for degradation[90]. By cleaving ubiquitin chains on HDM2 (or its mouse counterpart MDM2), USP7 counteracts HDM2 proteasomal degradation, leading to p53 suppression through increased ubiquitylation and degradation[91,92]. In theory, therefore, USP7 inhibition should trigger HDM2 degradation, p53 stabilisation and ultimately activation of apoptotic pathways in tumour cells[93]. Additional USP7 targets have also been described, such as PTEN, FOXO4 and FOXP3[34,94,95], suggesting alternative therapeutic mechanisms for USP7 inhibitors. USP7 has also recently been shown to promote DNA replication via acting as a deubiquitylase for the Ubl, SUMO[96].

The first published sub-micromolar USP7 inhibitor, HBX41108[97], was shown to be a rather non-specific inhibitor of DUBs[76]. Recently, more selective amidotetrahydroacridine derivatives such as HBX19818

and HBX28258 were identified, although these exhibited fairly low potency[98]. Despite this, HBX19818 was shown to covalently bind the USP7 catalytic Cys in preference to other cysteinyl groups, and to stabilise p53 and promote G1 arrest and apoptosis in cells[98]. Progenra's thiophen chemical series also provided relatively non-specific USP7 inhibitors, including the compounds P5091 and P22077[99]. In multiple myeloma cells, P5091 stabilised p53 and inhibited tumour growth, while in animal models, P5091 was well-tolerated, inhibited tumour growth, and prolonged survival[99]. More recent *in vivo* studies using P22077 within an orthotopic neuroblastoma mouse model showed significant inhibition of xenograft growth[100]. While these findings are encouraging, little is known about the binding modes of these compounds and whether they can be further optimised into more "drug like" entities. Recently, Almac Discovery and Genentech reported that fragment-based screens provided hits as starting points for USP7 discovery programmes[101]. Optimisation of one hit, ADC-01, assisted by X-ray crystallography, produced the non-covalent, highly selective USP7 inhibitor ADC-03 (Table 2).

The stability of p53 has also been recently reported to be regulated by the DUB, ATXN3[102]. ATXN3 was shown to bind and deubiquitylate p53, resulting in p53 stabilisation. Deletion of ATXN3 resulted in destabilisation of p53, while ectopic expression of ATXN3 induced expression of p53 target genes and promoted p53-dependent apoptosis. How and whether ATXN3 inhibitors could be exploited to treat cancer or other diseases remains to be established.

USP28 is another DUB that has recently been connected to p53, which functions together with the protein 53BP1 to promote p53-mediated transcriptional responses[103]. Furthermore, USP28 is mutated in human cancer cells, and is reported to antagonise the tumour suppressor FBW7[31], highlighting the potential for USP28 inhibitors in various cancers, especially colorectal[104]. USP28 is also reported to antagonise ubiquitin-dependent degradation of the oncogene product MYC as well as c-JUN and NOTCH[105]. While no USP28 inhibitors have yet been reported, it seems likely that drug-discovery activities are underway.

HIF1α and USP20: Another tumour suppressor protein, which has been linked to DUB activity, is the von Hippel-Lindau tumour suppressor protein (pVHL), which ubiquitylates hypoxia-inducible factor 1α (HIF1α) when cellular oxygen levels are normal, leading to the degradation of HIF1α. USP20, also known as VHL protein-interacting deubiquitinating enzyme 2 (VDU2), is reported to deubiquitylate a number of proteins, including HIF1α. USP20-mediated deubiquitylation of HIF1α prevents proteasomal degradation, allowing for transcription of hypoxic response genes. Thus,

inhibition of USP20 has potential for suppressing proliferation of hypoxic tumour cells. GSK presented brief details of its search for USP20 inhibitors at a conference in 2012 (Table 2)[106].

EGFR and USP8: Ubiquitylation serves as a signal that delivers membrane receptors from the cell surface to lysosomes, and in mammalian cells this has been most intensively studied for epidermal growth factor receptor (EGFR). Upon EGF binding, activated EGFR is rapidly internalized and transported, via early and late endosomes, to lysosomes where EGFR is degraded. USP8, also known as UBPY, deubiquitylates EGFR on early endosomes, rescuing EGFR from degradation[107,108]. In several cancers, including lung, breast and glioblastoma, EGFR is amplified or mutated in the tyrosine kinase domain, resulting in deregulation of receptor signalling that drives uncontrolled proliferation of tumour cells[109]. USP8 inhibitors (e.g. HBX90659) of a similar structural class to those identified for USP7[110] have been reported (Table 2). Moreover, a derivative of these compounds was shown to be efficacious in mouse models of lung cancer[111].

TGF-β and USP15: USP15 regulates the TGF-β (transforming growth factor beta) pathway and is believed to be important for the proliferation of glioblastoma cells[22]. USP15 binds to the SMAD7–SMAD E3 ligase complex and deubiquitylates and stabilises the type I TGF-β receptor, leading to enhanced TGF-β signalling. The *USP15* gene is amplified in glioblastoma, breast and ovarian cancers, and high expression of USP15 correlates with high TGF-β activity[22]. Depletion of USP15 reduces the oncogenic capacity of patient-derived glioma-initiating cells due to the diminished TGF-β signalling, suggesting therapeutic potential for development of USP15 inhibitors. In addition, USP15 has been shown to deubiquitylate receptor-activated SMADs (R-SMADs)[112], another set of TGF-β signalling pathway components.

Other oncogenic DUBs: The DUB UCHL1, normally expressed only in neurons and neuro-endocrine tissues[113,114], is highly expressed in many cancers, with its expression correlating with poor prognosis[115]. While there are reports that UCHL1 has a tumour suppressive role, most evidence supports its role as an oncogene[115]. Indeed, in a transgenic mouse model with constitutively activated UCHL1, sporadic tumours developed in many tissues[116]. Moreover, *in vitro* tumorigenesis studies showed that UCHL1 expression stimulated oncogenesis and an invasive phenotype[117-119], while UCHL1 depletion had anti-tumour effects and blocked cell migration in a lung cancer cell line[117]. The precise mechanism by which UCHL1 contributes to tumorigenesis remains unclear, although reports suggest that it contributes to cell survival signalling, cell cycle regulation, DNA repair, and regulating pools of

free ubiquitin in ways that affect protein degradation and function[115]. UCHL1 inhibitors have been described, the most potent being isatin acyl-oximes (LDN-57444, Table 2) with some selectivity over UCHL3[120]. In addition, a series of pyridinones have been identified as moderate UCHL1 inhibitors[121]. Enzyme kinetic studies revealed that these compounds are uncompetitive inhibitors and are selective for UCHL1, exhibiting no inhibition of other cysteine hydrolases tested. A weak tripeptide fluoromethyl ketone (FMK) inhibitor was subsequently shown through crystallographic studies to bind within the UCHL1 active site, irreversibly modifying the active-site cysteine[122]. Mission Therapeutics has also developed several series of potent and selective UCHL1 inhibitors[123,124]. While no UCHL1 inhibitors have demonstrated anti-tumour activity *in vivo*, inducible depletion of UCHL1 has been shown to cause disease regression in an orthotopic multiple myeloma model[125].

Another DUB associated with oncogenesis is USP22, the catalytic subunit of a deubiquitylase module in the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex. The best-characterised substrates for SAGA include several acetylation sites in histone H3 and a ubiquitylation site in histone H2B, post-translational modification of which regulates gene expression[29]. USP22 has strong links to oncogenesis[29], having been identified in microarray screens as part of an 11-gene 'death from cancer' signature for highly aggressive, therapy-resistant tumours. USP22 was later shown to act as an oncogene product, regulating cell cycle progression, proliferation and apoptosis[126]. Increased expression of USP22 has been connected with poor prognosis in several cancers including liver[127], colorectal[127], breast[128], oesophageal squamous-cell carcinoma[129] and oral squamous-cell carcinoma[130]. If USP22 DUB activity can be linked to survival and progression of these cancers, then inhibitors may provide attractive prospects for new therapies.

Cancer immunotherapy

Given the role of ubiquitin modifications and DUBs in many inflammatory processes (see below) as well as the renewed interest in targeting the immune system to fight cancer, the anti-neoplastic potential of therapeutically inhibiting DUBs involved in the immune system is being investigated. Amongst these is USP7, which positively regulates the stability of FOXP3, a critical transcription factor controlling the differentiation of regulatory T cells (Treg)[34]. In a search for DUBs that contribute to GATA3 stabilisation in Foxp3-expressing cells, both USP7 and USP21 were shown to upregulate GATA3-mediated activity using a reporter assay[131]. Furthermore, depletion of USP21 in Treg cells resulted in downregulation of FOXP3, compromised expression of Treg signature genes and impaired their suppressive activity[132]. As Treg cells restrict anti-tumour immune responses and promote tumour survival[133], these results suggest that depletion of FOXP3 in Treg cells by targeting USP7 and USP21

offer promise for anti-cancer immunotherapies. In this regard, Mission Therapeutics is investigating USP7 as an immuno-oncology target and has developed USP7 inhibitors (Mission Therapeutics Pipeline available from: http://missiontherapeutics.com/programmes/).

DUBs in neurodegenerative disease

Identification of ubiquitin in protein aggregates associated with neurodegenerative pathologies such as neurofibrillary tangles in Alzheimer's disease, Lewy bodies in Parkinson's disease or intranuclear inclusions in heridetary polyglutamine expansion disorders, has prompted much interest in understanding how ubiquitylation and deubiquitylation affect such aggregates[134]. DUB function in the central nervous system has been described in detail elsewhere[135,136], therefore below we focus on a select number of DUBs connected to neurodegenerative disease.

Mitochondrial quality control

Mitochondrial dysfunction and UPS impairment have been described as hallmarks of aging[137], and have been implicated in the etiopathogenesis of many age-related diseases, particularly neurodegenerative disorders such as Alzheimer's and Parkinson's. In accord with this connection, ubiquitylation has close links to mitochondrial function, with the UPS maintaining mitochondrial homeostasis by regulating organelle dynamics, the mitochondrial proteome and mitophagy[138]. Conversely, mitochondrial dysfunction can impair cellular protein homeostasis by generating oxidative damage. Notably, mutations in the ubiquitin E3 ligase Parkin are causally associated with certain cases of familial Parkinson's disease[139]. As Parkin ubiquitylates mitochondrial components, thus promoting turnover of mitochondria by lysosome-mediated mitophagy, defective mitophagy and accumulation of defective mitochondria that cause enhanced oxidative stress could be an underlying cause of Parkinson's disease[140,141]. A corollary of this is that Parkin activation – or inhibition of factors counteracting Parkin – could provide opportunities for disease alleviation.

A screen for DUBs that oppose Parkin function identified the mitochondrial-associated DUB USP30 as an antagonist of Parkin-mediated mitophagy[142,143], with USP30 depletion significantly decreasing mitochondrial numbers in cells, a phenotype that was rescued by wild-type but not catalytically inactive USP30. Furthermore, USP30 depletion *in vivo* provided stress protection in *Drosophila melanoga*ster models of Parkinson's disease (*park^{25}* or *pink1^{B9}*). In line with such findings, USP30 depletion in human HeLa cells led to elongated and interconnected mitochondria[144], suggesting a role for USP30 in regulating mitochondrial fusion/fission. Current models invoke USP30 functioning under normal physiological conditions to prevent inappropriate mitophagy. However, in response to stresses

such as membrane depolarization, Parkin is recruited to mitochondria to promote mitophagy[145]. Accordingly, under conditions of mitochondrial dysfunction – such as are caused by defects in Parkin (or its positive regulator PINK1), USP30 is thought to counteract clearance of damaged mitochondria, leading to a build-up of metabolically and energetically deficient cells[142]. It is thus hypothesised that, in the context of certain mitochondrial dysfunctions, USP30 inhibition would have therapeutic benefits. So far, only one chemical inhibitor of USP30 has been described, 15-oxospiramilactone (Table 2), which induced mitochondrial elongation in *Mfn1*-knockout mouse fibroblasts, with no effect on cell viability[146]. Mission Therapeutics is exploring USP30 inhibition for the treatment of Parkinson's disease and other mitochondrial disorders, and has published several patent applications describing USP30 inhibitors[124,147].

Two other DUBs connected to mitophagy are USP8 and USP15. Notably, USP8 depletion was found to delay Parkin translocation onto depolarized mitochondria, as well as mitochondrial clearance, and USP8 displayed an ability to remove K6 ubiquitin chains from Parkin *in vitro*[148]. In addition, USP8 has been shown to remove ubiquitin K63 chains from α-synuclein[149], a protein known to aggregate, often in a ubiquitylated form, in neuronal inclusion bodies (Lewy bodies) associated with neurodegenerative diseases such as Parkinson's disease. Depletion of USP8 in either human cells or *Drosophila* resulted in increased lysosomal degradation of α-synuclein[149]. Meanwhile, USP15 was identified as a Parkin-interacting protein that co-localizes with mitochondria[150]. In cells over-expressing Parkin, over-expression of wild-type but not catalytic-dead USP15 strongly inhibited mitophagy[143,150]. Furthermore, depleting endogenous USP15 enhanced mitophagy in HeLa cells, in a human dopaminergic neuronal cell line and in primary fibroblasts from human patients[150]. USP15 does not deubiquitylate Parkin under basal conditions or when cells are treated with mitochondrial depolarizing agents. It also does not appear to affect Parkin translocation to mitochondria[150], although it can oppose Parkin-mediated mitochondrial ubiquitylation. Finally, USP15 loss in *Drosophila* was found to rescue both locomotor defects and accumulation of dysfunctional mitochondria in flight muscles of *parkin* knock-out flies[143]. Collectively, these findings highlight the potential for USP8 and USP15 inhibitors in Parkinson's disease and perhaps other diseases associated with mitochondrial dysfunction.

Further highlighting connections between Parkinson's disease and DUBs, ATXN3 has been shown to interact with Parkin in a manner that counteracts Parkin auto-ubiquitylation[151]. In addition, USP7 was recently shown to remove K63-linked ubiquitin chains from α-synuclein[149], a protein that aggregates and accumulates in Lewy bodies, which are hallmarks of Parkinson's disease.

USP14

As described above, USP14 removes ubiquitin from certain substrates targeted to the proteasome, thus rescuing such substrates from degradation and maintaining free ubiquitin pools[54,152]. IU1 (Table 2), a reversible small-molecule USP14 inhibitor, was shown to target the USP14 catalytic site[46] and promote degradation of several over-expressed proteins whose accumulation is linked to neurodegenerative diseases, such as Tau, TDP-43 and ATXN3. Notably, IU1 only promoted degradation in *Usp14*$^{+/+}$ murine embryonic fibroblasts[46] but not in *Usp14*$^{-/-}$ cells, suggesting that this compound functions specifically through USP14. Furthermore, IU1 reduced accumulation of menadione-induced oxidized proteins and ameliorated menadione or hydrogen peroxide-induced cell death in human HEK293 cells[46]. Proteostasis Therapeutics (in collaboration with Biogen) is developing USP14 inhibitors for the clearance of aggregation-prone proteins, including α-synuclein in Parkinson's disease and Tau in Alzheimer's disease (http://www.proteostasis.com/product-pipeline/usp14/), and has published several patent applications describing USP14 inhibitors[153-155].

Despite the growing interest in USP14 as a therapeutic target in cancer and neurodegeneration, the fact that its loss causes severe morbidity and postnatal lethality requires further investigation, especially in regards to its role in neuromuscular junctions: the neuromuscular phenotype of USP14 deficient ax^{J} mice is rescued by neuronal-specific expression of USP14[156]. Furthermore, the extent to which USP14 contributes to the clearance of proteins involved in neurodegeneration *in vivo* remains controversial[157]. The development and use of USP14 inhibitors in disease-relevant models may shed further light on such issues, and hopefully will define potential therapeutic windows for USP14 inhibition in disease settings.

USP16

Down syndrome is a congenital disorder driven by triplication of human chromosome 21, on which the *USP16* gene resides. USP16 has been reported to regulate cell-cycle progression and gene expression through deubiquitylation of histone H2A[158]. Defects in haematopoietic stem-cell self-renewal in a Down syndrome mouse model were rescued by reducing USP16 expression to levels similar to those in control mice[159]. In addition, USP16 over-expression in normal human fibroblasts and neural progenitors lead to reduced cell expansion[159], similar to the strong proliferation defects observed in human Down syndrome fibroblasts[160]. Thus, USP16 is a key regulator that controls stem cell self-renewal and senescence in Down syndrome, suggesting that inhibitors of USP16 might provide therapeutic benefits to such individuals.

DUBs in immunity and inflammation

Pathogens are recognised by several families of pattern-recognition receptors (PRR), and activate various signal-transduction cascades via the retinoic acid–inducible gene 1–like receptor (RLR), nucleotide-binding oligomerization domain-like receptor (NLR) and the toll-like receptor (TLR)[161]. These signalling events mediate induction of inflammation that is important for recruiting immune cells to sites of infection. Ubiquitylation is a critical post-translational modification in this process[161]. Non-degradative K63- and M1-linked ubiquitin chains mediate the key upstream event of recruiting the TGFβ-activated kinase (TAK1) and the IκB kinase (IKK) complexes, respectively[162]. K63 polyubiquitination activates the TAK1 kinase complex, which phosphorylates IKKβ at key serine residues in the activation loop, resulting in IKK activation and transcriptional activation of target genes which include mediators of immune and inflammatory responses as well as feedback inhibitors of the NF-kB pathway[163]. Negative regulators include DUBs that cleave K63 and linear chains such as A20, CYLD and OTULIN (also known as FAM105B or Gumby)[161,164,165].

The *TNFAIP3* gene, which encodes the A20 protein, is probably the best-characterized DUB linked to inflammation[166]. A20 plays a key role in restricting TLR signalling and maintaining immune homeostasis through deubiquitylation of NF-κB signalling factors such as NEMO, RIPK1 and TRAF6[167]. In addition, A20 can bind polyubiquitin chains through its zinc finger domain, allowing for interaction with ubiquitylated NEMO protein. This ubiquitin-induced recruitment of A20 to NEMO is sufficient to block IKK phosphorylation by its upstream kinase TAK1, preventing NF-κB activation[168]. Thus, A20 deficiency promotes local or systemic inflammation *in vivo*, underscoring why inactivating *TNFAIP3* mutations have connections with both inflammatory and autoimmune syndromes[169].

CYLD is another DUB known to negatively regulate ubiquitylation of RIG-1 (one of the major RLRs) and RIG-I mediated IFN gene induction[170,171]. CYLD binds to RIG-I and inhibits ubiquitylation and signalling functions of RIG-I. CYLD also inhibits the ubiquitylation of TBK1 and IKKε which contributes to the negative regulation of IFN responses[171]. Consistently, CYLD deficiency causes constitutive activation of TBK1 and IKKε in dendritic cells. Despite enhanced RIG-I signalling, CYLD-deficient cells and mice are more susceptible to VSV infection due to attenuated signalling and antiviral gene expression induced by IFNβ, suggesting a positive role for CYLD in regulation of type I IFN receptor function[161].

Ubiquitin M1-linked chains are generated by the linear ubiquitin chain assembly complex (LUBAC) consisting of HOIP, HOIL-1 and SHARPIN. LUBAC is recruited to many immune receptors, and

ubiquitylates target proteins, including RIPK1, RIPK2, MyD88, IRAKs and NEMO[172,173]. Genetic loss of LUBAC components leads to immunodeficiency[174] and inflammatory phenotypes in mice[175-178], and mutations in LUBAC components also cause inflammatory conditions in humans[179,180]. Hence, loss of M1-linked chains imbalances immune signalling. OTULIN is the only DUB known to specifically cleave M1 linkages[181,182]. Accordingly, a homozygous hypomorphic mutation in human *OTULIN* has recently been shown to cause a potentially fatal auto-inflammatory condition termed OTULIN-related autoinflammatory syndrome (ORAS)[183].

Similar to ubiquitin, the Ubl ISG15 (interferon-stimulated gene 15) plays a key role in cellular signalling in response to pathogens. Conjugation of ISG15 to various cellular substrates is reversed by the interferon (IFN)-inducible isopeptidase USP18. USP18 is upregulated after viral infection, type I and type III IFNs, lipopolysaccharide, tumour necrosis factor alpha or genotoxic stress. In addition to its isopeptidase activity, USP18 negatively regulates type I and type III IFN signalling by blocking the type I IFN receptor 2 subunit[184].

Inflammatory and autoimmune disorders

Debilitating autoimmune diseases range from those with genetic components such as Crohn's disease, diabetes mellitus type 1, Graves disease and rheumatoid arthritis[185], to sporadic conditions including celiac disease, inflammatory bowel disease, multiple sclerosis, psoriasis, and systemic lupus erythematosus. In addition, chronic inflammatory diseases are characterised by a prolonged and persistent pro-inflammatory state, and include autoimmune disease as well as metabolic syndromes, neurodegenerative disease, chronic obstructive pulmonary disease and cardiovascular disease.

Following PRR stimulation, dendritic cells secrete various cytokines that regulate the differentiation of $CD4^+$ T cells to different subsets of helper T (Th) cells, including inducible Treg cells, T follicular helper cells, and Th1, Th2, Th9 and Th17 cells[186]. Th17 cells mediate pro-inflammatory functions through the secretion of pro-inflammatory cytokines, including IL-17A, IL-17F, and IL-22[187]. Moreover Th17 cells have been implicated in the development of autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus [188].

USP4 has been shown to stabilise the nuclear receptor RORγt in Th17 activated T cells, and has been proposed as a possible therapeutic target for rheumatoid arthritis[189]. Yang *et al.*, reported that USP4 is highly expressed in Th17 cells and its depletion resulted in decreased RORγt as well as IL-17A expression[189]. In addition, use of the reported USP4 inhibitor Vialinin A (Table 2) also diminished RORγt

and IL-17A expression[190]. Furthermore, expression of USP4, IL-17A and IL-17F mRNA have been shown to be significantly elevated in $CD4^+$ T cells from rheumatoid arthritis patients compared to healthy controls[189], providing further evidence for a role of USP4 in rheumatoid arthritis.

TRABID (also known as ZRANB1), is required for TLR-mediated expression of the inflammatory cytokines IL-12 and IL-23 in dendritic cells[191]. TRABID is proposed to deubiquitylate and stabilise the histone demethylase, JMJD2D, which regulates histone modification at the *Il12* and *Il23* promoters to facilitate recruitment of the NF-κB family member c-Rel[191]. Conditional deletion of TRABID in dendritic cells impairs IL-12 and IL-23 production and the generation of Th1 and Th17 subsets of inflammatory T cells, rendering mice refractory to the induction of experimental autoimmune encephalomyelitis (EAE)[191].

Another DUB associated with the activity of Th17 cells is USP18. Although this DUB has been extensively studied in the context of viral infection, Liu *et al.*, demonstrated that USP18 regulates the TAK1-TAB interaction, which is required for Th17 differentiation and autoimmune response[192]. Consistent with this, USP18-deficient mice were resistant to EAE[192].

T cell receptor signalling has been shown to be facilitated by the DUB, CEZANNE1 (OTUD7B), which binds and deubiquitylates zeta-chain associated protein (ZAP70), thus preventing the interaction of ZAP70 with negative-regulatory phosphatases[193]. ZAP70 is a cytoplasmic protein tyrosine kinase that plays a critical role in T-cell signalling. ZAP70 is recruited to phosphorylated sites on the T cell receptor where it is subsequently phosphorylated by the SRC kinase LCK. Phosphorylation of ZAP70 is required for full activation and downstream phosphorylation of adaptor proteins, which facilitate T cell signalling[194]. In addition, CEZANNE1 deficient mice exhibit attenuated T cell responses to bacterial infection and were refractory to EAE[193]. While young CEZANNE1 knockout mice had similar naïve and memory-like T cells compared to wild-type mice, older mice deficient for CEZANNE1 had reduced IFN-γ producing Th1 cell subsets[193].

Similar to Th17 cells, Th1 cells have the capacity to cause inflammation and autoimmune disease. The development, differentiation and function of Th1 cells is driven by the T-box transcriptional factor T-bet, which promotes Th1 immune response primarily through promoting expression of the cytokine IFN-γ[195]. The DUB USP10 has been shown to deubiquitylate and stabilise T-bet, resulting in enhanced secretion of IFN-γ[196]. In addition, USP10 mRNA expression was found to be elevated in PBMCs from patients with asthma compared to healthy donors[196].

While it is currently unclear why so many DUBs are involved in the regulation of immune responses, it is possible that different DUBs function in distinct cell types. Many published studies are based on cell lines and over-expression systems, and the expression of endogenous DUBs in various immune cells will be an important area for future investigation. Similarly, the generation of genetic models and the development of inhibitors for Cezanne1, TRABID, USP4, USP10 and USP18 will help determine their therapeutic potential.

DUBs with links to infectious diseases

As described below, there is growing interest in DUBs as potential therapeutic targets for various infectious diseases of man and other animals. Such potential is being explored both by developing compounds that inhibit the activity of pathogen-encoded DUB-like proteins, or target host-cell DUBs that control the pathogen life cycle or infectivity.

Viral infections

Ubiquitylation is important for modulation of protein–protein interactions, including the activation of innate immune signalling pathways, so perhaps not surprisingly, various viruses encode DUBs as a strategy to inhibit ubiquitin and ISG15-dependent antiviral pathways[197]. Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are two of the six known human coronaviruses. Both are highly pathogenic, with the potential for human-to-human transmission, and contain papain-like cysteine proteases termed SARS-CoV PLpro and MERS-CoV PLpro, respectively. In addition to processing viral polyprotein, these proteases remove ubiquitin and ISG15 from host cell factors, resulting in antagonism of the host antiviral immune response[198]. Hence, both SARS-CoV PLpro and MERS-CoV PLpro have been proposed as important antiviral targets. The X-ray structures of both proteases have shown similarity to the USP family of DUBs[199-201].

OTU domain-containing proteases from diverse RNA viruses, including the nairoviruses Crimean-Congo hemorrhagic fever virus and Dugbe virus, the papain-like protease (PLP2) domain of the arterivirus equine arteritis virus, and the protease (PRO) domain of the tymovirus turnip yellow mosaic virus can hydrolyze ubiquitin and ISG15 from cellular target proteins[197,202]. Many positive-strand RNA viruses, including arteriviruses and tymoviruses, encode polyproteins that are post-translationally cleaved by internal protease domains. In accord with this, both arterivirus PLP2 and tymovirus PRO

are critically required for viral replication due to their primary role in polyprotein maturation[197]. Thus, viral OTU proteases may represent promising therapeutic targets.

Bacterial infections

Bacteria employ a repertoire of effector proteins that target the eukaryotic ubiquitin system to promote bacterial pathogenicity. Pruneda *et al.* have recently characterised protease activity from human bacterial pathogens including *Salmonella* (SseL)*, Escherichia* (ElaD)*, Shigella (ShiCE), Chlamydia* (ChlaDUB1)*, Rickettsia* (RickCE), and *Legionella* (LegCE)[203]. LegCE showed no proteolytic activity; SseL, ElaD, and ShiCE demonstrated ubiquitin-specific protease activity; while ChlaDUB1 and RickCE cleaved both ubiquitin and, to a lesser extent, NEDD8-modified peptides. Interestingly, these DUBs encoded by human pathogens showed strong preference for K63-linked chains, only targeting K48 and K11 chains at later time points or higher enzyme concentrations. Therefore, bacterial DUBs are potential therapeutic targets.

Parasitic infections

In addition to expressing DUBs that target host functions, similar to viruses and bacteria, eukaryotic parasites also possess Ubl pathways of their own. The use of ubiquitin-based activity probes to identify DUBs in *Plasmodium falciparum* led to the identification of PfUCH54, which was shown to have deubiquitylating activity and also an ability to remove adducts of the Ubl, NEDD8[204]. Further investigation of the parasite *Toxoplasma gondii* using a similar strategy identified four DUBs, one of which was orthologous to mammalian UCHL3[205]. Structural studies on PfUCHL3 explained the dual specificity of the enzyme, and PfUCHL3 was found to be required for parasite survival[206]. Distinct differences in the ubiquitin binding site between PfUCHL3 and its human counterpart suggest that this parasitic DUB can be selectively targeted by inhibitors. Based on the above findings, it will be of great interest to further explore anti-infective opportunities for DUB inhibitors.

Therapeutic challenges, emerging technologies and compounds

Despite the significant and growing attractiveness of DUBs as drug targets, DUB-focused drug discovery has been challenging, with researchers in this arena facing various obstacles. First, while DUBs have clear catalytic pockets that *a priori* appear suitable for drug development, a key challenge has been to identify potent compounds that show selectivity amongst related DUBs and have properties commensurate with their development for clinical use. Second, ubiquitylation and deubiquitylation are intracellular processes that, at least at present, are only amenable to classical small-molecule chemical approaches. Third, because most DUBs execute the transfer of ubiquitin

molecules via a reactive thiol group, most standard assays used to identify inhibitors are prone to non-selective redox or alkylating false positives[207]. Fourth, the mechanisms-of-action of DUB enzymes are often complex, involving regulation of enzymatic activity through allosteric effects and/or substrate-mediated catalysis, with many DUBs alternating between active and non-active conformations (see below)[208,209]. This makes it challenging both to design predictive biochemical assays and develop drug-like compounds. Finally, DUBs often display specificity for ubiquitin chains as well as the target proteins. Hence, to optimise the likelihood of identifying genuine inhibitors, it is prudent to develop bespoke primary screening and secondary assays that recapitulate the most physiological substrate and ubiquitin-linkage setting for each DUB.

Despite the above issues, DUBs are fundamentally catalytically-driven proteins with known enzymatic functions, and as such present researchers with the opportunity to identify small-molecule inhibitors either within the active site or at adjacent allosteric pockets. Indeed, over the past few years there has been an increasing rate of progress in successfully screening for and evolving small-molecule DUB inhibitors, with the most developed of these now moving towards or into clinical evaluation (for examples, see Table 2).

Understanding DUB-substrate interactions

Understanding the mechanism-of-action of individual DUBs is important when initiating any screening and subsequent drug-discovery campaign. DUBs are generally isopeptidases that, in most cases, catalyse a proteolytic reaction between a lysine ε side chain and a carboxyl group corresponding to the ubiquitin C-terminus[209]. The last two C-terminal amino acid residues are glycines (Gly75-Gly76) that lack side chains, resulting in a narrow linker on either side of the isopeptide bond, which is mirrored in a long and narrow DUB catalytic cleft[209]. Moreover, cysteinyl-protease DUB catalytic activity tends to rely on two or three crucial residues comprising a catalytic diad or triad, generally constituted by a His side-chain that, by lowering the *pKa* of the catalytic Cys, leads to a nucleophilic attack on the ubiquitin-substrate isopeptide linkage[12]. Collectively, these properties bring complexity to identifying selective small-molecule inhibitors that target DUB catalytic sites and are likely to restrict the breadth of series that are suitable for developing potent and selective DUB inhibitors.

The Proteostasis thiophene pyrimidine-cored USP14 inhibitors are known to bind in the ubiquitin pocket and prevent the ubiquitylated substrate binding[210]. However, the majority of historical and current DUB drug-discovery programmes have focused on chemical series that include the provision of an active “warhead” that forms a reversible or irreversible covalent adduct with the DUB catalytic cysteine. The high reactivity of some of these warheads, which include oxidative, alkylating and

arylating moieties[210], is likely to limit drug selectivity, may hamper the development of acceptable pharmacokinetic and pharmacodynamics parameters, and may also pose risks of idiosynchratic toxicities in patients.

For this reason, less reactive warheads are being explored that are closely related to warheads utilised by non-DUB cysteine protease inhibitors in the clinic. For example, the USP8 inhibitor identified from a library of amidomethyl methyl acrylates (Compound 6)[211] contains a Michael acceptor group also found in Rupintrivir, an inhibitor of rhinovirus 3C protease and a GSK cathepsin C inhibitor[210]. In addition, USP9X inhibitors WP1130 and EOA1342143[212] contain a Michael acceptor group similar to that found in a Principia Biopharma Bruton's tyrosine kinase (BTK) inhibitor[210]. However, these examples are few in number, and the compounds are weak DUB inhibitors. Mission Therapeutics has discovered covalent active-site series that are 'drug-like', unrelated to any previously described DUB inhibitor, and which achieve sub-micromolar cell-based potencies and exhibit good oral bioavailability[123,124,147].

Allosteric regulation: implications

Most peptidases, including many cysteine proteases, recognise a small linear polypeptide motif and cleave either before or after the peptide bond[213]. DUBs, however, are more complex. Most DUBs, cleave an isopeptide linkage between the side-chain of a lysine residue and ubiquitin's carboxyl-terminal glycine, with the isopeptide linkage providing specificity and flexibility to the mechanism of proteolysis[214]. Also, DUBs need to accommodate a substantial globular post-translational modification (ubiquitin, Ubl, or ubiquitin/Ubl chains) into their catalytic site[215]. Furthermore, unlike most other cysteine peptidases, the catalytic triad of cysteinyl peptidase DUBs is not usually in a "functional" configuration, with allosteric regulation being required to render DUBs fully functional and processive. Such allosteric regulation can be substrate-mediated (e.g. OTULIN)[181], triggered by intra-molecular reorganisation (e.g. USP7)[216] or induced by key cofactors (as for USP1)[74]. In addition, several DUBs are associated with multi-protein complexes such as the proteasome[217], p97/VCP[218], or the COP9 signalosome[219]. These associations can allosterically regulate the affinity of DUBs for their substrates[208,220] and in some instances DUBs coexist in the same complex as the ubiquitylation machinery[221]. The above issues must therefore be carefully considered when establishing screening and compound-evaluation assays for a DUB. Some DUB inhibitors have been suggested to target allosteric sites, such as the USP1 inhibitor ML323[78].

Screening technologies

Approximately twenty years ago, a general assay was established for measuring DUB enzyme activity based on the substrate, ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub-AMC). This substrate is efficiently cleaved/hydrolysed by various DUBs, releasing a highly fluorescent AMC moiety. While this assay has been used in various DUB inhibitor screens, for example to identify USP1[222] and USP7[207,223,224] inhibitors, one significant drawback is that it is prone to fluorescence interference exhibited by many small molecules[225]. Moreover, AMC and alternative tags such as Rhodamine and TAMRA, which have been employed because they are less prone to fluorescence artefacts, contain a peptide linkage and thus differ quite significantly from most natural DUB substrates. Processing of such substrates thus requires the DUB to function in a non-physiological manner, thereby potentially diminishing prospects for identifying compounds that will operate in cellular or therapeutic settings.

A further challenge for development of DUB inhibitor screening assays is oxidative hydrolysis of the active-site cysteinyl residue of purified DUBs in biochemical buffers. This sensitivity requires use of protective reducing agents such as dithiothreitol (DTT), usually in millimolar concentrations, to maintain DUB enzymatic activity. Altering the concentration or type of reducing agent (for example, 2-mercaptoethanol, cysteine, glutathione or TCEP) can considerably affect inhibition obtained for hit compounds[207]. Following a high-throughput screen to identify USP7 inhibitors, Wrigley *et al.*, (2011) evaluated the ability of compounds to inhibit USP7 in the presence of different reductants[207]. Many compounds showed the greatest inhibition in the absence of any reductant, being less potent in the presence of cysteine or glutathione, and least potent in the presence of DTT or TCEP. A further subset of molecules showed an alternative profile, only demonstrating inhibition in the presence of DTT or TCEP. A final set of molecules only inhibited USP7 when no additional reductant was added. Together, these data demonstrate the critical nature of the reducing environment on DUB activity and inhibition. Thus, most screens based on high concentrations of reducing agents and using first-generation fluorescent substrates generate high false-positive rates, an issue that has likely been the most significant challenge in identifying genuine and selective DUB inhibitors.

Indeed, the non-selective nature of some DUB inhibitors is highlighted in biochemical selectivity-profiling assays, with relatively few DUB inhibitors reported in the literature showing promise in such studies[76]. Ritorto *et al.*, (2014) used MALDI-TOF mass spectrometry to screen for DUB activity and specificity, by systematically assessing the specificities of 42 recombinant human DUBs against di-ubiquitin isomers with all possible chain linkages (M1/linear, K6, K11, K27, K29, K33, K48 and K63-linked)[76]. Subsequently, they screened a panel of 32 DUBs against nine reported DUB inhibitors. Their

findings demonstrated that none of the compounds displayed strong selectivity towards a single DUB, and that many inhibited most DUBs on the panel.

Novel technologies based on chemically-synthesised DUB substrates containing isopeptide linkages, ubiquitin chains and/or assay technologies less prone to false positives such as luminescence, time-resolved fluorescence or mass spectrometry are advancing screening campaigns and therefore now being exploited[76,226-228]. For example, a ubiquitin-aminoluciferin substrate was used with a variety of DUBs to demonstrate a suitable assay window for high-throughput screening[207,229]. Subsequently, USP2 was used as a representative DUB to demonstrate statistical robustness of this reagent in a screening campaign for inhibitors. We believe that such developments are crucial to optimise the prospects for identifying and developing DUB inhibitors for ultimate clinical use.

Monitoring DUB activity/inhibition

A key issue when studying DUBs and their modulation, in cells, is understanding substrate specificity. Some DUBs have preferences for mono-ubiquitylated substrates, while others favour specific ubiquitin chain-types, chains bearing mixed linkages, or mixed chains containing ubiquitin and Ubls[230,231]. Furthermore, many DUBs have some specificity for the substrate protein itself, with this being mediated through mechanisms often involving regions of the DUB distinct from its catalytic site. DUB substrates can be determined by biochemistry, yeast-2-hybrid interactions, proteomic profiling and genetics[232], but this is often challenging and time-consuming. Clearly, the ability to directly monitor DUB activity within a native biological system is essential to understanding the physiological and pathological role of individual DUBs as well as the effects of DUB inhibition[233].

DUB activity in cells can be monitored by chemical probes that generate readily detectable covalent complexes with the DUB catalytic site (recently reviewed in Hewings *et al.*, 2017)[234]. Activity probes label DUBs based on their catalytic site thiol group[235], with DUB reactivity towards such probes depending on the type of electrophilic warhead fused to ubiquitin. In addition to profiling DUB levels/activity and catalytic inhibition, activity probes have also been used to identify DUBs by affinity purification/mass spectrometry[236]. More recently, activity-based probes (ABPs) bearing a fluorescent reporter tag have been generated to replace the initial tags (e.g. the HA epitope) to allow fluorescent imaging instead of detection by immunoblotting[226,227]. While production of ubiquitin ABPs was historically based on a trypsin-catalysed transpeptidation to modify ubiquitin at its carboxy terminus with a vinyl sulfone group, recent approaches involve the full-chemical synthesis of ubiquitin ABPs[226,237]. This advance allows incorporation of modified amino acid residues at any position in the

ABPs, whether natural or not. Mass spectrometry has become an important tool to monitor ubiquitin adducts as well as changes in ubiquitin levels[232,238]. Indeed, combining ABPs with immunoblotting or mass spectrometry can generate powerful tools for monitoring DUB activity and inhibition by small molecules[98,239] as well as assessing drug-enzyme target engagement in cells or tissues. For example, Altun *et al.* (2011), used ABPs to demonstrate the selectivity of P22077 for USP7 in cells, in contrast to PR-619 which inhibited a broad range of DUBs[239]. In addition, Reverdy *et al.*, (2012) demonstrated the cellular selectivity of HBX19818 for USP7 against a panel of DUBs using ABPs and immunoblotting[98].

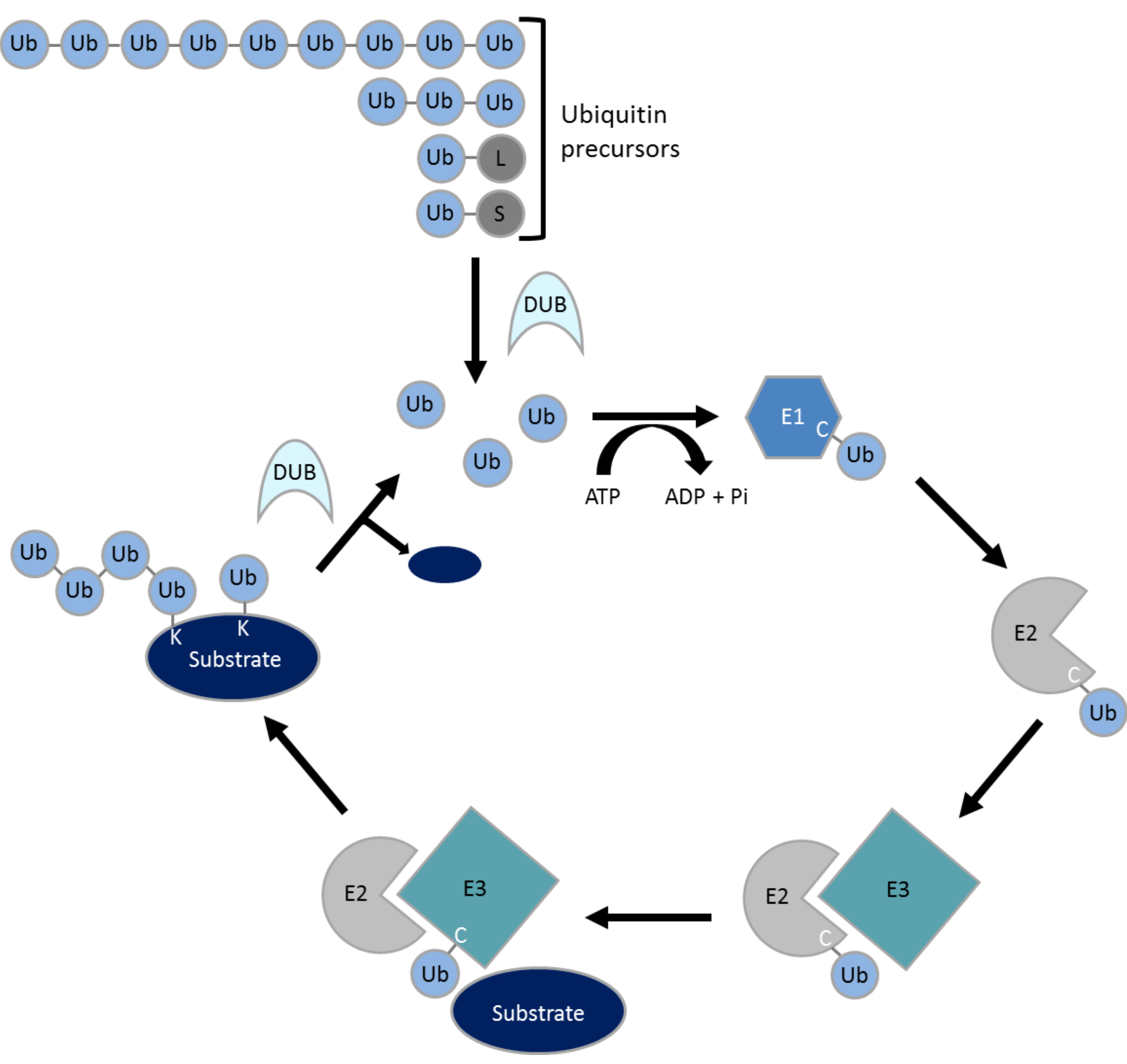
Activity-based proteomic probes have facilitated the development of pharmacologically active enzyme inhibitors. This approach represents a cell-based assay in which treatment with the inhibitor is performed on intact cells, allowing for a range of cellular enzymes to be assessed simultaneously[239]. Competition assays between an inhibitor and the ABP lead to a reduced labelling profile for the ABP, with loss of signal for ABP-labelled target enzymes allowing assessment of the specificity of inhibition. The limitation to this approach, however, is the number of enzymes successfully labelled by the ABP and the representation of active enzymes in the cellular proteome. ABPs were used to characterise the DUB inhibitors PR-619 and P22077 by immunoprecipitation combined with identification and label-free quantification by mass spectrometry based proteomics[239]. Using this approach, quantitative data for 25 cellular DUBs was obtained. PR-619 was confirmed as a broad DUB inhibitor, whereas P22077 was found to be a selective inhibitor of USP7 and USP47 that may therefore provide the basis for exploring therapeutic opportunities in oncology (see preceding sections and Table 2).

Concluding remarks

During the past decade, we have witnessed dramatic advances in our understanding of DUB functions, mechanisms-of-action, regulation and disease linkages. In parallel, there have been major improvements in DUB biochemical assays and screening technologies, leading to the development of increasing numbers of small-molecule DUB inhibitors whose selectivity is now being explored, and where possible refined. Such inhibitors are providing the basis for drug-like molecules suitable for clinical evaluation and are also providing versatile tools to further investigate DUB cell biology, regulation and biochemical mechanisms, as well as to test therapeutic hypotheses in disease models. Although still too early to predict the extent DUBs will deliver on their broad therapeutic potential, the next few years certainly seem set to produce further exciting developments in the arenas of DUB biology and drug-discovery.

Figure legends

Figure 1. The ubiquitylation cascade and the deubiquitylase family of proteins. a, Schematic of key events in ubiquitylation and deubiquitylation. The E1 enzyme activates ubiquitin in an ATP-dependent manner, resulting in a covalent thioester linkage between ubiquitin and the E1 cysteine residue. Ubiquitin is then transferred to an E2 conjugating enzyme forming a thioester linkage with the catalytic cysteine. Finally, an E3 ligase mediates transfer of ubiquitin from the E2 to a substrate, usually via a lysine side-chain. In subsequent rounds, ubiquitin molecules can be conjugated to the N-terminal amino group or lysines on ubiquitin itself to form chains. DUBs remove ubiquitin molecules from substrates or process ubiquitin precursors to generate free ubiquitin pools.



b, DUB phylogenetic tree. Sequences for full-length DUB and SENP proteins were aligned with COBALT, a constraint based alignment tool for multiple protein sequences, and subsequently visualised with FigTree v1.4.3.

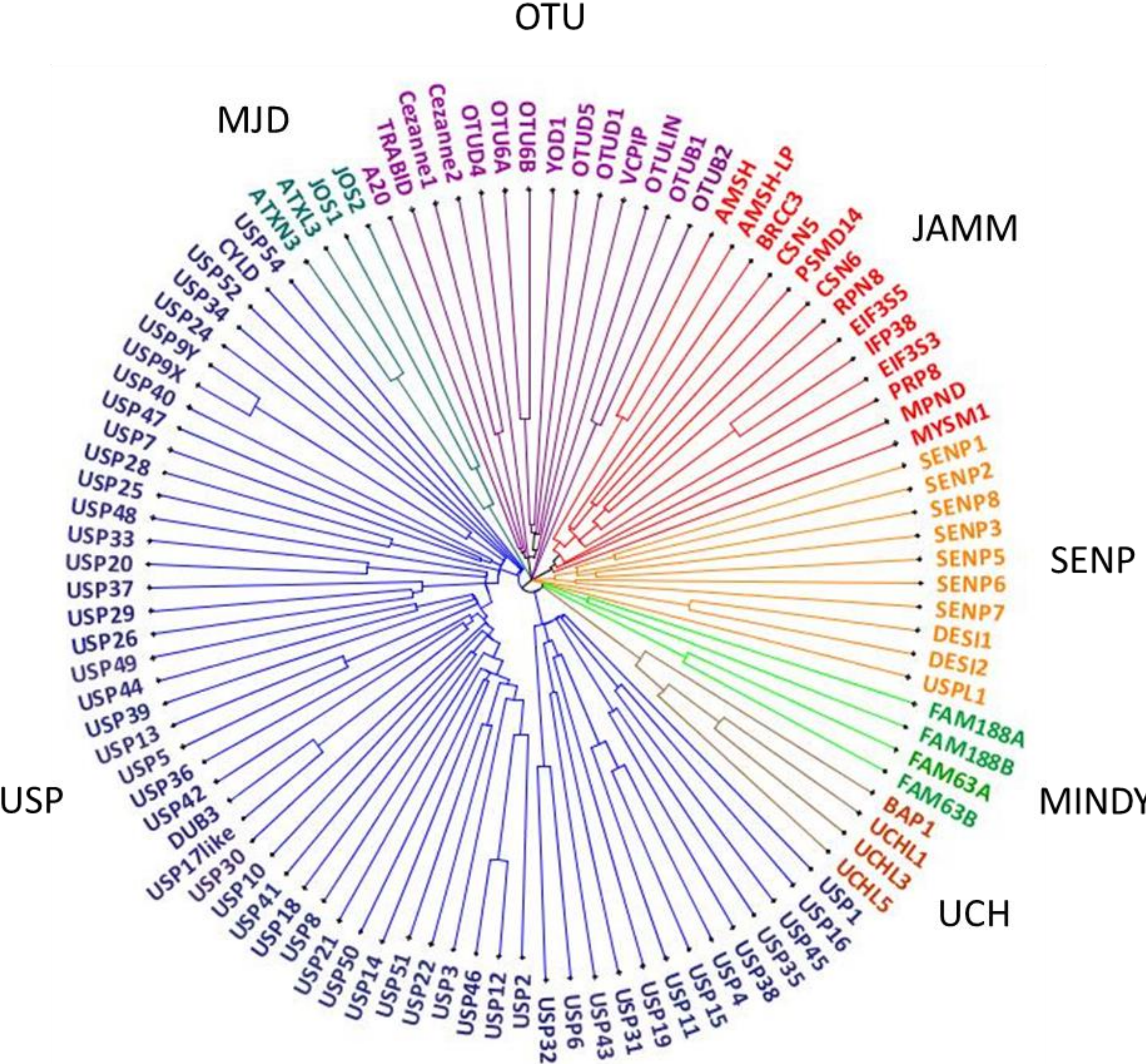


Figure 2. Various roles of DUBs in oncology. Selected, representative examples of DUBs (light blue ovals) involved in distinct cellular pathways and regulation of various ubiquitylated substrates (dark blue boxes) related to oncology. The proteasome and associated DUBs facilitate protein turnover and recycle ubiquitin. USP28 regulates turnover of the oncogene product c-Myc, ATXN3 controls stability of the tumour suppressor p53, and USP7 regulates p53 and its E3 ubiquitin ligase HDM2. USP1, USP4 and USP11 have important roles in DNA damage repair, while USP9X regulates CLASPIN and is linked to replication stress and checkpoint signalling. BAP1 and USP22 participate in chromatin remodelling by deubiquitylating histones, and UCHL1 plays a role in AKT signalling. These are representative examples only and not meant to be exhaustive. Examples of small-molecule compounds targeting these DUBs are shown.

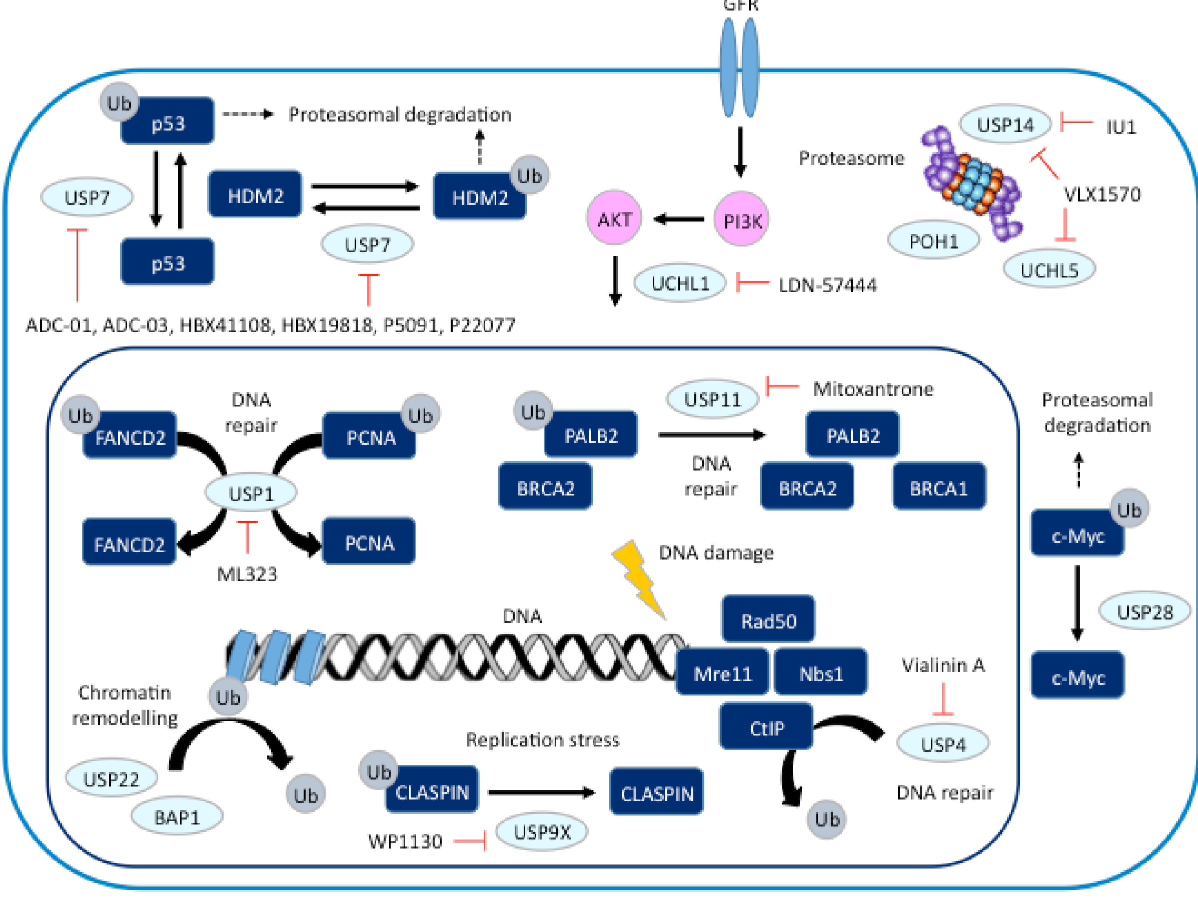


Table 1. DUBs associated with human disease.

Oncology					
Process Targeted	DUB	Target	Rationale	Disease expression	Reference
Proteasome	POH1	Many	General protein turnover	Liver	41
	USP14	Many	General protein turnover	Lung, Ovarian	47, 240
	UCHL5	Many	General protein turnover	Esophageal, Ovarian	240, 241
DNA Repair	USP1	FANCD2, PCNA	Fanconi Anemia pathway, translesion synthesis	Osteosarcoma	73
	USP4	CTIP	Homologous recombination	Lung, breast, liver	242-244
	USP11	PALB2	Homologous recombination	Breast	245
	USP9x	CLASPIN	Replication checkpoint	Colon, cervical, kidney, breast, prostate, brain, sarcoma	21
Oncogene and Tumour suppressors	ATXN3	p53, HDM2	p53-expressing tumours	Promotes p53-mediated apoptosis	102
	CYLD	NF-κB	Unclear	Mutated in cylindromatosis and multiple myeloma, reduced expression in colon, liver and melanoma	23, 280-283
	UCHL1	AKT	Unclear	Osteosarcoma, colon, breast, lung, kidney, myeloma	252-258
	USP6		Unclear	Translocated in aneurysmal bone cysts	284
	USP7	p53, HDM2	HDM2-overexpressing tumours	Leukemia, ovarian, lung	246-249
	USP8	EGFR	Regulates recycling of receptor tyrosine kinases including EGFR	Lung, mutated in Cushing's syndrome	107, 18, 19, 285
	USP15	Type I TGF-β receptor, R-SMADs	Regulation of TGF-β signalling	Glioblastoma, breast, ovarian	22, 112
	USP20	HIF1α	Sensitise hypoxic tumour cells	ND	290
	USP28	FBW7, c-MYC, JUN, NOTCH	APC-driven cancers	Colon, ovarian	250, 251
Epigenetics	BAP1	Histone H2A, HCF-1	Epigenetic deregulation of tumours	Uveal melanoma, sporadic melanona, mesothelioma, kidney,	286-289
	USP22	Histone H2A	Epigenetic deregulation of tumours	Colon, breast, esophageal, lung, pancreatic	127-129, 259, 260
CNS disorders					
Process Targeted	DUB	Target	Rationale	Disease expression	Reference
Neurodegeneration	ATXN3	Parkin	Counteracts Parkin auto-ubiquitylation	Expansion of CAG repeats causes Machado-Joseph disease, also known as spinocerebellar ataxia-3	151
	USP7	α-synuclein, REST	Antagonizes ubiquitylation of α-synuclein, regulates REST signalling and neuronal differentiation	Expressed in brain,	149
	USP8	Parkin, K6-Ub chains	Regulates mitophagy by removing ubiquitin from parkin, regulates TrkA levels in a NGF-dependent manner	Expressed in brain, including dopaminergic neurons	148, 263-265
	USP14	Proteasome substrates	Increased clearance of proteins involved in neurodegeneration (tau or ataxin-3)	Mutations cause ataxia	44, 153-155, 262
	USP15		Opposes Parkin-mediated mitophagy	Glioblastoma, wide expression brain	143, 150
	USP30	Ub conjugates at mitochondrial surface, Parkin	Mitochondrial dysfunction, mitophagy	ND	142, 144, 146, 261
Down's syndrome	USP16	Histone H2A	Antagonizes self renewal and/or senescence in Down's syndrome	Expressed in mouse and human embryonic stem cells	158-160, 266
Inflammation, immunity and infectious disease					
Process Targeted	DUB	Target	Rationale	Disease expression	Reference
Negative regulation of the immune response	A20	NEMO, RIPK1, TRAF6	Inhibits NF-KB signalling	Regulated by TNFα, IL1β and LPS	167, 273, 274
	CYLD	RIG-1, TBK1, IKKε	Inhibits NF-KB signalling		170, 171
	OTULIN	RIPK1, RIPK2, NEMO	Inhibits NF-KB signalling		172, 173
	USP18		Functions in hematopoetic cell differentiation, removes ISG15 conjugates, negative feedback regulator of type I IFN signaling	Highly expressed in thymus and peritoneal macrophages, expression regulated by IFNγ	268, 269, 291
	USP25		Negatively regulates IL-17-triggered signaling, negatively regulates virus-induced type I IFN production, positive feedback regulation of innate immune responses against RNA and DNA viruses	Expression regulated by IFN/IRF7	270-272, 292, 303
T reg responses	USP7	FOXP3	Stabilises FOXP3 in regulatory T cells, negative regulator of TNFα-stimulated NF-κB activity	Expressed and regulated upon viral infections in B and T cells	34, 97-99, 239, 267, 293
	USP21	FOXP3	Stabilises FOXP3 in regulatory T cells		131
Th1 and Th17 responses	Cezanne1	ZAP70	Positive regulator of T cell receptor signalling, binds and deubiquitylates Zap70		193
	TRABID	JMJD2D	Positive regulator of IL-22 and IL-23 cytokine production		191
	USP4	RORγt, RIG-I, TAK1	Stabilises RORγT in Th17 cells, positively regulates RIG-I-mediated antiviral response, negative regulator of TLR/IL1R signalling, targets TAK1 to downregulate TNFα-induced NFκB activation	Highly expressed CD4(+) T cells from patients with rheumatic heart disease	189, 190, 275, 276, 294
	USP10	T-bet	Stabilises T-bet in Th1 cells	Highly expressed PBMCs from patients with asthma	196
	USP17	RORγt, RIG-I, IL33	Positive regulator of RORγt in Th17 cells, regulates virus-induced type I IFN signaling, regulates the stability and nuclear function of IL33	Cytokine-inducible	277-279, 295
	USP18		Regulates TAK1-TAB interaction required for Th17 differentiation		192

Table 2. DUB inhibitors in development. Chemical structures shown are representative only, and additional structures can be found in Kemp, 2016[210].

DUB	Inhibitor	Structure	Company/Institution	Disease indication	Stage of development	Reference
POH1			Cleave Biosciences	Oncology	Preclinical	63-65
UCHL1	LDN-57444		Brigham and Women's Hospital and Harvard Medical School	Oncology	Preclinical	120
UCHL5/USP14	VLX1570		Vivolux	Oncology	Clinical trial phase (now suspended)	296
USP1	ML323		University of Delaware and National Institutes of Health	Oncology	Preclinical	77, 78, 297
USP4	Vialinin A		Tokyo University of Agriculture	Oncology	Preclinical	190
USP7	ADC-01, ADC-03	Unknown	Almac	Oncology, Immuno-oncology	Preclinical	101
USP7	HBX41108 (shown right), HBX19818		Hybrigenics	Oncology, Immuno-oncology	Preclinical	97, 98
USP7	P5091 (shown right), P22077		Progenra	Oncology, Immuno-oncology	Preclinical	298
USP8			Hybrigenics	Oncology	Preclinical	110, 264
USP9x	WP1130		University of Michigan	Oncology	Preclinical	86
USP11	Mitoxantrone		Thomas Jefferson University	Oncology	Preclinical	82
USP20	GSK2643943A		GSK	Oncology	Preclinical	106
USP14	IU1 and analogues		Harvard College and Proteostasis Therapeutics	Neurodegeneration	Preclinical	46, 153-155, 299, 300
USP30	15-oxospiramilactone		Chinese Academy of Sciences	Neurodegeneration	Preclinical	146
USP2	ML364		National Institutes of Health	Inflammation	Preclinical	301
USP4	Vialinin A		Tokyo University of Agriculture and Shanghai Institutes for Biological Sciences	Inflammation	Preclinical	189, 190
USP10/USP13	Spautin 1		Shanghai Institute of Organic Chemistry and Harvard Medical School	Inflammation	Preclinical	302

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