# Critical Review

# Emerging Roles of Deubiquitinases in Cancer-Associated Pathways

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*Summary*

**Deubiquitinases (DUBs) are emerging as important regulators of many pathways germane to cancer. They may regulate the stability of key oncogenes, exemplified by USP28 stabilisation of c-Myc. Alternatively they can negatively regulate ubiquitin-dependent signalling cascades such as the NF-$\kappa$B activation pathway. We review the current literature that associates DUBs with cancer and discuss their suitability as drug targets of the future.** © 2009 IUBMB

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## INTRODUCTION

Over the past few decades, ubiquitination has emerged as one of the most versatile of post-translational modifications, with roles in the regulation of a diverse array of cellular processes such as cell-cycle control, DNA damage repair and membrane trafficking (*1*). Ubiquitin is conjugated through the sequential action of three components: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes and finally E3 ubiquitin ligases, which provide for specificity. Polyubiquitin chains are formed through the serial addition of ubiquitin molecules linked to lysine residues in the preceding ubiquitin. A total of seven internal lysine residues (K6, K11, K27, K29, K33, K48, and K63) allows for diversity of chain configurations.

Ubiquitination plays a central role in degradation of proteins both through proteasomal targeting and by direct sorting to the lysosome. However, it is now becoming clear that reversible ubiquitination is also a crucial mediator within intracellular signalling cascades as exemplified by Nuclear Factor-$\kappa$B (NF-kB)

signalling. Protein networks can be formed by interaction with specific ubiquitin binding domains of which there are at least 20 classes within the human genome (*2*).

Reversibility of ubiquitination is accomplished through deubiquitinases (DUBs). Approximately one hundred human DUBs fall into five classes; ubiquitin specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), ovarian tumour proteases (OTU), Josephins and the Jab1/MPN/MOV34 metalloenzymes (JAMM, also known as MPN$^+$). The structure and function of these enzymes has recently been reviewed elsewhere (*3, 4*). In this review, we will focus on their emergence as attractive anticancer targets and discuss what is known about their function within pathways germane to cancer. In general terms, DUBs may influence the stability of key oncogenes or they may negatively regulate ubiquitin mediated signalling (Fig. 1).

Both oncogenic and tumour suppressive functions have been ascribed to individual DUBs. However, DUBs may have multiple substrates, thereby making it difficult to determine if a DUB has a net oncogenic or tumour suppressive function in vivo (Fig. 1). Although knockout and overexpression models are helpful, there is evidence to suggest that function may vary between tissue types and stage of malignancy. For a subset of DUBs, mutations and/or altered expression in cancer specimens and cell lines have been described (Table 1). Initiatives such as the Catalogue Of Somatic Mutations in Cancer (COSMIC) (*87*) and collation of gene expression data through Oncomine (*88*) provide important resources for the association of DUBs with cancer.

## DEUBIQUITINASES AND RECEPTOR TYROSINE KINASE SIGNALLING CASCADES

Targeting receptor tyrosine kinases (RTKs) and their downstream effectors, has recently proven to be one of the most successful avenues in drug development, with several such targeted therapies already in clinical practice. Examples include trastuzumab (Herceptin) and imatinib (Gleevec), which have contributed significantly to the treatment of ErbB2 overexpressing breast cancer (*89*) and gastrointestinal stromal tumours (*90*) respectively. However, for the majority of inhibitors, only a

| DUB | Target |
|---|---|
| CYLD | TRAF6, BCL-3 |
| A20 | TRAF6, RIP1 |
| Cezanne | RIP1 |

| DUB | Target |
|---|---|
| USP7 | p53 |
| USP15 | APC, IκB |
| USP11 | IκB |

| DUB | Target |
|---|---|
| USP7 | MDM2 |
| USP28 | MYC |
| USP2 | FAS, MDM2 |

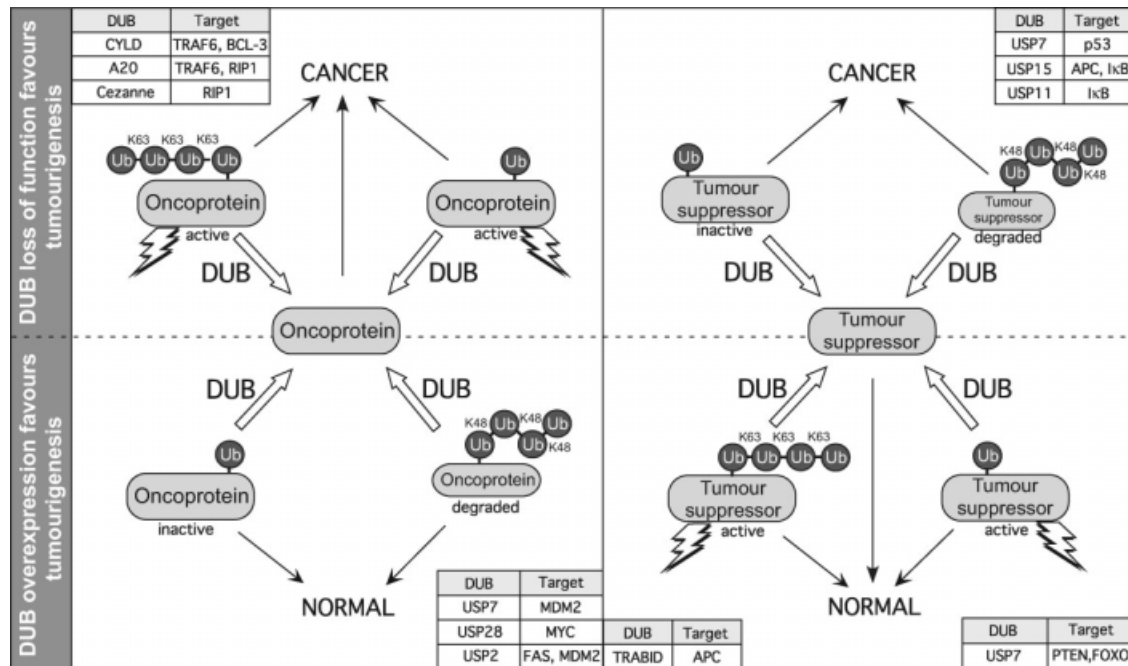| DUB | Target |
|---|---|
| TRABID | APC |

| DUB | Target |
|---|---|
| USP7 | PTEN,FOXO |

**Figure 1.** Deubiquitinases are important regulators of oncogenes and tumour suppressors. Both overexpression and loss of function of DUBs can promote cancer. Ubiquitination of oncoproteins and tumour suppressors can promote their destabilization by targeting them for degradation (*e.g.*, K48-linked poly-ubiquitination specifies proteasomal degradation), or regulate their activity (activation or inactivation). Activation here may refer to a variety of processes like translocation to the nucleus (*e.g.*, PTEN and FOXO), or engagement in signalling protein interaction networks (TRAF6, RIP1). Specific DUBs implicated in tumourigenesis are shown with their cognate targets. Note that not all targets are shown here.

small proportion of patients respond to treatment, and responses are often short lived. Innate or acquired resistance frequently occurs through up-regulation of other signalling pathways (such as the Met pathway in patients treated with EGFR inhibitors (*91, 92*)) or downstream activating mutations. A deeper understanding of the intricate regulation of RTK cascades is therefore essential for future rational drug development and in particular, combination therapy.

The significance of endocytic growth factor receptor trafficking in cancer is reflected in the multitude of aberrations in the endocytic machinery that have now been described (*93*). It is well established that ubiquitination of RTKs directs their lysosomal degradation and that mutants which escape ubiquitination are frequently transforming (*94*). Following receptor internalisation, ubiquitin-dependent receptor sorting occurs in the sorting endosome where receptors can be incorporated into lumenal vesicles destined for degradation in the lysosome or otherwise recycled to the plasma membrane (*95*).

Two endosomal DUBs exert opposite effects upon the fate of internalised epidermal growth factor receptor (EGFR). AMSH (associated molecule with the SH3 domain of STAM) is a member of the JAMM family of metalloproteases with specific activity against K63 ubiquitin linkages (*60, 96, 97*). Knockdown of AMSH leads to an increased degradation rate of

activated EGFR (*60, 98*). A working model to explain this observation suggests that AMSH deubiquitination of receptors at the sorting endosome leads to their recycling to the plasma membrane (*60, 99*). Thus, a balance between ubiquitinating and deubiquitinating activity will control receptor fate. Interestingly, in a review of the Oncomine database, AMSH expression was found to be elevated in lung, liver, bladder and colon cancer, consistent with a tumour promoting potential that may correlate with its endocytic function.

On the other hand USP8, also called UBPY, is required for the downregulation of EGFR and Met, the receptor for Hepatocyte growth factor (HGF) (*26, 98, 100, 101*). Its functions on the endocytic pathway are likely to be pleiotropic, but include the deubiquitination, and stabilisation of components of the endosomal sorting machinery, HRS and STAM (*26, 99*).

We recently reported an siRNA screen aimed at identifying DUBs that regulate the stability of ErbB2, a member of the EGFR family, which does not undergo activity dependent endocytosis (*67*). We identified POH1 (26S proteasome-associated pad1 homologue, also known as PSMD14; Rpn11 in yeast), a component of the proteasome lid, as a critical DUB controlling the ubiquitination status of ErbB2, albeit without necessarily affecting its turnover. Nevertheless, this highlights that one unanticipated effect of treatment with the proteasome inhibitor

**Table 1**
Deubiquitinases and cancer

| DUB name | Pathway/mechanism | Mutations and translocations | Published abnormalities in expression/protein level | Oncomine | |
|---|---|---|---|---|---|
| | | | | Upregulation | Downregulation |
| USP1 | Deubiquitinates and negatively regulates FANCD2 and DNA repair (5). Also shown to deubiquitinate and inactivate PCNA, an important component of the DNA repair translesion synthesis pathway (6). | None reported | Upregulated in hydatidiform mole (7). | Brain, liver, cervical, gastric, sarcoma. (13 others) | Leukaemia, testicular, ovarian, prostate |
| USP2 | Upregulated in response to androgen, leading to stabilization of fatty acid synthase (8). Additionally deubiquitinates and stabilizes MDM2 (9). | 1/2 bladder cancer cell lines[a] | Overexpressed in ovarian carcinoma (10). Overexpressed and associated with poor prognosis in prostate cancer (8). | Brain | Brain, renal (3), HNSCC (2), Colon (2), lung. (3 others) |
| USP3 | Deubiquitinates histone H2A/H2B. Knockdown leads to defects in cell cycle progression and increased DNA damage (11). | None reported | None reported | Brain, bladder, prostate, HNSCC, testicular. (2 others) | Brain, leukaemia |
| USP4 | Transforms NIH 3T3 cells (12). Although known to bind to Rb (13), its mechanism of action remains unknown. A recent study has indicated it negatively regulates the WNT signaling pathway (14). | 1/2 bladder cancer cell lines[a] | Upregulated in adrenocortical carcinoma (15), small cell and adenoncarcinoma of the lung (16). Downregulation in small cell lung cancer cell lines (17). | Myeloma (2), liver, melanoma, brain, bladder. (1 other) | Testicular, lung (3), HNSCC (5), renal, brain. |
| USP5 | Knockdown leads to an increase in level and activity of p53 (18). Mechanism thought to be indirect and involve accumulation of free ubiquitin chains. | None reported | None reported. | Lung, colon(2), leukaemia, ovarian(2), liver. (2 others) | Brain (3), lymphoma, cervical |

**Table 1**
(Continued)

| DUB name | Pathway/mechanism | Mutations and translocations | Published abnormalities in expression/protein level | Oncomine | |
|---|---|---|---|---|---|
| | | | | Upregulation | Downregulation |
| USP6 | Identified as oncogene through transformation of NIH 3T3 cells (19), mechanism unclear. Interacts with CDC2 and RAC1 to promote actin remodeling (20). TBC domain may regulate endosomal trafficking through activation of ARF6 (21). | Chromosomal translocations associated with aneurysmal bone cysts (22), usually but not exclusively, benign (23) | None reported | Myeloma, salivary gland, sarcoma | Brain (3), lymphoma, testis, myeloma, HNSCC. (1 other) |
| USP7 | Deubiquitinates and affects stability of both p53 and MDM2. Additionally involved in the regulation of localisation and activity of PTEN and FOXO (see text). | None reported | Increased expression in prostate cancer (24), However reduced expression reported in a study of NSCLC (25). | Colon, lung, testicular, myeloma, bladder (2). (5 others) | Brain, HNSCC, testicular, breast, melanoma. (3 others) |
| USP8 | Required for RTK downregulation following internalization, mechanism involving deubiquitination and stabilization of ESCRT components (26–28). | 1/11 lung cancer cell lines[a] | None reported | Brain (2), cervical, myeloma, lymphoma, brain. (1 other) | Leukaemia, lymphoma (2), testicular, lung (2) |
| USP9X | Interacts with and stabilizes beta-catenin (29, 30). Also promotes TGF-beta signaling through deubiquitination of SMAD4 (31). Additionally implicated in AMPK family kinase regulation. | None reported. | Overexpression reported in breast cancer (32). | Brain (2), gastric, cervical, colon, leukaemia. (3 others) | Brain, bladder, testicular, leukaemia (2), lymphoma (2) |
| USP11 | Deubiquitinates and stabilizes E7 (human papilloma virus (HPV) protein that targets Rb1-involved in pathogenesis of cervical cancer) (33). Interacts with BRCA2 (34) and stabilizes IkB (35). | None reported | None reported | Lung, myeloma, melanoma, HNSCC, skin. (1 other) | Brain (3), renal (2), testis, pancreas, HNSCC. (3 others) |

**Table 1**
(Continued)

| DUB name | Pathway/mechanism | Mutations and translocations | Published abnormalities in expression/protein level | Oncomine | |
|---|---|---|---|---|---|
| | | | | Upregulation | Downregulation |
| USP15 | Deubiquitinates E6 (HPV protein which leads to ubiquitination and degradation of p53-involved in pathogenesis of cervical cancer) (36). Implicated in stabilization of APC (37). Associates with COP9 signalosome and has been shown to deubiquitinate IκB (38). | None reported | Downregulated in paclitaxel resistant ovarian cancer (39). | Vulva, brain, breast, lymphoma. | Brain, bladder, testicular, liver, melanoma. (3 others) |
| USP17 | Negatively regulates the activity of ras-converting enzyme 1, thereby blocking ras membrane localisation and activation (40). | None reported | None reported | No available data | No available data |
| USP18 | Upregulates EGFR, mechanism uncertain but likely to occur at level of translation(41). Reported specificity for the ubiquitin-like modifier ISG15 (42). | None reported | None reported | Bladder, ovarian, lung, brain, prostate. (1 other) | None |
| USP20 | Deubiquitinates and stabilizes HIF1-alpha (43). | None reported | None reported | None | Lymphoma, leukaemia, testis, lung. |
| USP21 | Deubiquitinates histone H2A, activating transcription (44). | None reported | None reported | Leukaemia, bladder (2), liver, colon. | Testis, HNSCC. |
| USP22 | Deubiquitinates both Histone H2A and H2B, and is required for progression through cell cycle (45, 46). | None reported | Overexpressed and part of an 11-gene signature that predicts poor prognosis in a wide range of malignancies (47). | Lung (2), bladder, leukaemia, melanoma, salivary. (4 others) | Brain (3), leukaemia (2), colon, liver, lymphoma. |
| USP28 | Required for MYC stability(48). Additionally stabilizes Chk2 and 53BP1 in response to DNA damage (49). | Somatic mutation reported in case of lobular breast cancer (50). 1/101 renal cell lines[a]. | Overexpression in colon and breast cancer (48). | Testicular, cervical, HNSCC, myeloma. | None |

**Table 1**
(Continued)

| DUB name | Pathway/mechanism | Mutations and translocations | Published abnormalities in expression/protein level | Oncomine | |
|---|---|---|---|---|---|
| | | | | Upregulation | Downregulation |
| USP33 | Interacts with Robo1 and is required for Slit signaling (51, 52). | None reported | Overexpression in paediatric ALL (53). | Lung (2), lymphoma, Prostate (2), Myeloma, bladder. (1 other) | Brain (3), leukaemia (2), lung (3), breast. |
| USP39 | Involved in the mitotic spindle checkpoint, influences Aurora B expression, possibly through mRNA splicing (54). USP39 is catalytically inactive. | None reported | None reported | Leukaemia, ovarian, brain, lung, cervical. (6 others) | Brain, pancreas. |
| USP44 | Critical regulator of spindle checkpoint. Depletion of USP44 leads to defects in chromatin segregation (55). | 1/11 lung cancer cell lines[a] | None reported | Leukaemia. | Renal, brain, gastric, testis (2). |
| CYLD | Negative regulation of the NF-κB pathway (see text). | Mutations (catalytic domain) in Cylindromatosis and trichoepithelioma. 1/128 lymphoma[a] | Several reports of down regulation in cancers including lung (56), liver (57), colon (57), multiple myeloma (58). Downregulation associated with progression in melanoma (59). | Leukaemia, renal (2), lung, testis, myeloma. (2 others) | Brain, ovarian, lung (3), HNSCC (2), bladder. (8 others) |
| AMSH | Involved in endosomal sorting/trafficking and promotes recycling of RTK from multivesicular body (60). Also reported as a positive regulator of BMP signaling (61). | None reported | None reported | Lung, liver, bladder, leukaemia (2), colon. (6 others) | Leukaemia (2) |
| AMSH-LP | Potentiates TGFβ signaling through interaction with inihibitory I-SMADs (62) | None reported | None reported | Kidney (2), liver, brain, HNSCC. | Brain, testicular, leukaemia, brain. |

**Table 1**
(Continued)

| DUB name | Pathway/mechanism | Mutations and translocations | Published abnormalities in expression/protein level | Oncomine | |
|---|---|---|---|---|---|
| | | | | Upregulation | Downregulation |
| BRCC36 | Component of the BRCA1 and BRCA2 containing complex (BRCC) involved in maintaining the G2 checkpoint and in the response to ionizing radiation (63). | Gene locus is at a chromosomal break point associated with translocations in T-cell PLL (64). 3/36 lung cancer cell lines[a] | Overexpressed in breast cancer (63). | Lung, Testicular, colon, ovary, prostate. (6 others) | Brain (2). |
| MYSM1 | Deubiquitinates histone H2A and participates in androgen receptor dependant transcription (65). | None reported | None reported | Brain (2), testicular, HNSCC (2) | Brain. |
| POH1 | Component of the 19s proteasomal lid complex (66). Regulates ErbB2 ubiquitination, albeit without affecting its turnover (67). Deubiquitinates and stabilizes c-jun (68). | None reported | None reported | Lung (3), colon, gastric, HNSCC (2), brain. (8 others) | Leukaemia (2), breast, lymphoma, testicular. |
| BAP1 | Discovered through its interaction with BRCA1 (69), BAP1 has poorly understood tumour suppressive functions. A recent study has shown interaction with the cell cycle regulator HCF-1 to be critical (70). | Mutations and deletions in breast and lung cancer (69, 71, 72) | None reported | Testicular, lung (2), brain (2), kidney (2), cervical. (2 others) | Ovary, oesophageal, BCC, breast, lymphoma. (2 others) |
| UCHL1 | Function and mechanism of action uncertain (see text). | None reported (in cancer) | Upregulated in several malignancies (e.g. lung, colon) (73–75, 76, 77). However there have also been recent reports of methylation and reduced expression (see text). | Ovary, HNSCC (2), lung (3), gastric, oesophagus. (1 other) | Brain (2), ovarian, kidney, bladder, colon. (4 others) |
| UCHL5 | Reported to interact with SMADs and regulate TGFβ signaling (78). | None reported | None reported | Lung, breast, ovarian, vulva, parathyroid. | Brain, pancreas, breast. |

**Table 1**
(Continued)

| DUB name | Pathway/mechanism | Mutations and translocations | Published abnormalities in expression/protein level | Oncomine | |
|---|---|---|---|---|---|
| | | | | Upregulation | Downregulation |
| OTUB1 | OTUB1 interacts with estrogen receptor alpha and negatively regulates ER-alpha-mediated transcription (79) | None reported | None reported | Bladder (3), lung, prostate, HNSCC, breast. (3 others) | Brain (2), HNSCC (2), Testis, Cervical, renal, sarcoma. (4 others) |
| A20 | Negative regulator of NF-$\kappa$B signaling (see text). | Chromosomal deletions and inactivating mutations found in several lymphoma subtypes (80–83). 2 out of 11 lung cancer[a] | Study showing overexpression in Hodgkins and anaplastic B-cell lymphomas, with downregulation in other lymphoma types (84). | HNSCC (4), leukaemia (2), lung, brain (2), cervical. (6 others) | Bladder, ovary (2), lung, lymphoma (2), sarcoma. |
| Cezanne | Downregulates NF-$\kappa$B signalling through the deubiquitination and inactivation of RIP1 (85). | None reported | None reported | Liver, myeloma. | Ovarian. |
| TRABID | Positive regulator of WNT signaling required for TCF-mediated transcription (86). | 1/ 202 kidney cancer | None reported | Brain, testicular, leukaemia, oesophagus, liver. | Brain (2), leukaemia, liver, testicular, bladder. (2 others) |

Published data on DUB mutations and aberrations in expression remain sparse. A review of the COSMIC (Catalogue Of Somatic Mutations In Cancer www.sanger.ac.uk/genetics/CGP/cosmic (87)) and Oncomine (www.oncomine.org) (88) databases was therefore performed. Oncomine (Compedia bioscience, Ann Arbor, MI) was used for analysis and visualization of microarray data. Expression in cancer and normal tissue was compared, and studies in which statistically significant differences in expression ($P < 10^{-4}$) are collated above. Up to five cancer types are named; further studies meeting the aforementioned criteria are numerated but not named. Tumour subtypes were not analysed. Together with differences in stage etc, this may explain the observation of both over and under-expression within the same tumour group. Testicular refers to germ cell tumours of the testis.

[a]Mutations identified by COSMIC, expressed as a fraction of tumours of that type that were assessed. Arf6, ADP ribosylation factor 6; BAP1, BRCA1 associated protein 1; BRCC36, BRCA1/ BRCA2 containing complex subunit 3; HNSCC, Head and Neck Squamous Cell Carcinoma; Rb1, retinoblastoma 1; Robo1, Roundabout 1.

Bortezimib, may be an altered activity of ErbB2 receptors that could have clinical implications.

To date, the major established role of ubiquitin following RTK stimulation has been to promote lysosomal degradation of receptors. We recently screened for DUBs that may be involved in hepatocyte growth factor (HGF)-dependent cell scattering of A549 lung carcinoma cells (*102*). The implication of up to 12 DUBs in this process, independent of effects on Met receptor levels and down-regulation, suggests hitherto unappreciated roles of ubiquitin-linked events in regulating the outcomes of HGF-signaling. Although, the requirement for catalytic activity was not assessed, it is reasonable to suggest that this set of DUBs will contain some, which could be considered as anti-metastatic targets. The relevant substrates are, however, yet to be identified.

USP18 has recently been shown to influence EGFR protein expression levels, with a 50–80% reduction observed following USP18 knockdown (*41*). Conversely, overexpression of catalytically active USP18 resulted in an increase in EGFR expression. Amongst several RTKs tested (Met and ErbB2), only EGFR protein levels were affected and, although the exact mechanism remains uncertain, regulation appears to occur at the level of translation.

## CYLD, CYLINDROMATOSIS, AND NF-κB SIGNALLING

Cylindromatosis is a rare familial condition characterised by the development of multiple skin tumours, known as cylindromas, mostly on the scalp. Early work into its aetiology led to the discovery of CYLD, a member of the USP class of DUBs (*103*). Inherited in an autosomal dominant fashion, cylindromatosis has been shown to be due to mutations in the cyld gene. Individual tumours are characterised by loss of heterozygosity whereby the second copy of cyld is mutated and non-functional. The majority of mutations that have been described affect the catalytic domain (*104, 105*). A second familial condition, trichoepithelioma, is also caused by CYLD mutations, identical to those causing cylindromatosis but resulting in tumours of the hair follicle rather than the sebaceous gland as occurs in cylindromatosis. Malignant transformation in both conditions is very rare but has been described (*106, 107*).

In a series of groundbreaking experiments, Brummelkamp et al identified CYLD as a negative regulator of NF-κB signalling. Using a shRNA library targeting 50 DUBs to identify candidates involved in the regulation of cancer related pathways, they demonstrated that knockdown of CYLD enhanced NF-κB signalling following Tumour Necrosis Factor (TNF) stimulation (*108*). Two other groups identified CYLD in yeast two-hybrid screens for proteins interacting with NEMO (nuclear factor kappa B essential modifier), a key adaptor protein in NF-κB signaling (*109, 110*). This association of CYLD with the NF-κB pathway suggests the application of topical aspirin derivatives, which inhibit NF-κB activity, may be a valid therapeutic strategy in familial cylindrimatosis (*108*).

The role of CYLD in NF-κB signalling has been extensively reviewed elsewhere and is described only briefly here (*111–113*). Receptor stimulation leads to activation of the IκB kinase (IKK) complex, (comprising two catalytic subunits and the regulatory protein NEMO), which in turn phosphorylates IκB. Phosphorylated IκB dissociates from NF-κB, undergoes K48 polyubiquitination and is degraded by the proteasome leaving NF-κB to activate downstream proliferative signals (*114*). Several proteins involved in signal transduction (NEMO, TNF receptor-associated factor 2 and 6 (TRAF2 and TRAF6)), are modified by the addition of K63 linked polyubiquitin chains, which results in their activation. CYLD shows preferential activity for K63-linked polyubiquitin chains and its expression is upregulated by NF-κB, providing negative feedback regulation of this pathway through deubiquitination of NEMO, TRAF2, and TRAF6 (*109, 110, 115, 116*). CYLD has also been shown to deubiquitinate (and inactivate) the coactivator BCL-3 (*117*), which switches the transcriptional properties of NF-κB from repressive to activating, and is known to strongly promote cell proliferation and oncogenesis (*118, 119*). Recently, IκB kinase epsilon (IKKε), an oncoprotein overexpressed in 30% of breast cancers (*120*), which plays a critical role in interferon signalling pathways, has been shown to phosphorylate and inactivate CYLD (*121*). Significantly inactivation of CYLD was necessary for IKKε-driven transformation (*121*).

Emerging evidence indicates CYLD has yet other, NF-κB independent roles, which may be associated with malignancy, including (1) regulating mitotic entry and potentially cytokinesis (*122, 123*), (2) deubiquitination of TrkA, the receptor for neuronal growth factor (*124*), and (3) facilitating cell migration (*125*). Whilst in the latter cases, CYLD may function as a positive regulator of mitogenic and motogenic processes, it is loss of function that is more commonly associated with tumourigenesis and correlates with its negative role in the NF-κB pathway. There have been several reports of down-regulation of CYLD expression in cancer including lung (*56*), liver (*57*) and colon (*57*) cancer and multiple myeloma (*58*). Reduced expression has also been correlated with poor prognosis in melanoma (*59*). Furthermore, cyld$^{-/-}$ mice exhibit increased susceptibility to colon cancers on a background of induced colitis (*126*) as well as skin tumours (*117*).

A second DUB, A20 (also known as tumour necrosis factor-α-induced protein 3, TNFAIP3) negatively regulates NF-κB signalling. A member of the OTU class of DUBs, A20 exerts its regulatory activity through the removal of activating K63-linked ubiquitin chains from TRAF6 and Ral interacting protein 1 (RIP1) (*127, 128*). CYLD and A20 target an overlapping set of NF-κB pathway effectors (*e.g.* TRAF6, RIP1, NEMO) and it has been proposed that CYLD may largely be responsible for the constitutive inactivation of the cascade, whilst A20 protein levels and activity are induced in response to a negative feedback mechanism (*112, 113*). *In vitro*, A20 is only poorly active towards free K63- compared with K48-linked ubiquitin chains, but instead has been shown to remove these chains en bloc

from TRAF6 (*129, 130*). A20 has also been reported to possess E3-ligase activity, which adds K48-linked chains to RIP1 and TRAF6, although this function of A20 may also involve the E3-ligase ITCH (*131*). Thus A20 can edit ubiquitin chain modifications by co-ordinating removal of one type of ubiquitin chain (K63) promoting NF-κB activation from RIP-1 and its replacement with K48-linked ubiquitin chains specifying degradation. A20 is frequently inactivated in B-cell lymphomas (*132, 133*), and its restoration in A20 null cell lines leads to inhibition of cell growth and induction of apoptosis (*132*). These studies establish A20 as an important tumour suppressor in lymphoid malignancies.

Three further DUBs have been implicated in negative regulation of NF-κB signalling: the OTU DUB Cezanne (also known as OTUD7B), USP11 and USP15 (*112*). Cezanne is induced by TNF-alpha in cultured cells leading to down-regulation of NF-κB signalling through the deubiquitination and inactivation of RIP1, thereby providing a negative feedback loop in pro-inflammatory signalling (*85*). USP11, as well as its COP9-signalosome associated paralogue USP15, have both been shown to deubiquitinate and stabilise IκB, which binds and sequesters NF-κB in the cytoplasm (*35, 38*). However, in the case of USP11, a catalytically inactive mutant is still able to partially inhibit NF-κB activation, indicating a further, as yet unknown, mechanism of action.

The NF-κB pathway is constitutively active in a wide variety of cancers, and is intimately linked to cancer growth and metastasis (*134*). The involvement of five DUBs in NF-κB regulation underlines the importance of its tight regulation in normal cells, and in particular the role of reversible deubiquitination (*135*).

## USP9X, AMSH-LP, UCHL-5, AND THE TGFß PATHWAY

The Transforming Growth Factor (TGF)-ß pathway plays a dual role in tumour development: at early stages, this signaling cascade has a growth inhibitory effect whilst at later stages, the same growth factor promotes epithelial to mesenchymal transition (EMT) with pro-metastatic outcomes (*136*). Signal transduction in this pathway is primarily mediated by the SMAD effectors, and both SMADs as well as the TGFß receptors are regulated by reversible ubiquitination (*137*). USP9X (also called FAM) was identified as a positive regulator of TGFß signaling by means of a siRNA screen. It acts by removing monoubiquitin from the Co-SMAD, SMAD4, thereby permitting its association with phospho-SMAD2 and subsequent activation of TGFß/SMAD-responsive gene targets (*31*). USP9X has also been shown to control AMP-activated protein kinase (AMPK)-related kinase activity through direct removal of non-canonical K29/K33-linked ubiquitin chains (*138*). UCHL-5 (also called UCH37) has been reported to interact with the inhibitory SMAD7 and regulate TGFß-receptor ubiquitination, stability and TGFß-dependent transcription (*78*). Likewise, the closely related JAMM DUBs AMSH and AMSH-LP (AMSH-like pro-

tein) have been implicated as positive regulators in the bone morphogenetic protein (BMP) and TGFß responses respectively through their interaction with inhibitory I-SMADs (*61, 62*).

## USP4, USP15, TRABID, AND THE WNT SIGNALING PATHWAY

Various DUBs have been shown to influence the WNT signaling pathway, a key mediator of cell polarity, proliferation and cellular homeostasis (*139, 140*), which is widely implicated in malignancy, particularly colon cancer (*141*). WNT signaling increases the stability of ß-catenin by promoting its dissociation from the Axin/GSK-3/APC complex. This allows ß-catenin to enter the nucleus and activate TCF/Lef family transcription factors to promote specific gene expression. A siRNA screen of human DUBs identified USP4 as a suppressor of ß-catenin dependent transcription, possibly through direct deubiquitination of the transcription factor TCF4 (*14*). USP15, a COP9 signalosome associated paralogue of USP4, has also been implicated as a negative regulator of this pathway by stabilizing the tumour suppressor adenomatous polyposis coli (APC), thereby promoting the degradation of ß-catenin by the proteasome (*37*). In contrast to USP4 and USP15, the Otubain DUB, TRABID (TRAF-binding protein domain, also called ZRANB1) is a positive regulator of WNT signaling. It interacts with and deubiquitinates APC without affecting its stability, in line with its demonstrated specificity for K63-linked polyubiquitin chains (*86, 97*). TRABID is required for TCF-mediated transcription in cells with high WNT activity, including colorectal cancer cell lines in which it may affect assembly or activity of the TCF/ß-catenin transcription complex (*86*).

## USP7 AND THE TUMOUR SUPPRESSORS P53, PTEN AND FOXO

Initially described as a herpes simplex encoded protein required for lytic infection and termed Herpes associated USP (HAUSP), USP7 has subsequently been linked with the regulation of three vital tumour suppressors; p53 (*142*), PTEN (phosphatase and tensin homolog) (*24*), and FOXO (Forkhead box O) (*143*). The role of USP7 in p53 regulation is complex, as USP7 deubiquitinates both p53 and the E3 ligase MDM2 (murine double minute 2 homolog), which itself ubiquitinates and downregulates p53. Thus USP7 would be posited to have opposing effects depending on whether it predominantly deubiquitinates and rescues p53 or MDM2. Current evidence is that USP7 preferentially binds and deubiquitinates MDM2, thereby leading to increased degradation of p53, and consequent anti-apoptotic functions. In this scenario USP7 can be considered to be an oncogene (*144*). However, there may be situations in which the balance is reversed and USP7 stabilises p53 and acts as a tumour suppressor. For example, USP7 has been proposed to switch from MDM2 to p53 stabilisation in response to DNA damage and subsequent activation of the kinase ATM (ataxia

telangiectasia mutated) (*145*). Other DUBs also influence p53 stability including USP2, which is described further below (*9*).

The second prominent substrate for USP7 is PTEN, a lipid phosphatase and tumour suppressor which acts to terminate phosphatidylinositol 3-kinase (PtdIns 3-kinase) signalling through the hydrolysis of PtdIns(3,4,5)P$_3$ to PtdIns(4,5)P$_2$ (*146*). Possibly the second most important tumour suppressor after p53, PTEN is mutated in a wide range of malignancies. Interestingly, nuclear exclusion of PTEN has been shown to result in a more aggressive phenotype in malignancies (*147–149*), although the tumour suppressive functions of nuclear PTEN remain unclear (*150*). Monoubiquitination of PTEN promotes its shuttling into the nucleus where it is sequestered in PML bodies. USP7 in turn deubiquitinates PTEN and promotes its exclusion from the nucleus (*24*). The same study also showed overexpression of USP7 in prostate cancer with associated nuclear exclusion of PTEN.

The FOXO proteins (FOXO1, 3, 4, and 6) are transcription factors, which have tumour suppressive functions in mouse models. Phosphorylation of FOXOs, in response to PtdIns(3,4,5)P$_3$ mediated PKB/AKT activation, promotes their nuclear export, poly-ubiquitination and consequent proteasomal degradation. Conversely, mono-ubiquitination of FOXO3 and FOXO4 has been shown to result in nuclear retention and promote their transcriptional activity. As described above for PTEN, this ubiquitination is reversed by USP7 (*143*).

Ongoing work will hopefully decipher the various contributions of the different functions in vivo, clarifying the net result of inhibition of USP7 in individual cells and whole organisms. However, at least at face value, inhibition of USP7 would appear a promising anticancer strategy, by promoting stabilisation of p53. Early preclinical work on USP7 inhibitors has recently been reported (*151*).

## USP2, FATTY ACID SYNTHASE AND PROSTATE CANCER

In 2004, Graner et al identified the rat homologue of USP2 as an androgen regulated DUB (*8*) in a prostate cancer cell line. Expression of USP2 was upregulated in LNCaP cells exposed to dihydrotestosterone, and inhibited by the anti-androgen bicalutamide. USP2 was shown to associate with and stabilise fatty acid synthase, a protein, which is often overexpressed in biologically aggressive prostate cancer cells. Significantly, siRNA knockdown of USP2 resulted in apoptosis, which could be reversed by overexpression of fatty acid synthase (FAS) (*8*). However, a subsequent study, using a yeast two-hybrid screen, identified MDM2 as a further USP2 substrate. Unlike USP7, USP2 does not deubiquitinate p53 (*9*). Overexpression of USP2 was shown to reduce p53 stability, conversely suppression led to destabilisation of MDM2 and increased p53 level and activation (*9*). A recent study also reports a similar role for USP2 in the stabilisation of MDMX, another negative regulator of p53 and close structural homologue of MDM2 (*152*).

In a further study, increased expression of USP2 was found in 44% of prostate tumours studied (*153*). Overexpression of USP2 in prostate cancer cell lines protected cells from apoptosis and conferred resistance to chemotherapeutic agents, while siRNA knockdown led to apoptosis. Interestingly, gene profiling of prostate cancers overexpressing USP2 showed up-regulation of pathways associated with both known substrates, p53 and fatty acid synthase (*153*).

USP2 expression has also recently been shown to correlate with tumour progression and worse prognosis in oral squamous cell carcinomas (*154*). Whether this reflects a more widespread role for USP2 in carcinogenesis remains to be seen.

## USP28 AND C-MYC: REGULATING CELL PROLIFERATION

Aberrations in the expression and stability of the proto-oncogene c-myc, are widespread in malignancy (*155, 156*). MYC is a transcription factor that binds to ∼10–15% of the genome and acts as a core regulator of cell growth, proliferation and apoptosis (*157*). MYC is ubiquitinated and rapidly degraded leading to a short half-life and thereby allowing exquisite control of its cellular level (*158*). Although mutations affecting phosphorylation and subsequent ubiquitination of MYC have been described, stabilisation of MYC may also occur in the absence of mutations. Identified through a large-scale shRNA screen, USP28 has recently been shown to control the stability of MYC by antagonising the activity of the SCF$^{FBW7}$ ubiquitin ligase (*48*). Significantly, shRNA knockdown of USP28 led to a decrease in MYC in all members of a panel of cancer cell lines, and an associated inhibition in growth. The authors also demonstrated increased expression of USP28 in a significant proportion of colon and breast carcinomas. This study identifies a novel mechanism by which cancer cells may upregulate MYC. USP28 may well prove amenable to inhibition and provide a means of targeting malignancies in which MYC is up-regulated. In a different study, however, USP28 was shown to stabilise Chk2 and p53 binding protein1 (53BP1) in response to DNA damage and was required for DNA damage induced apoptosis (*49*). USP28 inhibition may therefore be expected to result in significantly different outcomes, depending on which pathways are activated.

## UCHL1

Ubiquitin C-terminal hydrolase –L1 (UCHL1), a member of the UCH class of DUBs, is one of the most well studied DUBs in view of its association with neurodegenerative conditions, including Parkinsons Disease, and a wide range of malignancies. Despite this, its *in vivo* functions remain enigmatic.

Normally almost exclusively expressed in neurones, the neuroendocrine system and the gonads, aberrant expression has been described in non-small cell lung cancer (*73, 74*), invasive colorectal cancer (*75*), pancreatic cancer (*159*), osteosarcoma

(76), and oesophageal cancer (77). Overexpression has also been associated with tumour progression, size and invasiveness (75, 159) There have been several reports indicating hypomethylation of the uchl1 gene leads to increased expression in Gall bladder cancer (160), as well as association with progression and metastasis in colorectal cancer (161). On the other hand there have also been reports of methylation mediated silencing of uchl1 in progression of oesophageal squamous cell carcinoma and gastric cancer (162, 163) and in pancreatic cancer cell lines (164). Furthermore, overexpression of UCHL1 has been associated with apoptosis in a breast cancer cell line (165). Although mutations have been described, these have so far been associated with Parkinsons disease rather than cancer, for which altered expression appears to be more common.

Kim et al. (166) recently demonstrated a role for UCHL1 in migration and invasiveness of the non-small cell lung cancer cell line H157, through siRNA knockdown studies. Additionally they suggested that UCHL1 knockdown resulted in decreased activation of AKT and downstream effectors. Bheda et al have also recently described upregulation of $\beta$-catenin signalling by UCHL1 (167). Moreover, they also showed reciprocal upregulation of UCHL1 expression by $\beta$-catenin, suggesting a positive feedback loop in transformed cells. In a second study, the same authors used a microarray approach to profile genes affected by siRNA knockdown of UCHL1 and have identified several genes involved in apoptosis, cell cycle, proliferation and migration whose expression was altered (168). Although the data described earlier indicate important roles for UCHL1 in malignancy, the mechanism by which this occurs remains uncertain. Structural analysis indicates a prominent loop covering the active site (169), thereby preventing the binding of large protein substrates and limiting activity to small peptide chains (170). It has been hypothesised that UCHL1 may, however, act at protein termini or on unfolded substrates (169).

## USP1 AND FANCONI ANAEMIA

Fanconi Anaemia (FA) is a rare congenital syndrome characterised by skeletal abnormalities, microcephaly, progressive bone marrow failure and a predisposition to malignancy later in life (171). Inherited as an autosomal recessive or X-linked disease, the clinical phenotype of patients with FA varies widely. However, all variants share a high frequency of chromosomal abnormalities and sensitivity to DNA intrastrand crosslinking agents (172). Thirteen complementation groups have been described (designated FA-A, -B etc), each characterized by mutation of a gene involved in a common pathway that regulates the sensing, signaling and repair of DNA intrastrand crosslinks (173).

Central to the FA pathway, FANCD2 (fanconi anaemia, complementation group D2) is monoubiquitinated by a nuclear multiprotein complex incorporating at least eight other FA proteins (173). This event is dependent on phosphorylation of serine 331, which has been shown to be mediated by CHK1 (174). Monoubiquitination of FANCD2 and PCNA (proliferating cell

nuclear antigen) promotes DNA repair through chromatin association and enhanced recruitment of enzymes involved in translesion synthesis respectively. In a siRNA screen, Nijman et al identified USP1 as a novel regulator of FANCD2, with USP1 inhibition leading to accumulation of monoubiquitinated FANCD2 (5). Whilst both FANCD2 and PCNA (6) are substrates of USP1, only deubiquitination of FANCD2 is necessary for DNA repair (175), reflecting the need for a dynamic population of FANCD2. Significantly, USP1 knockout mice exhibit a similar, albeit more severe, phenotype to most FA mouse models, with stunted growth, chromosomal instability and sensitivity to DNA cross-linking agents (176). The severity of the phenotype and the high perinatal lethality likely reflect involvement of other substrates, including PCNA, the ubiquitinated form of which was found to be elevated in these mice.

## DUB INHIBITORS

The stage is set to begin thinking about the development of small molecule inhibitors of DUBs. This will be facilitated by the development of suitable high throughput screening (HTS) platforms. Most current screens use fluorescent substrates such as Ub-AMC (177) or a sandwich of green fluorescing protein (GFP)-ubiquitin and terbium, which undergoes fluorescence resonance energy transfer (178). One limitation is that these assays will not be suitable for those DUBs, which do not process linear chains (e.g. AMSH) (97). Isopeptide linked substrates are required.

Proof of principle has been achieved through the identification of first generation inhibitor molecules from chemical libraries, which show some specificity. Two unrelated small molecule inhibitors targeted against the SARS coronavirus DUB papain like protease (PLpro) were identified from High-throughput screening (HTS), and one of these was subsequently optimized to achieve nanomolar inhibition (179, 180). HTS of small molecule libraries against UCHL1 identified several hits with selectivity for UCHL1 over the related UCHL3 (181). Small molecules showing some selectivity for USP7 and USP8 respectively have also been reported (182). HB41,108, is a USP7 inhibitor developed by Hybrigenics, recently shown to inhibit USP7 in the sub-micromolar range, leading to p53 stabilisation and activation with subsequent apoptosis and inhibition of cancer cell growth (151). Selectivity has so far been demonstrated with relatively small panels of enzymes. More effort needs to be placed upon the development of panels of DUB enzymes for characterisation of selectivity.

## CONCLUDING REMARKS

Cancer, although defined by shared clinical characteristics, is driven by a remarkable diversity of genetic and epigenetic aberrations in fundamental cellular processes. Future therapeutic strategies will depend on the ability to define the genetic makeup of individual tumours and to inhibit specific activated

pathways. This is in turn reliant on a thorough understanding of the regulation of these pathways. As discussed in this review, members of the DUB superfamily have been implicated in many of these processes. They provide novel targets for drug development, and may also prove useful biomarkers for activation of specific pathways. The early involvement of pharmaceutical companies, combined with a wealth of techniques built up through drug development in analogous areas, has given research in this field a head start. However, the clinical utility of targeting DUBs remains to be proven and will be the subject of extensive future research.

## ACKNOWLEDGEMENTS

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