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Regulation of cellular responses by deubiquitinating enzymes: an update

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1. ABSTRACT

The conjugation of ubiquitin as either a monomer or as a chain has long been known to regulate the stability, localisation, trafficking and/or function of many intracellular proteins. However, the recent explosion in our knowledge of the enzymes responsible for the removal of ubiquitin suggests they also play an important role in the regulation of many processes. Here we examine what is known about the role of deubiquitinating enzymes (DUBs), with particular emphasis upon their impact on cellular responses to external stimuli. In addition, we look at the evidence that although these enzymes are heavily outnumbered by those responsible for ubiquitin conjugation, that these enzymes may still be important cellular regulators, due to their ability to play multiple roles which can be cell type and cell context specific.

2. INTRODUCTION

It is now apparent that ubiquitination plays a fundamental role in the regulation of multiple intracellular processes. Initially this was thought to be mediated through the targeting of proteins for proteasomal degradation, but the realisation that ubiquitin may take many forms (mono, multi-mono, lysine 6/11/27/29/33/48/63 linked chains), that may or may not be dependent on the proteasome (1), has led to the expansion of its recognised roles.

Initially, efforts focused upon the enzymes responsible for the conjugation of ubiquitin to target proteins and how this regulates cellular processes. Ubiquitin is conjugated to proteins via a multi-step process that is controlled by a series of enzymes. Ubiquitin initially forms a thiol-ester bond with a ubiquitin-activating enzyme

Table 1. DUBs and receptor trafficking

Process	DUB	Substrate/s	Function
Receptor trafficking	AMSH	EGFR, ERBB2, ERBB3, c-met, PAR2, DOR, CXCR4	Thought to promote recycling back to the plasma membrane
	USP8	EGFR, ERBB2, ERBB3, c-met, PAR2, DOR, CXCR4	Promotes receptor transport to the lysosome.
	USP20	Beta-2 adrenergic receptor	Promotes receptor recycling to the plasma membrane.
	USP33	Beta-2 adrenergic receptor	Promotes receptor recycling to the plasma membrane.
	USP10	CFTR	Promotes recycling to the plasma membrane.
	UCH-L3	ENaC	Promotes recycling to the plasma membrane.

E1), before being transferred to a ubiquitin-conjugating, or ubiquitin carrier enzyme (E2) via a transthiolation reaction. Finally, a protein ligase (E3) catalyses the transfer of ubiquitin from the E2 to a lysine residue within the target protein (2). The identification of signature (RING FINGER, U-box or HECT) domains (3) has led to the identification of multiple proteins with E3 ligase activity and has prompted a dramatic expansion in our understanding of the role of ubiquitin in the regulation of numerous proteins and processes.

The removal of ubiquitin potentially plays as important a role in the regulation of these proteins and processes, but progress was initially slow in regards to determining the identity and function of the deubiquitinating enzymes (DUBs). However, recent efforts using bioinformatics has led to a dramatic expansion in the number of known DUBs (4) and when combined with studies examining the proteins with which these enzymes interact (5) has now led to an exponential increase in our knowledge of their regulation and function.

Currently, five deubiquitinating enzyme families have been identified including the Ubiquitin C-terminal Hydrolases (UCHs), the Ubiquitin Specific Proteases (USPs), the Machado-Joseph Disease Protein Domain Proteases (MJDs), the Ovarian tumour Proteases (OTUs) and the JAMM Motif Proteases (6).

The UCHs were the first family of DUBs to be identified and in humans there are four of these cysteine proteases (UCHL1, UCHL3, UCHL5 and BAP1) (6). The catalytic domains are highly homologous and have been reported to favour the cleavage of small protein substrates (~20-30 amino acids) from ubiquitin as well as potentially playing roles in ubiquitin recycling (7). Few further clues to their function can be gained from their structure as UCHL1 and UCHL3 contain little more than the characteristic UCH domain and UCHL5 and BAP1 only show the presence of an additional coiled-coil domain (6).

The largest family of DUBs are the USPs, consisting in humans of approximately 56 members. They are again cysteine proteases identified by the inclusion within their catalytic domain of two well conserved motifs known as the Cys and His boxes (6). Unlike the UCH's, these proteases vary greatly in size and structure, with a host of other domains having been identified in various members including zinc fingers, ubiquitin-like domains and various ubiquitin interacting motifs (6).

The OTUs are identified by the presence of an OTU domain (8). There are approximately 15 putative members of this family (6), although only a handful have been shown to display deubiquitinating activity (9, 10, 11) and the role of many of these enzymes is as yet unclear. However, several members display the presence of zinc finger, ubiquitin-like and ubiquitin interacting motifs, suggesting some connection to the ubiquitin pathway (6).

The only confirmed member of the MJD family is Ataxin-3 (12), although a number of homologous proteins have been proposed to represent putative family members (13). Ataxin-3 is also a cysteine protease, but shows little homology to the UCH, USP and OTU families.

The JAMM domain proteases are the only metalloproteases known to act as DUBs. There are a number of JAMM domain containing proteins, but not all of these have been identified as DUBs and it is unclear whether this will be their only function (6).

3. CELLULAR FUNCTIONS OF DEUBIQUITINATING ENZYMES

DUBs play many roles within the cell contributing to everything from housekeeping functions, such as regulating the function of the proteasome (14) and recycling free ubiquitin, to performing specific roles in the regulation of intracellular signalling pathways or the cell cycle (6). These functions all involve the removal of ubiquitin from either a substrate protein or another ubiquitin molecule onto which they have been attached and can result in a number of outcomes depending on the ubiquitin modification involved. These outcomes can include the rescue of a substrate protein from either proteasomal or lysosomal degradation, the re-localisation of a protein within the cell, or the activation/deactivation of the protein concerned (6).

In this review we concentrate on the DUBs that regulate intracellular signalling and discuss their influence on receptor trafficking and recycling and the processes that can be triggered by extracellular stimuli, such as cell cycle progression or cell movement. Then, we will examine how DUBs contribute downstream in the regulation of intracellular signalling and transcription. Finally we will discuss the role DUBs play in disease and discuss how the current evidence suggests many DUBs have multiple substrates and possibly perform multiple tasks.

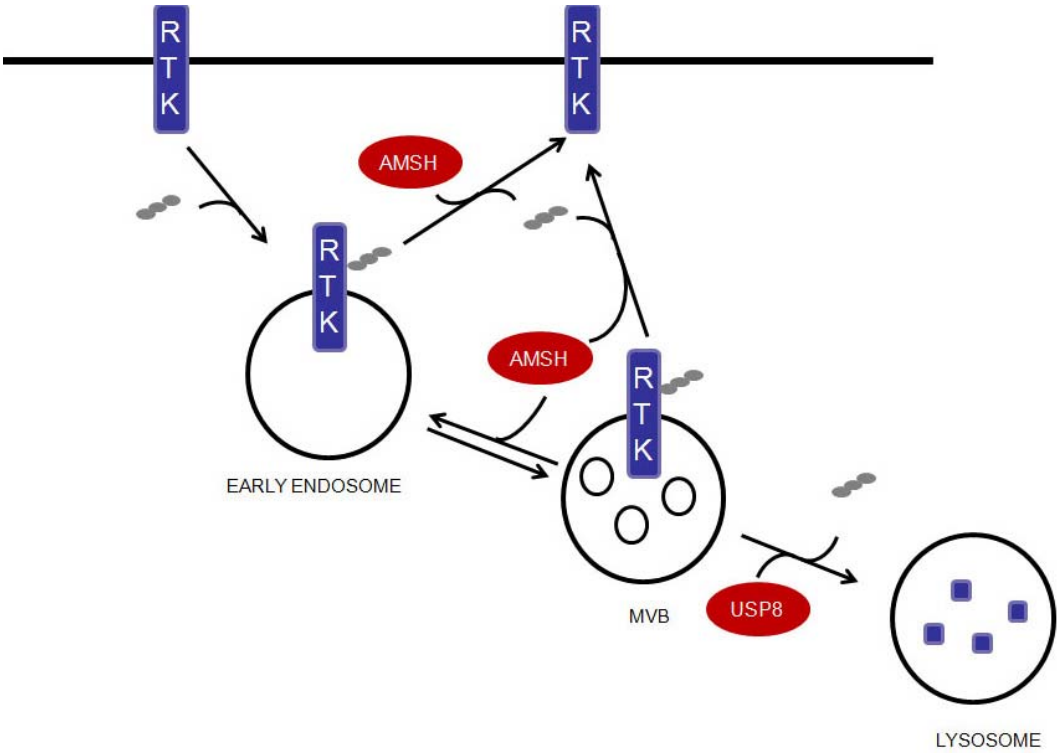

Figure 1. Regulation of receptor internalisation. Many extracellular receptors are internalised upon binding to ligand in a ubiquitin (illustrated as grey circles) dependant process which causes them to be incorporated into early endosomes from where they can be moved on to multi-vesicular bodies (MVB) and then the lysosome, or recycled back to the cell surface. The deubiquitinating enzymes AMSH and USP8 regulate this process with removal of ubiquitin by AMSH apparently prompting recycling of these receptors back to the surface and USP8 removing ubiquitin to allow their entry into the lysosome and eventual degradation.

3.1. Regulation of receptor trafficking

Cellular responses are often regulated by the binding of an extracellular ligand to a cell surface receptor. To allow proper signalling and control of the response these receptors usually undergo endocytosis and are either, de-activated and recycled back to the surface, or trafficked to the lysosome where they are destroyed (15). Ubiquitination has been shown to regulate the passage of many receptors through these events and DUBs have also been demonstrated to regulate them (15) (Figure 1 and Table 1).

Much of the work in this area has focussed on the receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) and has led to the identification of AMSH (associated molecule with the Src homology 3 domain of STAM [signal-transducing adapter molecule]) and USP8 (also known as UBPY) as DUBs which contribute to the endocytic trafficking of EGFR (15). Upon activation, EGFR is ubiquitinated (16), internalised and then interacts with the Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate)-STAM complex in early endosomes before being trafficked to the lysosome for degradation (17). Both AMSH and USP8 interact with this complex and regulate EGFR trafficking in different ways. AMSH appears to act prior to EGFRs committal to the lysosome and opposes this by deubiquitinating EGFR and prompting its recycling to the membrane before it reaches the multi-vesicular body (MVB) (18). However, AMSH has also been shown to interact with the ESCRT III complex which acts at a later stage in the MVB and it has been argued AMSH may have a role here, but this is as yet to be resolved. In contrast, USP8 deubiquitination is required for lysosomal targeting and degradation of EGFR (19, 20, 21, 22) suggesting it acts after the receptor has been committed to the lysosome and may deubiquitinate the receptor, allowing it to be transported into the lysosome, and so contributing to the maintenance of free ubiquitin levels (18) (Figure 1). AMSH and USP8 also play a role in regulating the trafficking of a number of other RTKs such as ERBB2, ERBB3, and hepatocyte growth factor receptor (c-met)(22, 23), as well as a number of G protein coupled receptors (GPCRs) such as protease activated receptor 2 (24), the delta-opioid receptor (DOR)(25) and the chemokine receptor CXCR4 (26).

USP20 and USP33 have recently been shown to regulate the trafficking of the beta-2 adrenergic receptor (27), another GPCR, suggesting other DUBs may also be involved in this process. As with EGFR, upon agonist binding the beta-2 adrenergic receptor is ubiquitinated and internalised into the endocytic compartment prior to lysosomal targeting and degradation. USP20 and USP33 both serve to deubiquitinate and cause its deactivation and

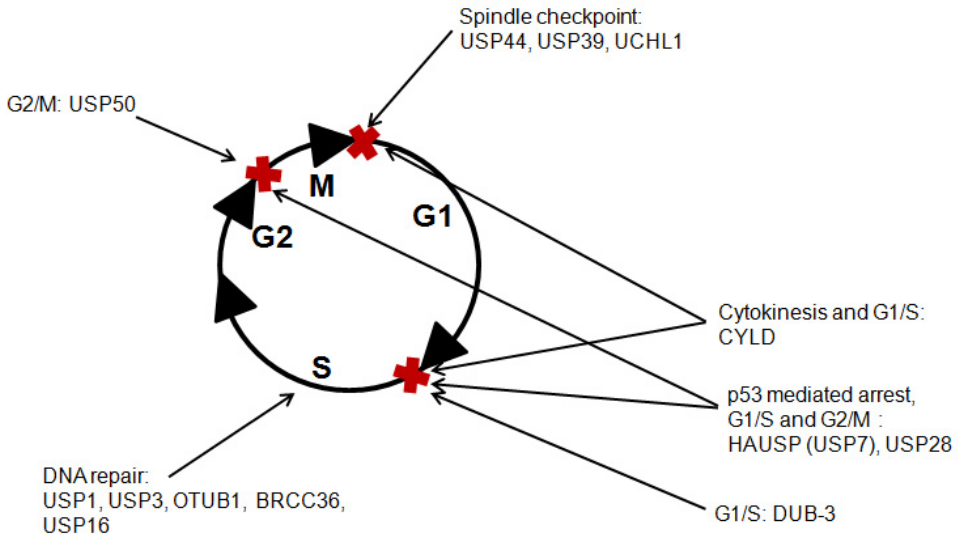

Figure 2. Cell cycle progression and deubiquitinating enzymes. Cell cycle progression requires exquisite control and proper function of the G1/S, G2/M and spindle checkpoints as well as functional DNA repair during S-phase. A number of deubiquitinating enzymes have been shown to be important for progression through the cell cycle due to their involvement in the regulation of the G1/S (CYLD, USP7, USP28, DUB-3), G2/M (USP50, USP7, USP28) or spindle (USP44, USP39, UCHL1) checkpoints as well as their regulation of a number of DNA repair mechanisms (USP1, USP3, OTUB1, BRCC36, USP16) which are important during S-phase.

recycling to the plasma membrane where it can again be activated (27).

In addition to their involvement in receptor trafficking, DUBs have now also been implicated in the internalisation and recycling of ion channels. The cystic fibrosis trans-membrane conductance regulator (CFTR) is a chloride channel that is part of the ABC transporter superfamily and recent studies would suggest that CFTR is ubiquitinated and targeted to the lysosome after internalisation from the surface of the airway epithelium (28). USP10 mediated deubiquitination promotes recycling of the CFTR to the plasma membrane and rescues it from lysosomal degradation (28).

The epithelial sodium channel (ENaC) is also ubiquitinated and targeted for lysosomal degradation upon internalisation (29), and this is antagonized by UCH-L3 which deubiquitinates ENaC and causes it's recycling to the plasma membrane (29). Indeed, loss of UCH-L3 activity caused a loss of ENaC from the apical surface of these cells suggesting that this mechanism may be important for the maintenance of polar membrane expression of these channels (29).

Collectively these observations suggest that the DUBs play an important role in the regulation of receptor and ion channel stability, internalisation and recycling, and thus ultimately influence how cells respond to extracellular stimuli. Moreover, as this is currently an area of intense research interest, our current assessment of the role of DUBs in receptor endocytosis and recycling is likely to be in its infancy.

3.2. Cell cycle progression

Ubiquitination has been extensively associated with the control of cell cycle progression, in most cases through its regulation of the destruction of checkpoint proteins which must be removed to allow progression through different phases of the cell cycle (30). However, recent studies would suggest DUBs also have essential roles in the precise control of cell cycle checkpoints and regulation of the DNA damage repair machinery, all of which allow proper progression through the cell cycle (Figure 2 and Table 2).

In particular, USP28 interacts with 53BP1, a protein which binds to p53 and is necessary for the DNA damage associated cell cycle checkpoint. USP28 stabilises 53BP1 and other checkpoint proteins such as Chk2, Nbs1 and ATRIP (31) and its depletion interferes with proper checkpoint control.

USP28 has been demonstrated to deubiquitinate the c-Myc proto-oncogene and protect it from degradation, thus driving cell proliferation (32). However, USP28 dissociates from with c-Myc upon DNA damage (33) suggesting that this triggers a switch in USP28 from a positive to a negative regulator of cell cycle progression.

CYLD was originally identified as a tumour suppressor gene and its action was attributed to its ability to regulate the NF-kappaB pathway (34). However, it has now become apparent that CYLD has other substrates and also functions during the cell cycle to control progression through G1/S and cytokinesis (35). In particular, CYLD is found in the peri-nuclear region during G1/S transition where it interacts with Bcl-3 and delays G1/S progression (35), as well as negatively regulating histone deacetylase 6

Table 2. DUBs and cell cycle progression

Process	DUB	Substrate/s	Function
Cell cycle progression	USP28	53BP1, Chk2, Nbs1, ATRIP, c-Myc	Involved in the control of the DNA damage checkpoint.
	CYLD	Bcl-3, HDAC6	Controls progression through G1/S and cytokinesis.
	USP17	RCE1	Required for G1/S progression
	USP44	Cdc20	Required for the mitotic spindle checkpoint.
	USP39	Aurora B kinase mRNA	Required for the mitotic spindle checkpoint.
	UCHL1	Tubulin?	Regulation of mitotic spindle checkpoint. May act as a dimer and have E3 ligase activity.
	BAP1	BRCA1, HCF-1	Can positively and negatively regulate cell cycle progression depending on context.
	USP50	HSP90? Wee1?	Role in G2/M checkpoint?
DNA damage repair	USP1	FANCD2, PCNA	Regulates initiation of fanconi anaemia DNA damage pathway and translesion DNA synthesis.
	USP7	Mdm2, MdmX	Stabilises Mdm2/MdmX and causes p53 turnover.
	USP10	p53	Counteracts p53 nuclear export and degradation.
	OTUB1	UBC13	Blocks histone ubiquitination.
	BRCC36	H2A, H2AX	Deubiquitinates histones and blocks DNA repair.
	USP3	H2A, H2B	Role in DSB repair.
	USP16	H2A	Necessary for cell cycle progression after DSB repair.

HDAC6) leading to an increase in acetylated alpha-tubulin and microtubules (36). CYLD also appears to interact with HDAC6 in the midbody during cytokinesis and as a result regulates the rate of cell division (35).

Expression of the cytokine inducible DUB USP17 (also known as DUB-3) is tightly regulated during the cell cycle and the loss of USP17 impedes G1/S progression (37). USP17 regulates the processing and function of a number of small GTPases which are important for G1/S progression through its regulation of the protease Ras converting enzyme 1(RCE1) (38).

USP44 was originally identified in a shRNA screen to identify proteins involved in the spindle checkpoint. USP44 depletion led to an almost complete loss of the spindle checkpoint and it has been demonstrated that USP44 acts by counteracting the activation of the anaphase promoting complex (APC) (39). APC is activated when associated with ubiquitinated Cdc20 and USP44 counteracts this by deubiquitinating Cdc20, allowing it to remain in a complex with Mad2 (39).

USP39 was identified during a similar siRNA screen as being required for the full activity of the mitotic checkpoint (40). USP39 lacks the required cysteine and histidine residues within its active site and as a result is inactive. However, depletion of USP39 rendered cells unable to arrest in response to certain circumstances and it has now been shown to stabilise the mRNA of aurora B kinase, as well as possibly other genes involved in the mitotic checkpoint (40).

UCHL1, which is expressed during M-phase and associates as a dimer with the mitotic spindle, has also been implicated in the control of the mitotic spindle checkpoint. As a dimer, UCHL1 has previously been proposed to function as an E3 ligase and it has been suggested that its role in this checkpoint may involve the ubiquitination of tubulin so regulating its polymerisation (41).

BAP1 has been suggested to be a negative and positive regulator of cell proliferation. Originally it was identified due to its association with the tumour suppressor BRCA1 and it was thought to negatively regulate cell cycle progression as its expression enhanced the BRCA1 mediated cell cycle block (42). Indeed, inactivating mutations were found in tumour cells, and its restoration in these cells resulted in a proliferation block (43). However, it has now been implicated as a positive regulator of cell proliferation as it deubiquitinates host cell factor-1 (HCF-1) and its knockdown blocks cell proliferation rather than enhancing it (44). This could suggest that BAP1, like USP28, can influence the cell cycle differently depending on the presence or absence of DNA damage.

Recently USP50 has been proposed to potentially play a role in the G2/M checkpoint (45). USP50 was found associated with HSP90 and its loss was shown to enhance the bypass of the G2/M checkpoint produced in response to CDC25B expression. Subsequently USP50, which is recruited to the nucleus upon DNA damage, was shown to prevent Wee1 accumulation in the nucleus and thus to block cell cycle progression, through an HSP90 dependent mechanism (45).

Another area where DUBs have been implicated is in the regulation of DNA damage repair (Figure 2 and Table 2). The Fanconi Anaemia (FA) pathway is involved in the repair of DNA-crosslink damage, which can arise during DNA replication, and takes its name from a rare clinical syndrome characterised by chromosome instability and an increased susceptibility to leukaemia and squamous cell carcinomas (46). Eight proteins have been identified that play a role in this pathway and one of these, FANCD2, is thought to be monoubiquitinated (47). Monoubiquitination of FANCD2 targets it to nuclear foci where it associates with other repair proteins and initiates DNA repair. The mutations associated with FA in many cases lead to the loss of FANCD2 monoubiquitination and thus disrupts this DNA repair pathway (48). Knockdown of USP1 leads to increased monoubiquitination of FANCD2 and increased resistance to DNA-crosslinking agents

Table 3. DUBs and cell migration

Process	DUB	Substrate/s	Function
Cell migration	USP14	CXCR4	Rescues CXCR4 from degradation.
	AMSH	CXCR4	Regulate CXCR4 recycling and degradation.
	USP8		
	USP17	RCE1?	Required for chemotaxis.
	ATXN3L, UCHL1, USP3, USP6, USP15, Cezanne, USP50, VCPIP1, USP1, USP30, USP33, USP47	???	Impede Met receptor mediated cell scattering.

indicating USP1 can remove FANCD2 monoubiquitination and inhibit the initiation of DNA repair through this pathway (49). USP1 has also been implicated in the control of another DNA repair pathway through its deubiquitination of PCNA. The sliding clamp PCNA assembles into a trimeric ring which encircles DNA and can recruit the replication machinery as well as specialised translesion DNA synthesis (TLS) polymerases which can bypass damaged template DNA during replication. Following DNA damage the latter are recruited to monoubiquitinated PCNA (50). In the absence of DNA damage USP1 removes the monoubiquitin from PCNA, but following DNA damage USP1 undergoes auto-cleavage allowing the accumulation of monoubiquitinated PCNA (51). Regulation of USP1 stability therefore may allow the initiation of both this and the FA pathway thereby acting as a molecular switch for multiple DNA repair pathways.

After DNA damage p53 is up-regulated and causes a cell cycle arrest. The levels of p53 are controlled by proteasomal degradation via the action of a number of ligases, most importantly Mdm2 (52). Mdm2 interacts with a homologous protein MdmX which is thought to stabilise Mdm2 by blocking its auto-ubiquitination (53). Upon DNA damage it has been proposed that MdmX is phosphorylated targeting it for proteasomal degradation (54). This destabilises Mdm2 leading to a rise in the levels of p53. The deubiquitinating enzyme HAUSP (USP7) was originally shown to deubiquitinate p53 and as a result it was thought to stabilise this molecule (55). However, subsequent studies would suggest HAUSP is a negative regulator of p53 levels and its main function is to deubiquitinate Mdm2 and MdmX stabilising these molecules and therefore down-regulating p53 levels (56).

Usp10 has more recently been shown to deubiquitinate p53 and counteract its nuclear export and degradation in response to its ubiquitination by Mdm2 (57).

OTUB1 has been shown to arrest the ubiquitination of proteins in response to double strand breaks (DSBs). DSBs result in the ubiquitination of histones and other associated proteins to allow the initiation of DNA repair. However, OTUB1 appears to block this ubiquitination, rather than removing it, as its action does not require its deubiquitinating activity. This is potentially thought to be due to its association with E2 enzymes such as UBC13, although the exact mechanism by which ubiquitination is blocked still remains unclear (58).

The JAMM family member BRCC36 associates with a complex known as the BRCC (BRCA1 and BRCA2 Containing Complex). This complex includes BRCA1, BRCA2, RAD51 and BRCC46 and is required for DSB DNA repair. Ubiquitination of the histones H2AX and H2A with K63 chains at sites of DSBs provide a docking site for the DNA repair proteins containing ubiquitin interacting motifs (UIMs). BRCC36 has been proposed to counteract the ubiquitination of H2AX and H2A terminating DNA repair (59).

Depletion of USP3 by RNAi leads to a delay of S-phase progression and the accumulation of DSBs as well as the initiation of the DNA repair response (60). USP3 removes monoubiquitin conjugates from histones H2A and H2B. Monoubiquitinated H2A accumulates with H2AX at DSB sites and as a result USP3 is thought to potentially play a role in the repair of DSBs and the maintenance of genome integrity during the cell cycle (60).

USP16 (Ubp-M) is also thought to remove monoubiquitin from histone H2A upon successful completion of DSB repair (61) and its depletion results in slowed proliferation and defects in mitosis suggesting its presence is necessary for proper cell cycle progression (62).

3.3. Regulation of cell migration

Recent studies would suggest that in addition to cell proliferation, DUBs can also influence cell movement in response to a number of stimuli (Table 3). In particular, over-expression, or loss, of USP14 can regulate CXCR4 mediated cell migration, even though it has no effect upon CXCR4 mediated ERK activation (63). The mechanism behind this is unclear although USP14 rescues CXCR4 from degradation.

AMSH and USP8, as previously mentioned, can also influence trafficking and recycling of CXCR4 upon internalisation and as such potentially regulate cell movement in response to CXCR4 stimulation (26).

In addition, we have observed that USP17 is induced in response to CXCR2 and CXCR4 engagement and depletion of USP17 blocks both chemokine induced cell migration and chemokinesis (64). This results from the failure of the relevant GTPases (Cdc42, Rac1, Rap1) to re-localise upon CXCR2/4 engagement, and since each of these GTPases has a CAAX box, may result from the improper processing of this motif as previously observed (38).

A siRNA library, targeting the majority of the known DUBs, was also used to examine hepatocyte growth factor induced cell scattering, a process involving the loss of cell to cell contacts and subsequent cell motility. This

Table 4. DUBs and intracellular signaling

Process	DUB	Substrate/s	Function
Intracellular signaling	CYLD	TRAF2, TRAF6, RIP1, RIG-I, NEMO, Lck, Dvl	Regulates NF-kappaB activation in some contexts. Regulates JNK activation in response to TNFR and IL-1/TLR. Regulates TCR signaling. Regulates Wnt/beta-catenin signaling.
	A20	RIP1, TRAF6, RIP2, NEMO	Regulates NF-kappaB activation.
	Cezanne	RIP1	Blocks NF-kappaB activation.
	USP31	TRAF2, P65/Rel	Blocks NF-kappaB activation.
	UCHL1	???	Regulates NF-kappaB activation.
	USP11	???	Regulates NF-kappaB activation.
	USP17	RCE1	Regulates Ras/MEK/ERK signaling.
	USP9x	Smad4	Regulates TGF-beta signaling.
	UCH37	TGF-betaR	Regulates TGF-beta signaling.

study observed that 12 of these DUBs may be necessary for this Met receptor mediated process to proceed (65). Three separate inhibitory phenotypes were observed, the presence of large flat cells (ATXN3L, UCHL1, USP3, USP6, USP15, Cezanne), the loss of cell to cell contacts (USP50, VCPIP1) and those that resembled un-stimulated or Met depleted cells (USP1, USP30, USP33, USP47). The exact mechanisms underlying these phenotypes remain to be elucidated, but again suggest that DUBs can influence cell motility and thus migration.

3.4. Regulation of intracellular signalling

During the last few years a huge effort to identify DUB substrates has implicated many of these enzymes in the regulation of a wide range of different signalling pathways.

However, the best characterised signalling pathway in respect to ubiquitination is that leading to the activation of the transcription factor NF-kappaB (Figure 3 and Table 4). It has been demonstrated that K63 linked polyubiquitin chains are conjugated to multiple signalling intermediates within a number of pathways and that this ultimately allows the activation of NF-kappaB (66). The activation of NF-kappaB is initiated in response to ligation of the tumour necrosis factor receptor (TNFR), the Interleukin 1 (IL-1)/Toll-like receptor (TLR) family, the T-cell receptor (TCR) and intracellular receptors such as RIG-I and NOD2 (66). Engagement of IL-1/TLR family members leads to the oligomerisation and trans-auto-ubiquitination of TRAF6. TRAF6 exhibits E3 ligase activity and can assemble K63 linked polyubiquitin chains (67, 68) and, in addition to its auto-ubiquitination, it acts to polyubiquitinate RIP1 and NEMO (IKKgamma) (69). In response to TNFR signalling cIAP1/2 is recruited via TRAF2 and then cIAP1/2 polyubiquitinates RIP1 with K63 linked chains (70, 71). Activation of receptors such the TCR and B-cell receptor (BCR) triggers MALT1 to complex with BCL10, CARMA1 and TRAF2/6 leading to the K63 linked polyubiquitination of MALT1, BCL10 and NEMO, although the ligase responsible is as yet unknown (72). Activation of members of the NOD-like family, such as the intracellular receptor NOD2, leads to the K63 linked ubiquitination of RIP2, although there is debate regarding which ligase is responsible (73). Finally, activation of RIG-I like receptors can also lead to NF-kappaB activation and RIG-I has been found to be polyubiquitinated with K63 linked chains upon activation (74).

In all these pathways the K63 linked polyubiquitination of the various intermediates results in the recruitment of other constituents of the signalling pathway which bind to K63 linked ubiquitin chains. In particular, the protein kinase TAK1 is recruited through its association with TAB2 and TAB3 and this allows the phosphorylation and activation of IKKbeta which is also recruited as part of the IKK complex (68). The active IKK complex then phosphorylates I-kappaB triggering its polyubiquitination with K48 linked chains by the E3 ligase beta-TRCP and subsequently the degradation of I-kappaB (75). Once the NF-kappaB transcription factor is released from I-kappaB, its nuclear localisation signal is unmasked and it translocates to the nucleus and triggers transcription.

A number of DUBs have been shown to play a role in the regulation of NFkappaB activation. CYLD, a member of the USP family, was originally identified as a tumour suppressor gene mutated in cylindromatosis (34), and was subsequently implicated in NF-kappaB regulation. CYLD acts by removing K63 linked polyubiquitin chains from numerous signalling intermediates including TRAF2, TRAF6, RIP1, RIG-I and NEMO (12, 76, 77) and thus blocks the activation of NF-kappaB. This, in conjunction with its induction in response to NF-kappaB activation, suggested it was part of a classical feedback loop that terminated NF-kappaB activation (78). However, it is now recognised that CYLD may not play as specific a role in NF-kappaB regulation as first thought and that it also appears to play roles in the regulation of several other pathways. Firstly, CYLD was shown to block JNK activation in response to TNFR and IL-1/TLR signalling and whilst this role appears to be universal, its regulation of NF-kappaB activation appears to only to be important under specific circumstances (79). In particular, CYLD -/- mice were found to show no defect in NF-kappaB signalling in bone marrow derived macrophages, but they have been shown to have defects in their B-cell populations due to constitutive activation of NF-kappaB. Secondly, the CYLD -/- mice were also shown to have a defect in T-cell development which suggested CYLD is a positive regulator of signalling through the T-cell receptor (TCR) (80). This has been suggested not to be due to any influence on the NF-kappaB pathway, but to result from the removal of both K48 and K63 linked polyubiquitin chains from Lck allowing its active form to interact with its downstream substrate ZAP-70 (80), although it has also been suggested CYLD may affect TCR signalling by regulating TAK1

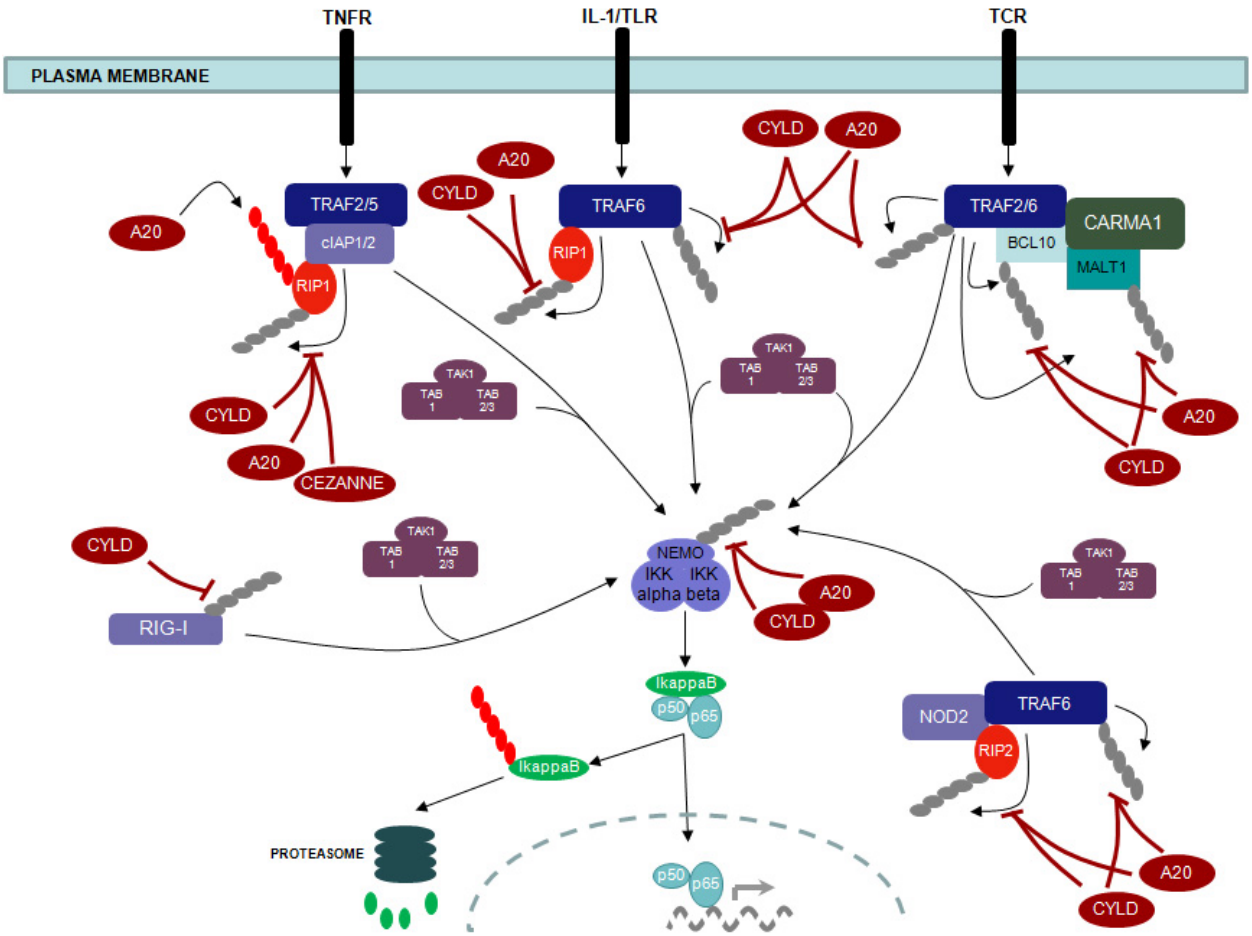

Figure 3. Regulation of NF-kappaB activation. Ubiquitin ligases, in response to signalling through the IL-1/TLR, TNFR, TCR, RIG-I and NOD like receptor pathways, catalyse the addition of Lys63 linked chains (shown in grey) to TRAF2/6, RIP1/2, MALT1, BCL10, NEMO and RIG-I respectively. This allows the recruitment of other proteins including TAK1 in complex with TAB2/3 to allow the eventual activation of the IKK complex. IKK then targets IkappaB for beta-TRCP mediated Lys48 linked ubiquitination (shown in red) and proteasomal degradation allowing NF-kappaB to move to the nucleus and initiate transcription. Deubiquitinating enzymes (CYLD, A20, CEZANNE) de-conjugate Lys63 linked chains from TRAF2/6, RIP1/2, MALT1, BCL10, NEMO and RIG-I to block NF-kappaB activation. E3 ligase activity of A20 also catalyses the addition of Lys48 linked chains to RIP1 resulting in its degradation through the proteasome.

ubiquitination (81). CYLD-/- mice have also been shown to be more susceptible to chemically induced skin tumours and this is thought to result from increased nuclear activity of Bcl-3 associated p50 or p52 and not NF-kappaB (p50/p65) activation (82). Thirdly, and most recently, CYLD has been shown to negatively regulate signalling in the Wnt/beta-catenin pathway through the removal of K63 linked polyubiquitin chains from Dvl (83). These studies suggest that, rather than being the universal regulator of NF-kappaB first hypothesised, CYLD has a limited role in NF-kappaB signalling as well as several other cell type specific roles which allow it to influence a number of different signalling pathways.

Another DUB implicated in NF-kappaB signalling is A20, a member of the OTU family. A20 was originally identified due to the fact it was up-regulated following Tumour Necrosis Factor alpha (TNF-alpha) treatment and that mice deficient in A20 developed severe inflammation associated with hypersensitivity to TNF-alpha and LPS and exhibited sustained NF-kappaB activation (84). A20 was shown to block NF-kappaB activation by TNF-alpha by removing K63 linked polyubiquitin chains from RIP1 kinase (70). Moreover, it was also shown, in addition to its deubiquitinating activity, to uniquely exhibit E3 ligase activity due to the presence of a Ring Finger domain which also acts to conjugate K48 linked polyubiquitin chains to RIP1 and target it for proteasomal degradation (70). A20 has since been reported to deubiquitinate a number of other targets including TRAF6, RIP2 and NEMO and as a result has been implicated in the regulation of NF-kappaB activation through the TNFR, IL-1/TLR, NLR and TCR pathways (85, 86). In addition, the spontaneous inflammation observed in mice lacking A20 would suggest that, unlike CYLD, it may play an essential role in the regulation of these pathways.

Table 5. DUBs and transcriptional control

Process	DUB	Substrate/s	Function
Transcriptional control	USP22	H2B, H2A	Regulates transcriptional activation of GAL genes. Required with ATXN7L3 and ENY2 for full androgen receptor transcriptional activity.
	2A-DUB	H2A	Androgen receptor co-activator.
	USP21	H2A	???
	USP7	???	Required for proper PRC1 function.
	USP11		

In addition to A20 and CYLD, a number of other DUBs have been implicated in the regulation of NF-kappaB. Cezanne is another member of the OTU family which blocks NF-kappaB activation through the deubiquitination of RIP1 (13, 87) and it has now been proposed that Cezanne may remove K11 chains preferentially (88). USP31 interacts with TRAF2 and p65/RelA, to target K63 linked polyubiquitin chains and to block NF-kappaB activation in response to a number of stimuli (89). However, its exact mode of action is as yet unclear. DUBA has also been implicated as a negative regulator of NF-kappaB activation and thus type 1 interferon (IFN-I) production through its ability to deubiquitinate TRAF3 (90). Finally, UCHL1 has been suggested to regulate NF-kappaB activation in the vasculature, although no mechanism has been proposed (91) and USP11 is thought to regulate NF-kappaB activation through its control of IkappaB kinase alpha (IKK-alpha) transcription in a p53 dependant manner (92). This further emphasises the importance of ubiquitination, and particularly DUBs, in the regulation of NF-kappaB activation.

To further demonstrate the importance of ubiquitination in the proper regulation of NF-kappaB activation it has also been shown that this regulation can be used to circumvent the immune response. In particular, the *Yersinia* virulence factor YopJ acts as a deubiquitinase to remove ubiquitin from TRAF2, TRAF6 and IkappaB-alpha blocking the activation of NF-kappaB as a mechanism to evade the host immune response (93). In addition, non-structural protein 2 (nsp2), an OTU protein expressed by the porcine reproductive and respiratory syndrome virus, can counteract IkappaB-alpha degradation and inhibit NF-kappaB activation (94).

The role of DUBs is not just limited to NF-kappaB activation pathway. The DUB/USP17 family of DUBs were originally identified in mice as cytokine inducible immediate early genes (DUB-1/2) (95) and subsequently a human family member, USP17, has been demonstrated to be cytokine inducible and to modulate cytokine induced Ras/MEK/ERK signalling (38, 96). This results from USP17's modulation of the protease RCE1 which is involved in the processing of the Ras C-terminal CAAX motif (38, 97).

USP9x (Also known as FAM) removes monoubiquitin from Smad4 allowing it to interact with phosphorylated Smad2 and facilitate TGF-beta signalling (98). UCH37 deubiquitinates and stabilises the TGF-beta receptor and so potentiates TGF-beta signalling (99).

A recent study has utilised morpholino knockdowns of the majority of the known zebrafish DUBs to examine their potential roles in development. 57 of the 85 DUBs examined in this way were necessary for proper development suggesting that these enzymes are potentially essential for embryogenesis (100). Interestingly, two of the different phenotypes observed in conjunction with these knockdown experiments were suggestive of effects on the notch (otud7b, bap1, uchl3) and BMP (otud4, usp5, usp15, usp25) signalling pathways (100), although the mechanisms underlying these effects are as yet unknown.

The work outlined demonstrates that DUBs play a significant role in intracellular signalling, particularly in the regulation of NF-kappaB activation. However, new signalling functions are constantly being added and it would appear we have much to learn in regards to the influence of DUBs on intracellular signalling.

3.5. Transcriptional control

Ubiquitination of the histones H2A and H2B is essential for transcriptional control and a number of DUBs have been implicated in its regulation (Figure 4 and Table 5). It is not entirely clear whether their ubiquitination has a positive or negative effect upon transcription (recently reviewed by Attanassov and colleagues) (101) as the modification of histones is a complicated and highly regulated process involving a balancing act between ubiquitination, methylation and acetylation. However, the data in regard to the DUBs would suggest they work by facilitating transcriptional elongation.

H2B is deubiquitinated by Ubp8, the yeast ortholog of USP22, which is part of the Spt-Ada-Gcn5 acetyltransferase complex (SAGA) and this deubiquitination positively regulates transcriptional activation of GAL genes (102). However, there is still some debate as to whether the ubiquitination of H2B has a positive or negative effect upon transcription, although it may play different roles in distinct phases of transcriptional elongation (103). USP22 has also recently been proposed to be necessary for cell cycle progression as part of this complex, as its knockdown blocked the cell cycle at G0/G1 (104) and USP22 expression has been associated with tumour progression, something which clearly fits with this observation (105).

H2A is the preferred ubiquitination substrate in mammalian cells with between 5% and 15% of total H2A thought to be ubiquitinated (101). It is not clear whether ubiquitination of H2A has a positive or negative effect, although studies of the DUBs which target H2A would suggest that ubiquitination negatively regulates transcription.

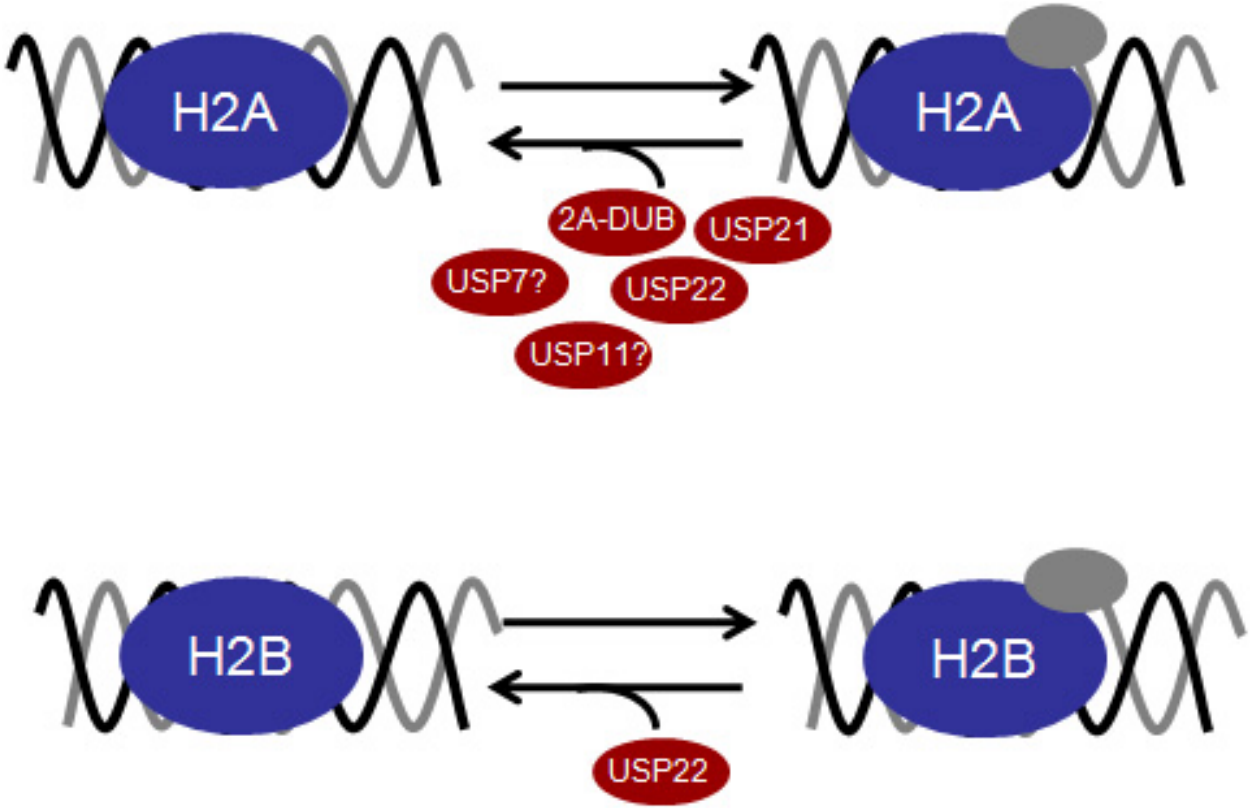

Figure 4. Regulation of Transcription. Transcriptional activity is up-regulated by the addition of monoubiquitin (shown in grey) to the histones H2A and H2B. A number of deubiquitinating enzymes, as illustrated (2A-DUB, USP21, USP22, USP7, USP11), can remove this monoubiquitin and so negatively regulate transcription of surrounding genes.

2A-DUB has been identified as an androgen receptor (AR) co-activator which deubiquitinates H2A at the promoters of AR target genes (106). USP22 is also required with ataxin7-like 3 (ATXN7L3) and human ortholog of enhancer of yellow 2 (ENY2) for the full transcriptional activity of the AR (107).

USP21 has also been shown to remove ubiquitin from H2A, and therefore relieve transcriptional repression of genes in regenerating mouse liver (108).

USP7 and USP11 have been shown to associate with the polycomb repressive complex 1 (PRC1) which is responsible for the repression of transcription of a number of genes. Both are required for proper PRC1 function, at least in regards to the INK4a tumour suppressor gene (109). Interestingly, PRC1 represses transcription, at least in part, by ubiquitinating H2A.

4. DUBS AND DISEASE

There is clear evidence that DUBs play a role in the initiation and progression of a number of diseases. In particular, several DUBs have been implicated in cancer as either potential tumour suppressors or oncogenes.

CYLD was originally identified as the tumour suppressor gene mutated in the rare familial cylindromatosis (FC) syndrome (34). It has subsequently been shown to be mutated in Brooke-Spiegler syndrome (BSS) and multiple familial trichoepithelioma (MFT) (110). These are all tumours of skin appendages and it has been suggested that they all may represent phenotypic variants of the same tumour type (110). Its action was originally put down to its effect on NF-kappaB, but now it is thought its role in cancer may be more related to its regulation of the cell cycle through Bcl-3 and HDAC6 (35).

A20 has also been proposed to potentially be a tumour suppressor as the absence of A20 expression has been observed in some cases of non-hodgkins lymphoma including cases of diffuse large B-cell lymphoma, Burkitt's lymphoma and T-cell lymphomas including anaplastic large cell lymphoma (111). In addition, cases of ocular adnexal marginal zone B-cell lymphomas consistently show a reduction in A20 expression (112) and a number of inactivating mutations of A20 have been found in marginal zone lymphomas (113).

USP7 has also been proposed to be a potential tumour suppressor due to its regulation of p53. However, although it may represent a potential therapeutic target, apart from a study indicating that its expression is down-regulated in non-small cell lung cancer little evidence supports a direct role for USP7 in cancer (114).

In contrast a number of DUBs have been proposed to be potential oncogenes due to their over-

expression in tumours. Unp (USP4) is over-expressed in both lung cancers and adrenocortical carcinomas (115, 116) and its overexpression can transform NIH3T3 cells in a nude mouse assay (117). USP6 (Tre-2 or Tre17) was originally identified as an oncogene as its expression could transform NIH3T3 cells (118) and has subsequently been shown to be over-expressed in cases of Aneurysmal bone cysts (ABC) as a result of a chromosomal translocation as well as being over-expressed in Ewings sarcoma and osteoblastomas (119). USP22 expression has also been associated with tumour progression and to be predictive of therapy failure in cases of colorectal cancer (105).

USP17 expression has also recently been demonstrated to be up-regulated in a number of different tumour types, when compared to normal tissue (37) and it has been suggested USP17 may represent an oncogene (120). However, the finding that USP17 is expressed in a cell cycle regulated manner (37), in conjunction with previous studies which have demonstrated its constitutive expression blocks cell proliferation (38, 96), suggest USP17 is not an oncogene, but that its expression results from the fact the tumour cells are actively dividing.

In addition to their roles in cancer, DUBs have also been implicated in neurodegenerative diseases. Mutations of UCH-L1 have been identified in one family with a history of Parkinson's disease (PD) (121) and a particular polymorphism in UCH-L1 has been associated with decreased susceptibility to PD (122). In the gad mouse (gracile axonal dystrophy) homozygous truncating mutations were identified in the murine homologue of the UCH-L1 gene. These mice exhibit a neurodegenerative phenotype, but do not develop a PD-like phenotype (123). As a result the role of UCH-L1 in PD is unclear.

Mutations in Ataxin-3 have also been associated with another neurodegenerative disease, Machado-Joseph disease (124), and it is now thought Ataxin-3 is involved in protein quality control where the loss of its function may lead to the aggregation of proteins (125).

As mentioned throughout this review, the DUBs have also been shown to play a vital role in the regulation of many processes which are important for the regulation of the immune system, including the regulation of NF-kappaB activation in response to TLR/TCR/RIG-I/NOD-2, as well as the regulation of cell movement, cytokine and chemokine signaling. It is therefore surprising that a link between the DUBs and immune related pathologies has not been unearthed. However, recent publications would suggest that certain A20 alleles are associated with a number of different immune system associated pathologies such as Crohn's disease, Lupus and irritable bowel syndrome (126), suggesting its functionality may be important in these diseases. This could suggest that the lack of evidence linking the DUBs and immune related disorders may as yet materialise when we start to look more closely.

5. DUBS; ONE ENZYME, MULTIPLE ROLES?

Although we have seen an expansion in the numbers of known DUBs (now ~ 100) over the past few years they are still heavily outnumbered by the E3 ligases responsible for the conjugation of ubiquitin to substrates. This could suggest that, either each DUB is responsible for the regulation of numerous ubiquitinated proteins, that DUBs play no role in the regulation of many of the ubiquitinated proteins, or that the truth lies somewhere in between. Irrespective of this, it has become apparent from the recent expansion in our knowledge that DUBs appear, in many cases at least, to have multiple substrates and to play roles in the regulation of multiple different processes.

The best example to date is CYLD which was originally identified as a tumour suppressor and was initially reported to be a negative regulator of NF-kappaB activation (34). However, CYLD has now been shown to play a role in the regulation of cell cycle progression (35) and T-cell development (80) as well as regulating the activation of multiple signalling intermediates including JNK (79), Lck/ZAP-70 (80), Bcl-3 (82) and Dvl (83). CYLD, although not now thought to have a universal role in NF-kappaB signalling, does appear to be important in keratinocytes, B-cells and T-cells suggesting that its different roles may also be cell context/type specific.

USP1 is another example which removes monoubiquitin from both FANCD2 and PCNA and so prevents the initiation of two separate DNA repair pathways (49, 51). Following DNA damage USP1 undergoes auto-cleavage allowing the accumulation of monoubiquitinated FANCD2 and PCNA and prompting the initiation of both of their associated DNA repair pathways (51).

USP28 can deubiquitinate c-Myc and protect it from degradation, thus driving cell proliferation (32). However, upon DNA damage, USP28 dissociates from c-Myc and switches from a positive to a negative regulator of cell cycle progression by associating with 53BP1, a protein which binds to p53 and is necessary for the DNA damage associated cell cycle checkpoint (31).

AMSH and USP8 are both thought to play a role in the trafficking and recycling of multiple cell surface receptors (22, 23, 24, 25, 26) and this role is likely to expand as more receptors are examined.

These examples, and others, suggest DUBs may play multiple roles and that we should keep an open mind in regards to the function of these enzymes and not assign them a single specific substrate as their function may well depend upon their intracellular location as well as the cell type and context in which they are expressed.

6. CONCLUSIONS AND PERSPECTIVES

The recent explosion in our knowledge regarding the DUBs has highlighted many as potentially important regulators of multiple intracellular processes. However, the recent siRNA screens examining their roles in cell scattering (65) and development (100) suggest we may have only scratched the surface with regards to the potential roles these enzymes play, and when other knockdown screens are performed, their roles may far

outreach anything we have postulated to date. In addition, the observation that individual DUBs can have multiple roles in functionally diverse processes suggests there may be much to learn even about those DUBs which have already been extensively characterised. All of this, combined with the recent interest in DUBs as potential therapeutic targets (127, 128) would suggest these enzymes will be a research focus for many years to come and that we should be optimistic but cautious in regard to their potential.

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