De-ubiquitinating enzymes: intracellular signalling and disease

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Abstract
Ubiquitination is now accepted as an important process for regulating intracellular signalling and the realization that many known signalling molecules exhibit E3 ligase activity has led to great strides in our understanding of how these pathways are regulated. However, as most of the de-ubiquitinating enzymes have as yet no identified substrate, little is known about their potential role in the regulation of intracellular signalling. Here, we examine what is known about de-ubiquitinating enzymes and signalling, with particular emphasis on their role in the regulation of immune signalling and the initiation of DNA repair. In addition, we look at the evidence implicating these enzymes in the pathogenesis of diseases such as cancer and neurodegenerative diseases.

Introduction
Over the last decade, it has become apparent that ubiquitination plays a fundamental role in the regulation of many intracellular functions. Initially, this was thought to be mediated through its control of the proteasomal degradation of target proteins. However, the realization that ubiquitination may take many forms (mono, multi-mono and Lys6/Lys29/Lys48/Lys63-linked chains) that can be proteasome-dependent or -independent [1] has led to fundamental insights into endocytosis, cell biology and disease. In particular, research has focused on the enzymes responsible for the conjugation and de-conjugation of ubiquitin to and from 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 (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 combinations of E2 and E3 allow the conjugation of ubiquitin to specific recognized targets and the identification of large numbers of E3s through the presence of signature [RING (really interesting new gene) finger or HECT (homologous to E6-associated protein C-terminus)] domains has led to great strides in our understanding of how the ubiquitination of numerous proteins is regulated.

However, the removal of ubiquitin potentially plays as important a role in the regulation of cellular processes, and the recent increase in the number of known de-ubiquitinating enzymes through the use of bioinformatics and the identification of a number of new families has led to more interest being focused on how these enzymes may contribute to cellular regulation. In particular, several recent papers have identified a role for these enzymes in the regulation of intracellular signalling and in particular the activation of the transcription factor NF-κB (nuclear factor-κB) by CYLD, A20, Cezanne and USP31 (ubiquitin-specific protease 31) [3–9] and the initiation of DNA repair by USP1 [10,11].

To date, five families of de-ubiquitinating enzymes have been identified including the UCHs (ubiquitin C-terminal hydrolases), the USPs (ubiquitin-specific proteases), the MJDs (Machado–Joseph disease protein domain proteases), the OTUs (ovarian tumour proteases) and the JAMM (Jab1/MPN/Mov34 metalloenzyme) motif proteases [12].

The UCHs were the first de-ubiquitinating enzymes to be identified and in humans are a group of four cysteine proteases that are highly homologous within their catalytic domains. They have been reported to favour the cleavage of small protein substrates (~20–30 amino acids) from ubiquitin and are thought to play roles in ubiquitin recycling [13].

The USPs are the largest family consisting in humans of approx. 58 members. They are cysteine proteases that are identified by the inclusion within their catalytic domain of two well-conserved motifs known as the cysteine and histidine boxes [12].

The only confirmed member of the MJD family is ataxin-3 [14], although a number of ataxin-like proteins have been
Figure 1 | Ubiquitination and NF-κB activation

(A) Ubiquitin ligases (shown in green) catalyse the addition of Lys⁶³-linked chains (shown in grey) to TRAF-2/TRAF-6, RIP and IKKγ. This recruits TAK1 in complex with TAB2/3 to activate the IKK complex. IKK then targets IκB for βTRCP-mediated Lys⁴⁸-linked ubiquitination (shown in red) and proteasomal degradation. (B) De-ubiquitinating enzymes (shown in dark blue) de-conjugate Lys⁶³-linked chains from TRAF-2/TRAF-6, RIP and IKKγ to block NF-κB activation. E3 ligase activity of A20 also catalyses the addition of Lys⁴⁸-linked chains to RIP, resulting in its degradation through the proteasome.

proposed to represent putative de-ubiquitinating enzymes [15]. These are cysteine proteases that show little homology to the other families and little is known about ataxin-3 apart from its involvement in Machado–Joseph disease.

The OTUs are also cysteine proteases identified by the presence of an OTU domain [16] and there are approx. 14 putative members of this family [12]. However, only a handful have been shown to display de-ubiquitinating activity [5,8,17] and the role of all the OTU-containing proteins as de-ubiquitinating enzymes is unclear.

The JAMM domain proteases are the only metalloproteases known to act as de-ubiquitinating enzymes. There are a number of JAMM domain-containing proteins in humans, but only a few have been identified as de-ubiquitinating enzymes and it is unclear whether the removal of ubiquitin is the only function of the JAMM domain [12].

De-ubiquitinating enzymes and signalling

De-ubiquitinating enzymes and immune signalling

Ubiquitination has been demonstrated as a regulatory mechanism for many proteins involved in intracellular signalling. However, no pathway has been explored in as much detail as the activation of the transcription factor NF-κB (Figure 1). In particular it has been demonstrated that ubiquitin acts at multiple levels to regulate the activation of this transcription factor in response to signalling through the TNFR [TNF (tumour necrosis factor) receptor], IL-1 (interleukin-1)/TLR (Toll-like receptor) and TCR (T-cell receptor) pathways. Engagement of IL-1/TLR family members leads to the oligomerization and trans-auto-ubiquitination of TRAF-6 (TNFR-associated factor-6). This is accomplished via its RING finger domain, which exhibits E3 ligase activity and can assemble Lys⁶³-linked polyubiquitin chains [18,19]. TRAF-6 also polyubiquitinates IKKγ [IκB (inhibitory κB) kinase γ], resulting in the activation of the IKK complex [20]. In response to TNFR signalling, TRAF-2 is polyubiquitinated and can polyubiquitinate RIP (receptor-interacting protein), again through the assembly of Lys⁶³-linked polyubiquitin chains [6,21]. This results in the polyubiquitination of IKKγ, although the RING finger domain of TRAF-2 has been shown to be dispensable for this, suggesting an alternative mechanism to that of TRAF-6 [22]. The TCR pathway can also trigger polyubiquitination of IKKγ. It has been shown that upon TCR stimulation MAL1 complexes with BCL10 and CARMA1 and can act as an E3 ligase against IKKγ [23].

The conjugation of Lys⁶³-linked polyubiquitin chains to IKKγ results in the recruitment of the protein kinase TAK1 (transforming growth factor-β-activated kinase 1) through its association with TAB2 (TAK1-binding protein 2) and TAB3 and allows phosphorylation and activation of IKKβ [19]. This active complex can then phosphorylate IκB, triggering the addition of Lys⁴⁸-linked polyubiquitin chains by the E3 ligase βTRCP (β-transducin repeat containing protein) and the subsequent degradation of IκB [24]. The nuclear localization signal of the NF-κB transcription factor is then unmasked
causing it to translocate to the nucleus and trigger NF-κB-dependent transcription.

A number of reports suggest that de-ubiquitinating enzymes can negatively regulate NF-κB activation. The de-ubiquitinating enzyme CYLD was originally identified as a tumour suppressor involved in cylindromatosis [25], but has subsequently been shown to regulate NF-κB activation by removing Lys^48-linked polyubiquitin chains from TRAF-2, TRAF-6 and IκKα [3–5]. This, in conjunction with its induction in response to NF-κB activation, suggested that it acted in a classical feedback loop to terminate signals through this pathway [26]. However, further studies have suggested that its action is not as simple as first thought. In addition to its role in inhibiting NF-κB activation, it also blocks JNK (c-Jun N-terminal kinase) activation in response to TNFR and IL-1/TLR signalling. It has also been proposed that while its regulation of JNK activation is universal, it may play a role in inhibiting NF-κB activation in response to TNFR 

However, it has recently been reported that CYLD^−/− mice showed no defect in NF-κB signalling in bone marrow-derived macrophages. Moreover, a defect in T-cell development was observed suggesting CYLD acts as a positive regulator of signalling through the TCR [28]. A proposed mechanism for this is the removal by CYLD of both Lys^48- and Lys^63-linked polyubiquitin chains from Lck, allowing its active form to interact with its downstream substrate ZAP-70. In addition, it has been shown that CYLD^−/− mice are more susceptible to chemically induced skin tumours and that the increased proliferation in these cells is the result of increased nuclear activity of Bcl-3-associated p50 or p52 and not p50/p65 (NF-κB) [29].

Mutations of CYLD have only been associated with hair follicle and skin appendage tumours [30] and when taken in conjunction with the CYLD^−/− mice, data would suggest that CYLD may have a non-redundant role only in keratinocytes.

The second de-ubiquitinating enzyme implicated in NF-κB signalling is A20, a member of the OTU family. A20 was initially shown to be up-regulated following TNF treatment and mice deficient in A20 developed severe inflammation associated with hypersensitivity to TNF and LPS and exhibited sustained NF-κB activation [31]. Initially, it was shown that A20 acted to block NF-κB activation through the TNFR signalling pathway by removing Lys^63-linked polyubiquitin chains from RIP kinase [6]. In addition, it was discovered that A20 uniquely exhibits both de-ubiquitinating and E3 ligase activity. As well as its OTU domain it also contains a RING finger domain, which can act as a ligase to conjugate Lys^48-linked polyubiquitin chains to RIP upon removal of the Lys^63-linked chains leading to its targeting for proteasomal degradation [6]. The production of A20^−/− and TNF^−/− or A20^−/− and TNFR^−/− double deficient mice still resulted in spontaneous inflammation and suggested that A20 also played a role in TNFR-independent signals [7]. It has since been reported that A20 could de-ubiquitinate TRAF-6 and implicated A20 in the regulation of NF-κB activation through both the TNFR and IL-1/TLR pathways [7]. In addition, the spontaneous inflammation observed upon the removal of A20 would suggest that, unlike CYLD, it may play a more general role in the termination of these pathways.

In addition to A20 and CYLD, two other de-ubiquitinating enzymes have been implicated in the regulation of NF-κB. Cezanne is a de-ubiquitinating enzyme that shows homology to A20 and has been shown to block NF-κB activation, although the exact mechanism underlying this regulation has as yet to be reported [8]. USP31 has been shown to interact with TRAF-2 and p65/RelA, to target Lys^63-linked polyubiquitin chains and to block NF-κB activation in response to a number of stimuli [9]. However, as with Cezanne, its exact mode of action is as yet unclear.

Interestingly, the importance of ubiquitination in the regulation of NF-κB activation has also been shown for pathogens and this regulation can be used to circumvent the immune response. In particular, the Yersinia virulence factor YopJ has been shown to act as a de-ubiquitinase to remove ubiquitin from TRAF-2, TRAF-6 and IκBα, blocking the activation of NF-κB as a mechanism to evade the host immune response [32].

The role of de-ubiquitinating enzymes in the immune system is not just limited to NF-κB activation. USP18, which specifically de-conjugates the ubiquitin-like molecule ISG15, has been demonstrated to negatively regulate type 1 interferon signalling via the JAK (Janus kinase)–STAT (signal transducer and activator of transcription) pathway and play a role in regulating the innate immune response to viruses [33]. However, a recent report would suggest that this regulation is independent of its enzymatic activity and involves its ability to bind IFNAR2 [interferon (α, β and ω) receptor 2] and block the binding and activation of JAK1 [34].

De-ubiquitinating enzymes and small GTPase regulation

Another family of de-ubiquitinating enzymes implicated in immune signalling are the DUB/USP17s. They were originally identified in mice as cytokine-inducible immediate early genes (DUB-1/2) [35] and more recently a number of highly homologous human members have been identified (DUB-3/USP17) and shown to be cytokine inducible [36]. In addition, a number of family members have been implicated in the regulation of both cell proliferation and survival. In particular, DUB-1 and DUB-3 expressions block proliferation [36,37] and DUB-2 has been shown to increase cell survival in Ba/F3 cells upon cytokine withdrawal [38]. This suggests that this family is important for the regulation of both cell proliferation and survival, but until recently the mechanism underlying this regulation was unclear. However, we have now shown that DUB-3 can modulate signalling through the Ras/MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase/ERK pathway by its regulation of the processing of the Ras C-terminal CAAX box (J.A. Johnston and J.F. Burrows, unpublished work). Preliminary results would suggest that DUB-3 can also act to modulate other CAAX
Ubiquitination and DNA repair

Figure 2 | Ubiquitination and DNA repair

FANCD2 is mono-ubiquitinated, causing it to be recruited to nuclear foci for DNA repair. PCNA is mono-ubiquitinated, allowing it to associate with specialized translesion DNA polymerases and bypass DNA damage. USP1 removes mono-ubiquitin from PCNA and FANCD2. Upon DNA damage, USP1 is deactivated by autocleavage, activating DNA repair.

Another protein important for the DNA damage response is p53, which is up-regulated in response to DNA damage, causing cell-cycle arrest. The levels of p53 are controlled by proteasomal degradation via the action of a number of ligases, most importantly Mdm2 (murine double minute 2) [43]. Mdm2 interacts with a homologous protein, MdmX, which is thought to stabilize Mdm2 by blocking its auto-ubiquitination [44]. Upon DNA damage, it has been proposed that MdmX is phosphorylated, targeting it for proteasomal degradation [45]. This destabilizes Mdm2, leading to a rise in the levels of p53. The de-ubiquitinating enzyme HAUSP (herpesvirus-associated USP) (USP7) was originally shown to de-ubiquitinate p53 and as a result it was thought to stabilize this molecule [46]. However, subsequent studies would suggest that HAUSP is a negative regulator of p53 levels and its main function is to de-ubiquitinate Mdm2 and MdmX, stabilizing these molecules and therefore down-regulating p53 levels [47].

**De-ubiquitinating enzymes and DNA repair**

Another area where de-ubiquitinating enzymes have been implicated is in the regulation of DNA damage repair (Figure 2). The FA (Fanconi's anaemia) pathway is involved in the repair of DNA-crosslink damage and takes its name from a rare clinical syndrome characterized by chromosome instability and an increased susceptibility to leukemia and squamous cell carcinomas [39]. Eight proteins have been identified that play a role in this pathway and one of these, FANCD2 (FA complementation group D2), has been shown to be mono-ubiquitinated [40]. Mono-ubiquitination of FANCD2 targets it to nuclear foci where it is thought to associate with other repair proteins and initiate DNA repair. The mutations associated with FA in many cases lead to the loss of FANCD2 mono-ubiquitination and it is thought that this plays a vital role in the pathway [41]. Recently, it has been shown that knockdown of USP1 leads to increased mono-ubiquitination of FANCD2 and increased resistance to DNA-cross-linking agents. This study also indicated that USP1 can remove the mono-ubiquitin from FANCD2 and inhibit the initiation of DNA repair through this pathway [10].

The sliding clamp PCNA (proliferating-cell nuclear antigen) assembles into a trimeric ring which encircles DNA and can recruit the replication machinery as well as specialized TLS (translesion DNA synthesis) polymerases that can bypass damaged template DNA during replication. Following DNA damage the latter are recruited to mono-ubiquitinated PCNA [42]. In the absence of DNA damage, USP1 removes the mono-ubiquitin from PCNA, but following DNA damage USP1 undergoes autocleavage allowing the accumulation of mono-ubiquitinated PCNA [11]. This mechanism may allow the initiation of both this and the FA pathway, thereby acting as a molecular switch for multiple DNA repair pathways.

**De-ubiquitinating enzymes and disease**

There is clear evidence that de-ubiquitinating enzymes play a role in the initiation and progression of a number of diseases. In particular, several de-ubiquitinating enzymes have been implicated in cancer. CYLD was originally identified as the tumour suppressor gene mutated in the rare FC (familial cylindromatosis) syndrome which presents as tumours arising from hair follicles. It has subsequently been shown to be mutated in BSS (Brooke–Spiegler syndrome) and MFT (multiple familial trichoepithelioma) both of which present as tumours of skin appendages and it has now been suggested that FC, MFT and BSS may represent phenotypic variants of a single entity [30].

HAUSP is involved in the regulation of the important tumour suppressor p53, but the only evidence as yet to implicate it in cancer is a recent study indicating that in non-small cell lung cancer HAUSP expression is down-regulated in many tumours [48]. However, its ability to negatively regulate p53 levels has led to it being suggested as a potential therapeutic target.

Unp (USP4) has been shown to be overexpressed in both lung cancers and adrenocortical carcinomas [49,50]. Although the substrate of Unp is unknown, it has been shown to have the ability to transform NIH 3T3 cells in a nude mouse assay [51] and to interact with the retinoblastoma protein [52], an important tumour suppressor gene, suggesting it may well play a role in cancer.

USP6 (Tre-2 or Tre17) was originally proposed as a potential oncogene due to its ability to transform NIH 3T3 cells [53]. Subsequently, it has been shown that chromosomal translocations in cases of aneurysmal bone cysts result in overexpression of this protein in these tumours [54]. This combined with additional evidence suggesting overexpression in Ewings sarcoma and osteoblastomas would suggest that this protein plays a role in the formation of these tumours.
In addition to their roles in cancer, de-ubiquitinating enzymes have also been implicated in neurodegenerative diseases. Mutations of UCH-L1 have been identified in one family with a history of PD (Parkinson’s disease) [55] and a particular polymorphism in UCH-L1 has been associated with decreased susceptibility to PD [56]. 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 [57]. As a result, the role of UCH-L1 in PD is unclear.

Conclusions
The growing importance of ubiquitin in cellular regulation has focused much attention on the molecules that regulate its conjugation and de-conjugation. As a result we have significantly expanded the known pool of de-ubiquitinating enzymes. Currently, there are approx. 90 known de-ubiquitinating enzymes [12] and further studies may as yet expand this number.

It is obvious that these enzymes potentially represent an important class of regulatory proteins which play roles in a wide range of cellular processes from activation of the immune system to the initiation of DNA repair. However, less than a third of the known de-ubiquitinating enzymes have had specific substrates identified, suggesting that much work will be required to understand their importance.

References


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