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BALANCING ACT: DEUBIQUITINATING ENZYMES IN THE NERVOUS SYSTEM

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Abstract

Many pathways important to the nervous system are regulated by the post-translational conjugation of ubiquitin to target proteins. The reversal of ubiquitination, or deubiquitination, is equally critical to neuronal function. By countering protein ubiquitination, deubiquitinating enzymes (DUBs) help control neuronal fate determination, axonal pathfinding, and synaptic communication and plasticity. The significance of DUBs to the nervous system is underscored by links to various neurological diseases. Due to cell type or substrate specificity, certain DUBs may also represent therapeutic targets for neurodegeneration. Here, we review recent findings which have shaped our current understanding of emerging functions for DUB in the nervous system.

Ubiquitin and the Nervous System

The development and function of the nervous system depends on cellular processes ranging from DNA transcription to protein degradation and on conserved signaling pathways between and within cells. These diverse processes and pathways rely on key regulatory elements that dictate the “when” and the “how” of neuronal development, function and disease. One such element is the post-translational conjugation of ubiquitin (Ub; Glossary) to proteins. An evolutionarily conserved 8.5 kDa protein expressed in all eukaryotic cells, Ub modulates the function of target proteins to which it is conjugated.

Ub first captured the interest of neuroscientists due to its presence in proteinaceous accumulations in various neurodegenerative diseases including, neurofibrillary tangles in Alzheimer's Disease (AD), Lewy Bodies in Parkinson's Disease (PD), and intranuclear inclusions in hereditary polyglutamine disorders [1-5]. Conjugation of Ub to proteins, or ubiquitination, occurs through the action of three classes of enzymes: E1 (Ub Activating Enzyme), E2 (Ub Conjugating Enzyme) and E3 (Ub Ligase; see **Box 1**). Because Ub molecules can be attached to other Ubs through any one of 7 lysines to form chains, a target protein can be modified by a single Ub (mono-ubiquitination) or by a poly-Ub chain. Depending on the type of ubiquitination (i.e. mono-or poly-Ub, and/or the type of Ub-Ub linkages), the conjugated protein is subjected to different fates (**Box 1**). Ub-Ub linkages through lysine 48 (“K48”) generally target proteins for degradation, while Ub-Ub linkages

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through K63 are typically involved in non-degradative pathways, including protein sorting and DNA repair. Mono-ubiquitination of a protein is also usually associated with non-degradative pathways, such as changes in subcellular localization.

Like many other post-translational modifications, ubiquitination is reversible. Indeed, the process of deubiquitination is central for normal cellular functions. Deubiquitination is accomplished by deubiquitinating enzymes (DUBs; **Figures 1 and 2**). Some of the earliest work on DUBs was performed in the nervous system, including studies of a form of learning in Aplysia [6, 7] and of neuronal development in *Drosophila melanogaster* [8, 9]. The importance of specific DUBs to the nervous system was underscored by mutations in DUBs that cause neurological disorders in mice [10, 11] and humans [12]. Recently, the approach of targeting specific DUBs has emerged as a potential therapeutic route for some neurodegenerative diseases [13]. Many general reviews describe DUBs (for example [14-17]). Here, we focus solely on DUBs in the nervous system because of emerging evidence for their important functional roles in both the central and peripheral nervous system.

Deubiquitinating Enzymes and the Nervous System

The human genome encodes ~95 potential DUBs [18], categorized into five classes based on homologies within the catalytic domains (**Figure 1**). Many DUBs contain additional non-catalytic domains that mediate binding to poly-Ub chains with specific linkages, to specific substrates, or to functional protein partners [14]. By performing one or more of the proteolytic functions summarized in **Figure 2**, DUBs modulate various cellular pathways including cell growth [e.g. BRCA1 associated protein-1 (BAP1)], apoptosis [e.g. Ubiquitin Specific Protease 7 (USP7], endocytosis and endosomal trafficking ([e.g. Associated Molecule with the Src Homology 3 domain of STAM (AMSH) and USP8], and proteostasis (e.g. USP14) (**Figure 2)** [14, 16-19]. Considerable information has been collected on the enzymatic properties of some DUBs, including several implicated in the nervous system (reviewed in [16, 17]). For most DUBs, however, defined substrates and precise physiological functions remain unknown.

Since the 1980s evidence has increasingly established the importance of Ub-dependent pathways in neurons [20]. Recently it has become clear that DUBs, as central players in the regulation of protein ubiquitination, are involved in crucial nervous system functions. **Table 1** lists DUBs documented in the nervous system, though presumably many others are expressed in brain, spinal cord, and peripheral nerves. The following sections highlight DUBs for which evidence supports their importance in synapse development and function, neuronal pathfinding, and in neurological diseases.

UCHL1: Important for Synaptic Structure and Function

Ub availability to target proteins for degradation, or to other cellular pathways, is necessary for nervous system homeostasis and function. One DUB involved in the stabilization of mono-Ub is Ubiquitin Carboxyl-terminal hydrolase L1 (UCHL1), an abundant neuronal protein [21-23]. UCHL1 is known to interact strongly with and stabilize mono-Ub from degradation [22]. Mice lacking UCHL1 have reduced brain mono-Ub [22] and mice overexpressing UCHL1 have increased mono-Ub levels [22]. No clear substrates have been described for UCHL1 *in vivo*, but UCHL1 may deubiquitinate α-synuclein ([24], see below). UCHL1 also cleaves Ub from small adducts and from *Ubiquitin* gene products *in vitro* [21], presumably increasing mono-Ub availability.

An Aplysia UCHL1 ortholog (AP-Uch) was the first DUB linked to synaptic function [6]. Activity-based changes in synaptic plasticity, including Long-Term Potentiation or

Facilitation (LTP or LTF), are thought to be a molecular basis for learning and memory. Expression of the Aplysia UCHL1 ortholog, Ubiquitin carboxyl-terminal hydrolase (AP-Uch), increases in sensory neurons after LTF *in vivo*, and blockage of AP-Uch induction or function inhibits LTF [6, 7]. The DUB activity of Ap-Uch may serve to increase the steady-state levels of available Ub in neurons, presumably by recycling Ub during proteasomal degradation of proteins involved in LTF.

Mammalian UCHL1 also functions at the synapse. Investigations at the Neuromuscular Junction (NMJ) in wild type and *Uchl1* knockout mice indicate that UCHL1 is necessary for NMJ structure and function. *Uchl1* knockout mice, which develop normally, show spasticity and paralysis before dying prematurely [25]. Electrophysiological and structural examinations of the NMJ indicate that loss of UCHL1 reduces the number of neurotransmitter vesicles released by the neuron (quantal content) [25], which could result from reduced mono-Ub availability. As a result, synaptic plasticity is hindered and nerve terminals retract at some NMJs [25]. The hypothesis that UCHL1 maintains synaptic mono-Ub levels is supported by studies in hippocampal neurons: increased activity of UCHL1 leads to higher levels of mono-Ub, while pharmacological inhibition of UCHL1 has the opposite effect and alters synaptic structure [23]. While these studies revealed a critical role for UCHL1 at the synapse, they have not described fully the molecular functions of UCHL1 at the NMJ. One possibility is that reduced quantal content stems from decreased Ub recycling at the synapse, which would hinder Ub-dependent pathways.

The importance of UCHL1 to neuronal health is demonstrated by an in-frame deletion in the *Uchl1* gene, which causes late onset, progressive ataxia in gracile axonal dystrophy (*gad*) mice [11]. *gad* mice show a “dying-back” type of axonal degeneration similar to *Uchl1* knockout mice [25] but, unlike in many neurodegenerative diseases, neuronal perikarya do not degenerate in *gad* or in *Uchl1* knockout mice [25, 26]. *gad* and *Uchl1* knockout mice develop normally, suggesting that UCHL1 is important for neuronal maintenance but not development.

Possible links between UCHL1 and other neurodegenerative diseases have also been suggested. Soluble UCHL1 protein levels are reduced in the hippocampus of a transgenic mouse model of AD ($_{APP/PS}$1), which exhibits contextual learning deficits and impaired hippocampal LTP[27]. Soluble UCHL1 levels are also lower in post-mortem AD brains [26]. Such reductions are possibly the result of sequestration of UCHL1 in neurofibrillary tangles [26]. Overexpression of UCHL1 in transgenic AD mice alleviates cognitive defects and restores synaptic plasticity in a manner dependent on the catalytic activity of UCHL1 [27]. These and other findings reinforce the notion that UCHL1 has important functional roles at central synapses, including being involved in cognitive processes.

A missense mutation in UCHL1 (UCHL1^{I93M}) was reported in 1998 as the cause of dominantly inherited PD in one family [28], though no subsequent instances of this mutation have been reported [26, 29]. UCHL1^{I93M} has reduced DUB activity *in vitro* [28], thus it was initially hypothesized that partial loss of UCHL1 function may result in PD. However *gad* mice, which lack UCHL1 protein, do not develop neurodegenerative hallmarks of PD. To investigate whether UCHL1^{I93M} is indeed pathogenic, transgenic mice expressing *Uchl1*^{I93M} were generated [30]. *Uchl1*^{I93M} mice show loss of nigral dopaminergic neurons, a hallmark of PD, and develop Ub- and UCHL1-positive inclusions, though not Lewy Bodies (the histopathological hallmark of PD) [30]. These data support the hypothesis that UCHL1^{I93M} causes neurodegeneration, perhaps through a gain-of-function mechanism. Another missense mutation (UCHL1^{S18Y}) was reported in one study as being protective against PD [29], however, an inverse relationship between PD and UCHL1^{S18Y} does not exist in all populations [26, 29]. Thus, the significance of this polymorphism is currently

questioned. Finally, a recent *in vitro* study has linked UCHL1 function to PD: the DUB activity of a farnesylated, membrane-bound form of UCHL1 was found to rescue α-synuclein, a protein whose accumulation is associated with neurotoxicity and the development of PD, from lysosomal degradation [24]. If this finding is confirmed *in vivo*, it would suggest UCHL1 as a potential therapeutic target for α-synuclein-related PD. Severing UCHL1 from the membrane might increase degradation of α-synuclein, effectively reducing levels of this aggregation-prone protein and alleviating neuronal stress.

In summary, UCHL1 is important for synaptic stability and general neuronal health, and its dysfunction has been implicated in certain neurodegenerative diseases. UCHL1 may function primarily by maintaining available pools of mono-Ub in order for sufficient levels to be available for use in Ub-dependent pathways linked to synaptic integrity. The fact that USP14, another Ub recycler (see below), also acts at the synapse supports the notion that readily available mono-Ub is necessary for normal synaptic function. However, unlike USP14, which plays a developmental role, UCHL1 seems necessary for synaptic plasticity-related events in the adult rather than during development. Further studies of UCHL1 are required to understand the details of its molecular roles and its relevance in neurological diseases.

USP14: Recycling Ub at Synaptic Proteasomes

USP14 is one of three DUBs associated with the 19S proteasome, the other two being UCHL5 (also known as Uch37) and Proteasome 26S Subunit, non-ATPase, 14 (PSMD14, also known as POH1) [19]. A major function of 19S-associated DUBs is Ub recycling at the proteasome (**Figure 2**). The DUB activity of USP14 is markedly enhanced when bound to the proteasome [19, 31], implying that it functions primarily at the proteasome. A mutation in *Usp14* underlies the phenotype in the *ataxia* (*ax*^{J}) mouse [10, 32]. Homozygous *ax*^{J} mice suffer from early onset, progressive ataxia and tremor, reduced body and brain mass, paralysis and early death [31-33]. An insertion into intron 5 of *Usp14* leads to 90% reduction of USP14 protein in the brain of homozygous *ax*^{J} mice [10, 31, 32]. *ax*^{J} mice do not show neuronal loss [10], but have reduced mono-Ub levels, particularly in synaptosomal fractions [32]. Reduced Ub recycling at synaptic proteasomes in *ax*^{J} mice is likely to be the major underlying cause of the resulting phenotype.

USP14 is important for NMJ development. Recordings at the NMJ of *ax*^{J} mice reveal defective release of the neurotransmitter acetylcholine (ACh) [10], and loss of USP14 leads to developmental anomalies at the pre- and postsynaptic terminals of the NMJ [34]. The NMJs of *ax*^{J} mice are swollen and poorly arborized, with aberrant nerve terminals and an immature morphology of nicotinic ACh receptor clusters. These changes correlate with loss of mono-Ub in synaptosomal fractions and are linked to ataxia in *ax*^{J} mice [34]. Importantly, NMJ deficits in *ax*^{J} mice are due to USP14 loss at the neuronal, not the muscular, side of the synaptic junction [34].

USP14 is also important for central synaptic function [35]. In *ax*^{J} mice, cerebellar Purkinje cells have increased cell surface expression of $GABA_A$ receptors ($GABA_A$Rs), including at extrasynaptic sites, with a concomitant increase in inhibitory GABAergic currents, that effectively reduces cerebellar output [35]. $GABA_A$Rs are known to be ubiquitinated in neurons [36], and USP14, which directly interacts with $GABA_A$Rs, may deubiquitinate it [35]. Generally, mono-ubiquitination/deubiquitination controls the recycling of various receptors from the cell membrane [20], including $GABA_A$Rs [36]. Consequently, USP14 deficiency could perturb the turnover and cell surface distribution of important synaptic receptors, including $GABA_A$Rs.

Although an important functional role for USP14 in maintaining adequate monomeric Ub levels at the developing synapse has emerged, recent work also indicates that pharmacologically inhibiting USP14 can enhance proteasome function and the clearance of the neurodegenerative disease-linked proteins tau and ataxin-3 [13]. Thus, USP14 likely serves additional functions beyond maintenance of mono-Ub levels, including the proteasomal turnover of specific substrates.

Fat Facets and USP9X: Cell Differentiation and Synaptic Function

The first DUB linked to cell differentiation in the nervous system was the *Drosophila* USP class DUB, Fat Facets (Faf). Faf prevents over-neuralization of the developing eye. Flies genetically null for Faf, or expressing catalytically inactive Faf, have supernumerary photoreceptors due to aberrant differentiation of cells that normally acquire non-neural fates [8, 37]. Genetic manipulations have determined that Faf interacts with Liquid facets (Lqf), an orthologue of the mammalian epsins that are implicated in endocytosis [38, 39]. Decreasing Lqf enhances the phenotype of Faf mutants, while increasing Lqf renders Faf unnecessary [38]. Ubiquitination of Lqf is stabilized in Faf-less eyes, and Lqf co-immunoprecipitates with Faf from fly embryos [39]. These findings led to the conclusion that Faf directly controls the ubiquitination status of Lqf.

Deubiquitination of Lqf by Faf regulates Notch-Delta signaling in the developing eye [40]. Notch-Delta signaling is a highly conserved pathway specifying cell fate, and internalization of the Notch ligand, Delta, is important for Notch signaling. As an epsin, Lqf helps mediate Delta internalization; Lqf mutants fail to internalize Delta normally [41]. A model has emerged in which deubiquitination of Lqf by Faf promotes Delta internalization and Notch signaling, promoting non-neural fates for certain cells [38, 40]. In the absence of Faf, Notch-Delta signaling diminishes and cells that normally adopt a non-neural fate become neurons instead. While this pathway is well defined genetically, some molecular details remain unclear: Does Lqf deubiquitination by Faf rescue Lqf from degradation? Consistent with this possibility, Lqf protein levels are lower in Faf-null mutants [39]. In the absence of Faf, persistently ubiquitinated Lqf might be degraded, thereby reducing Delta internalization, impairing Notch signaling and promoting neuralization. Alternatively, Faf may alter Lqf activity. The ability of human epsins to interact with partners at the synapse is presumably regulated by mono-ubiquitination [20]. Consequently, Faf-dependent deubiquitination of Lqf may also control its activity by affecting its interactions.

Faf also plays a role at the *Drosophila* NMJ. Neuronal Faf overexpression leads to more synaptic boutons, increased synaptic span, and elaborate branching [9]. Although the NMJ is expanded by Faf overexpression, the postsynaptic response is reduced as a result of defective neurotransmitter release [9]. The E3 ligase Highwire interacts genetically with Faf: Highwire loss-of-function phenocopies Faf overexpression [9]. This E3-DUB genetic interaction suggests a delicate balance between ubiquitination and deubiquitination at the synapse. Highwire-dependent ubiquitination may regulate synaptic molecules and neurotransmitter release, and be counteracted by Faf and possibly other DUBs. The morphological effects of Faf overexpression at the NMJ require its substrate, Lqf [42], but the molecular details of this interplay are unclear.

The mammalian orthologue of Faf is USP9X. USP9X interacts with the Lqf orthologue, epsin-1, and co-localizes with it at the synapse [43]. A fraction of epsin-1 is mono-ubiquitinated. RNAi-mediated knockdown USP9X stabilizes ubiquitinated epsin-1 in cultured epithelial cells [43], suggesting an evolutionarily conserved role for USP9X in deubiquitinating epsin-1 and regulating its function or stability. Finally, the expression pattern of USP9X is altered in a toxin (MPTP)-induced mouse model of PD [44], but

whether USP9X contributes to PD pathogenesis is uncertain. More work is needed to understand the roles, substrates and activities of USP9X in the nervous system.

USP33: Important for Neuronal Pathfinding

Regulated axonal growth and pathfinding are essential to normal nervous system development. USP33 was recently shown to be critical for axonal pathfinding in mouse and chick embryos [45]. USP33 interacts with the axonal guidance receptor Roundabout (Robo) 1 [45], whose ligand is Slit. Slit proteins are secreted guidance cues important for commissural neuron pathfinding and midline crossing. The Robo1-Slit interaction helps ensure that axons do not re-cross the midline after crossing it once.

In vitro and *in ovo* experiments indicate a role for USP33 in commissural axon pathfinding. Knockdown of endogenous USP33 hinders the response of axonal growth cones to Slit, resulting in impaired midline crossing [45] similar to that seen in Slit knockout and Robo1 mutant mice [46]. In RNAi-knockdown studies, crossing defects are rescued by co-expression of RNAi-resistant USP33, provided it retains catalytic activity [45]. Cell-based assays support a model in which USP33 reverses Robo1 ubiquitination: knockdown of USP33 increases levels of ubiquitinated Robo1, while USP33 overexpression reduces ubiquitinated Robo1 levels [45]. USP33 likely controls Robo1 stability and/or availability upon Slit stimulation. Though direct deubiquitination of Robo1 by USP33 has not been reported, it is reasonable to speculate that USP33 deubiquitinates and thus rescues Robo1 from degradation during the Slit response. Alternatively, Robo1 deubiquitination may favor its localization to the cell membrane, reminiscent of the role USP33 plays in 7-transmembrane receptor signaling and trafficking [47]. Either possibility would increase the axon-guidance response to Slit. Future work may support a model wherein once axons cross the midline, USP33-mediated deubiquitination of Robo1 ensures that sufficient Robo1 is present at the cell membrane to interact with Slit, preventing re-crossing of the midline by developing axons.

Ataxin-3: a DUB with Protective and Toxic Properties

Ataxin-3, a DUB from the class of the Machado Joseph Disease (MJD) Proteases , first received attention as the disease protein mutated in the neurodegenerative disorder Spinocerebellar Ataxia Type 3 (SCA3, also known as MJD [48, 49]). SCA3 is caused by a polyglutamine-encoding CAG repeat expansion in the *ATXN3* gene. SCA3 is one of at least nine so-called polyglutamine neurodegenerative diseases, which also include Huntington's disease (HD) and at least five other SCAs [48, 49]. SCA3 is a progressive ataxia accompanied by difficulties in speech and swallowing, impaired eye movements, neuropathy and sometimes dystonia or parkinsonism. Degeneration can be widespread, but is usually most pronounced in the cerebellum, brainstem, substantia nigra, and globus pallidus interna [48, 49].

Ataxin-3 is a highly specialized DUB with ubiquitin interacting motifs (UIMs) flanking the polyglutamine domain (**Box 2**). *In vitro*, ataxin-3 binds K48- and K63-linked Ub chains at least four Ub long through its UIMs, preferentially cleaves chains longer than four Ub, and cleaves K63-linkages better than K48-ones [50, 51]. These properties suggest a Ub chain editing/proofreading function for ataxin-3, whereby ataxin-3 helps to determine polyUb chain length and type of Ub-Ub linkage on a substrate rather than fully deubiquitinate it. Ataxin-3 has been implicated in several pathways of protein quality control. Through its interaction with the proteasomal shuttle protein Valosin Containing Protein (VCP)/p97, ataxin-3 assists with proteasomal targeting of ER-Associated Degradation (ERAD) substrates, most likely by editing Ub chains on substrates [52, 53]. Ataxin-3 may regulate the Ub status of many proteins because over-expression of ataxin-3 in cultured, non-

neuronal cells reduces levels of highly ubiquitinated species [51]. Supporting this notion, brain lysates of *Atxn3* knockout mice show increased levels of ubiquitinated proteins [54].

Additional evidence for ataxin-3's role in protein quality control came from studies in *Drosophila* [55]. Exogenous expression of human wild-type ataxin-3 in Drosophila suppressed polyglutamine-related neurodegeneration in vivo, in a manner that was dependent on its Ub-binding and chain cleaving properties [55]. Temperature-sensitive mutants of proteasome subunits impair this rescue [55], implying that ataxin-3-mediated neuroprotection relies on proteasome activity. The exact mechanism by which ataxin-3 is neuroprotective in the fly remains unknown, and a neuroprotective role for ataxin-3 has not yet been established in mammals. While mice lacking ataxin-3 appear normal [54], ataxin-3 may be particularly important during proteotoxic stress [56], as occurs in polyglutamine diseases. Consistent with this view, cultured embryonic fibroblasts derived from *Atxn3* knockout mice poorly tolerate heat stress [56]. Finally, the *C. elegans* orthologue of ataxin-3, ATX3, is implicated in insulin-like growth factor 1 (IGF-1)-dependent aging: worms deficient in both ATX3 and VCP/p97 live longer than their wild-type counterparts [57]. Through its deubiquitinating activity, ATX3 likely regulates the ubiquitination status and proteasomal turnover of IGF-1 signaling components. However, it is unclear how ATX3 roles in IGF-1 signaling are tied to the *C. elegans* nervous system.

A key function of ataxin-3 is probably to triage proteins for degradation: 1) Ataxin-3 interacts with several protein quality control E3 ubiquitin ligases, including Carboxyl-terminus of HSC70-Interacting Protein (CHIP) [58], Ubiquitination factor E4B (E4B/Ufd2) [59] and parkin [60]; 2) Ataxin-3 interacts with the proteasome and the proteasome shuttle proteins VCP/p97 and Human Homologue of Rad23 (hHR23)A [53, 59, 61] and 3) Ataxin-3 preferentially binds Ub chains at least four Ub long and readily cleaves longer chains, but is inefficient at cleaving shorter, K48-linked chains [51]. Thus, ataxin-3 may assist E3 ligases in forming the desired type of Ub chain on a substrate: through the proofreading functions of ataxin-3, the Ub chain would tend to be K48-linked and no longer than four Ub, making it a good proteasomal target [62]. Since ataxin-3 interacts with the proteasome and proteasomal shuttle proteins, it probably also facilitates delivery of ubiquitinated substrates to the proteasome (**Box 2**).

The mechanisms driving polyglutamine-mediated neurodegeneration in SCA3 are uncertain. *In vitro*, the Ub binding and cleaving capabilities of ataxin-3 appear not to be affected by polyglutamine expansion [50, 51, 63]. In cultured non-neuronal cells, however, polyglutamine-expanded ataxin-3 is less efficient at reducing levels of ubiquitinated species [51], suggesting that some of its functions are likely to be affected by aberrant polyglutamine expansion. Supporting the notion that polyglutamine expansions alter ataxin-3 function, it was recently reported that pathogenic ataxin-3 targets the E3 ligase parkin for autophagic degradation, unlike wild-type ataxin-3, which rescues it through deubiquitination [60]. As is the case for several other polyglutamine proteins [e.g. 64], polyglutamine expansion in ataxin-3 may lead to both a partial loss-of- and a toxic gain-of-function.

DUBs as Therapeutic Targets

The ubiquitin-proteasome system (UPS) has long been an attractive therapeutic target, due to its important role in protein quality control and the regulation of other cellular pathways. Currently, the proteasome inhibitor Bortezomib (Velcade®) is used to treat cancers including multiple myeloma and mantle cell lymphoma [65]. However, proteasome inhibition has undesirable side effects, due to the fact that its indiscriminate inhibition affects all cellular processes that normally rely on ubiquitin-dependent protein degradation.

DUBs offer significant advantages as potential therapeutic targets within the ubiquitin enzymatic pathway, due to their cell-type and substrate-specificity. Indeed, DUBs have recently emerged as attractive targets for the design of novel therapeutics to treat various malignancies [66] and neurodegenerative diseases [13]. Structural studies have revealed differences in the catalytic pockets of DUBs, some of which appear inoperative unless bound to substrate [16, 66, 67], raising the possibility of DUB-specific inhibitors. In fact, high-throughput screens have recently identified a small molecule inhibitor for USP7, a DUB linked to p53 stability [66]. This cyanoindenopyrazine derivative inhibits USP7 catalytic activity and stabilizes p53 in vitro, leading to apoptosis [68]. The efficacy of this molecule *in vivo* has not yet been reported.

With respect to DUBs important in the nervous system, a screen was recently conducted to identify USP14 inhibitors [13]. As discussed above, USP14 functions in synaptic integrity by recycling mono-Ub at the proteasome. Recently, however, USP14 was also shown to rescue proteins targeted for degradation by deubiquitinating them at the proteasome [13]. Mouse embryonic fibroblasts lacking USP14 show enhanced clearance of several disease-related proteins, including tau and polyglutamine-expanded ataxin-3, and overexpression of catalytically inactive USP14 increases protein degradation [13]. The screen for modulators of USP14 isolated several specific inhibitors [13], including 1-[1-(4-fluorophenyl)-2,5-dimethylpropyl-3-yl]-2-pyrrolidin-1-ylethanone (IUI). Treatment with IUI enhances the clearance of several disease-causing proteins and improves cellular resistance to oxidative stress [13]. By inhibiting the catalytic activity of USP14, IUI presumably prevents USP14 from rescuing some ubiquitinated proteins brought in close proximity to the proteasome. It remains to be determined if IUI can prevent neurodegeneration. Treatment of adult mice that express toxic proteins, such as polyglutamine-expanded ataxin-3, with IUI may increase turnover of the toxic species and promote survival. However, full or near-complete pharmacological inhibition of USP14 could be detrimental to neuronal function, due to its importance in synaptic development and function.

While the search for DUB-centered therapies is still in its infancy, structural studies and enzymatic assays together should accelerate rational drug design. Indeed, structure-based assays conducted with UCHL1 have identified potential activators and inhibitors [67, 69], but their efficacy in cells has not yet been reported.

Concluding Remarks

The development and integrity of the nervous system depend on the close regulation of numerous Ub-dependent pathways by specific DUBs (**Table 1**). Balanced ubiquitination/deubiquitination and continued availability of mono-Ub are important for synapse structure and function. Specific DUBs that are currently known to play a role at the synapse include UCHL1, USP14, and Faf/USP9X. Besides ensuring that mono-Ub is available at the synapse, DUBs likely control the turnover of specific proteins involved in synaptic function and/or plasticity. For example, USP14 probably rescues some synaptic proteins from proteasomal degradation, while Faf probably deubiquitinates proteins involved in neurotransmitter release at the NMJ. Inter-neuronal signaling during development also requires controlled protein ubiquitination/deubiquitination, as exemplified by the role of USP33 in axonal pathfinding and that of Faf in cell differentiation. Finally, as long-lived post-mitotic cells, neurons cannot dilute toxic proteins by disseminating them to daughter cells via mitosis, thus, Ub-dependent protein quality control may be especially vital to neuronal homeostasis. Consequently, mutations in DUBs that maintain mono-Ub availability (eg. UCHL1, USP14), or that participate directly in protein quality control (eg. ataxin-3) can cause neuronal dysfunction and neurodegeneration.

DUBs operate in the crowded cellular milieu where interactions with other proteins surely modulate activity. According to a recent proteomic analysis of human DUBs and their associated protein complexes [70], DUBs can have a myriad of partners that regulate DUB location, activity, and specificity. Many DUBs interact with E3 ligases [reviewed in 14 and, 15; 70], and not only to counter E3-mediated ubiquitination of substrates. Some DUBs, like ataxin-3, may edit Ub chain linkage and length during Ub chain formation, working with E3 ligases to target proteins to the correct cellular pathway. As Ub chain editors, certain DUBs could regulate diverse cellular pathways depending on the macromolecular complexes in which they reside and on their subcellular localization.

Many questions about DUB functions in the nervous system remain unanswered (Box 3). For example, future research needs to investigate whether DUB localization, enzymatic activity, substrate specificity and interactions in neurons are activity-dependent. In addition, some DUBs might be critical for excitatory synapses, while others function selectively at inhibitory synapses. Since many DUBs possess multiple protein-protein interaction domains, perhaps the same set of DUBs support different synapses (or neurons), but rely on separate protein partners to modify their activity and specificity. Finally, most work conducted on DUBs in the nervous system has neglected a critical cellular component, glial cells. Future research may discover glial-enriched or glial-specific DUBs that support neuronal function indirectly by assisting with glial function, homeostasis, and gliotransmission. To date, few DUBs have been investigated in detail in the nervous system, but future studies are likely to reveal critically important functions for many DUBs in both neurons and glia.

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Glossary

Ataxia	Gross lack of coordination. A neurological symptom that usually indicates a dysfunction of neural pathways involved in motor coordination.
Deubiquitinating enzymes (DUBs)	A family of cysteine and metallo-proteases that cleave chemical bonds between ubiquitin molecules or between ubiquitin and another protein.
K48-linked ubiquitin chains	Ubiquitin-ubiquitin linkages that generally target substrate proteins for degradation by the proteasome.
K63-linked ubiquitin chains	Ubiquitin-ubiquitin linkages that generally target proteins non-degradative pathways.
Mono-Ubiquitination	Ubiquitin conjugation that involves a single moiety of ubiquitin. This type of ubiquitination can alter the sub-cellular localization of a protein and regulate cellular processes such as receptor trafficking and gene transcription.
Poly-ubiquitination	Ubiquitin conjugation that involves chains of >1 ubiquitin moiety. A variety of different types of ubiquitin chains are known (eg. K6, K7, K11, K27, K29, K33, K48, and K63-linked ubiquitin chains).

Proteasome	A large molecular complex found in all eukaryotic cells, the proteasome selectively degrades poly-ubiquitinated proteins. It consists of the 20S particle, which proteolyses proteins, and one or two regulatory 19S particles .
Ubiquitin	A small regulatory protein that alters the interaction properties of proteins to which it is conjugated.

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Box 1: A Primer on Ubiquitination

During the first step of Ub conjugation, ATP is expended as the catalytic cysteine of E1 (Ub activating enzyme) forms a thiol-ester intermediate with the terminal glycine of Ub (G76; see Figure IA and B). Next, Ub is transferred as a thiol-ester bond to the catalytic cysteine of an E2 (Ub conjugating enzyme). Finally, in the presence of an E3 (Ub ligase) Ub is typically transferred to the lysine residue of a target protein via an isopeptide bond, though it can be conjugated to other amino acids [71, 72]. Substrate specificity is determined by E2s and E3s.

Proteins can be mono-ubiquitinated or poly-ubiquitinated. The fate of a ubiquitinated protein depends on the type of ubiquitination: mono-ubiquitination of a membrane protein, such as the AMPA receptor, can lead to its internalization. Protein ubiquitination is reversed by DUBs, which can completely deubiquitinate a protein or edit the extent of ubiquitination (see Figure IA and **Figure 3**).

Ub is an 8.5-kDa polypeptide with seven lysines that enable isopeptide bond formation between different Ub moieties. Seven types of Ub-Ub isopeptide linkages can be formed (Figure IB), all of which are found in cells [73-76]. Different chains adopt different conformations: K63 linkages are more linear while K48 and K11 linkages are more compact [77-80]. The differing morphology of specific Ub-Ub linkages helps determine their recognition by Ub-binding domains, targeting them to different pathways. For example, K48-linked poly-Ub chains generally target proteins for proteasomal degradation, whereas K27 linkages are associated with autophagy, and K63-linked Ub chains are linked to several processes, including autophagy, DNA repair and endosomal sorting.

Proteins conjugated to a chain of four or more K48-linked Ub are recognized by the 26S proteasome [62]. The 26S proteasome consists of a barrel-shaped macromolecule comprising four stacked rings of seven subunits each (the 20S component), capped on one or both sides by the 19S complex, which contains proteins that participate in Ub recognition, deubiquitination, and protein unfolding (Figure IC). Ub attached to substrates is rescued from degradation by the action of three 19S-associated DUBs: UCHL5, USP14 and PSMD14 [19]. The target protein is unfolded, fed into the 20S component and degraded in an ATP-dependent manner.

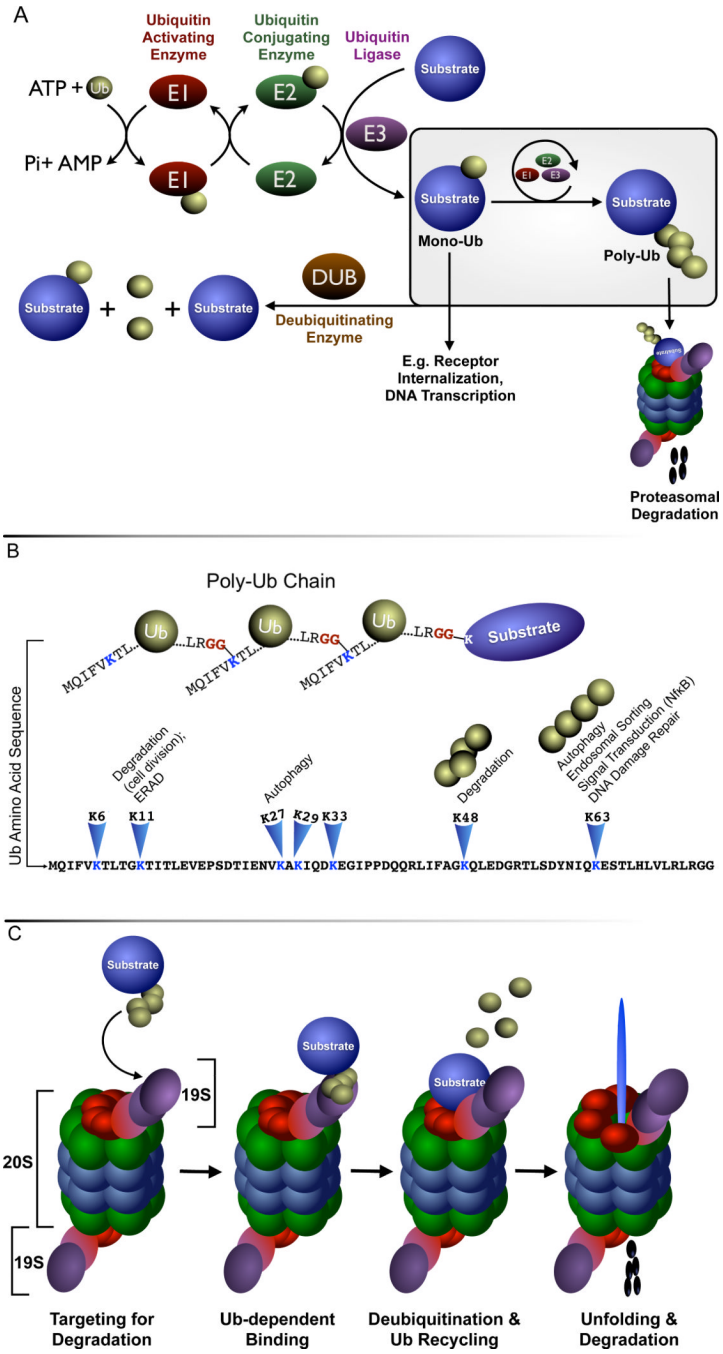
Box 2. Ataxin-3: structural and functional domains

Ataxin-3 offers a good example of how complementary biochemical, cell and animal studies lead toward a comprehensive understanding of enzymatic properties and cellular functions of a DUB. Ataxin-3 consists of an N-terminal, structured catalytic domain and a C-terminal portion that is flexible and mostly intrinsically unstructured. The catalytic domain comprises the catalytic site and two Ub-binding sites. The C-terminal portion contains Ub-Interacting motifs (UIMs) flanking a polyglutamine tract. Structural and biochemical studies of ataxin-3 have determined: 1) The structure of the catalytic domain (gray ribbon structure with catalytic residues depicted in red; [81, 82]). 2) Details of ataxin-3's interactions with mono-Ub and poly-Ub [51, 83-85]; 3) The catalytic preference of ataxin-3 to cleave poly-Ub chains longer than four Ub and K63-linkages over K48-linkages [51]; and 4) That ataxin-3 interacts with the proteasomal shuttle proteins, HHR23A and VCP/p97 [53, 59, 61].

According to structural data, the catalytic pocket of ataxin-3 is well defined and the catalytic residues are aligned in a productive conformation [81]. Ub-binding sites 1 and 2 on the catalytic domain of ataxin-3 assist the catalytic pocket with access to isopeptide bonds. Site 1 is necessary for ataxin-3 activity; mutating it renders ataxin-3 inactive [84]. Cellular studies have demonstrated that ataxin-3 regulates ERAD substrate turnover through its interaction with VCP/97 [52, 53]. Importantly, VCP/97 and ataxin-3 together control aspects of longevity in *C. elegans*, perhaps by regulating the turnover of IGF-signaling components [57]. Ataxin-3 shuttles between the nucleus and the cytoplasm, concentrates in the nucleus upon various stressors, and is important for cell viability during heat stress [56]. Abnormal expansion of the polyglutamine tract occurs in the neurodegenerative disorder SCA3. Expansion may perturb some cellular functions of ataxin-3 and/or induce novel functions with deleterious consequences for the brain, but notably it does not appear to affect Ub chain binding or cleavage. Similar to other polyglutamine disease proteins [48, 49], expanded ataxin-3 is most pathogenic when it is concentrated in the nucleus [86].

Box 3. Outstanding questions

- Neurons are highly polarized cells, thus, it will be important to determine which DUBs reside in different neuronal compartments.
- Which DUBs are shared among excitatory and inhibitory synapses, or among sensory and motor neurons? Also, certain DUBs may be essential to the central nervous system, while others may function more in the peripheral nervous system.
- Which DUBs function in an activity-dependent manner? Depending on the activity of neurons, DUB re-localization to different sub-cellular compartments, or the interaction of DUBs with new macromolecular complexes, may regulate DUB activity or substrate specificity.
- What is the development expression pattern of DUBs in the nervous system? Are there particular subclasses of DUBS that are expressed at high levels only during development, or only in the adult?
- Little is known about DUB roles in receptor cargo trafficking in the nervous system. AMSH is necessary for postnatal neuronal survival in mice (Table 1). What are the neuronal functions of AMSH, and what other DUBs are involved in receptor trafficking in the nervous system?
- Which class of DUBs are more promiscuous in substrate recognition? By contrast, which DUBs function relatively selectively, and have relatively few substrates?
- Are there glial-specific DUBs, and how do these DUBs support neuronal function?



Box 1 Figure I. Ubiquitination and the ubiquitin-dependent proteasome system

(A) Key enzymatic steps resulting in protein ubiquitination. (B) At least seven different types of polyubiquitin chains can form, resulting in differences in the subsequent destination and fate of the ubiquitinated protein. (C) The 26S proteasome and ubiquitin-dependent protein degradation.

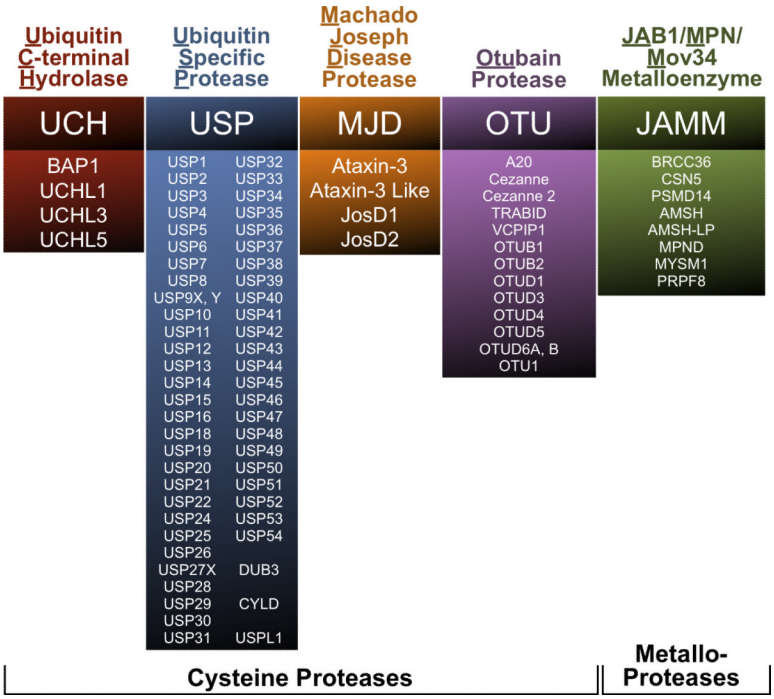


Figure 1. Human DUBs

Based on homology in the catalytic domain, DUBs are categorized into UCH, USPs, MJD protease, OTUs and JAMM subclasses [18]. All are cysteine proteases except the JAMM class of DUBs, which are zinc-dependent metallo-proteases [16-18]. The human genome encodes nearly 95 DUBs, each belonging to one of the above five categories. Some DUBs show preference or specificity for certain Ub-Ub linkages or a specific Ub chain length [16].

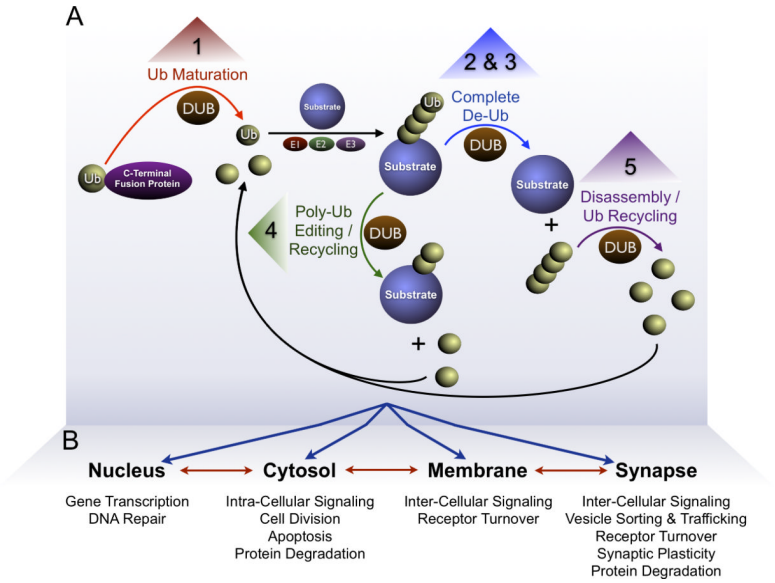
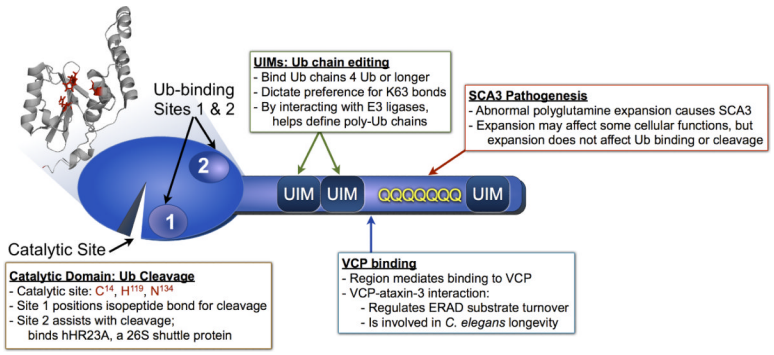


Figure 2. Proteolytic Functions of DUBs

A) As enzymes, DUBs mediate several proteolytic functions: 1) Process Ub precursors to their mature form. *Ubiquitin* genes encode C-terminally extended forms of Ub fused either to ribosomal subunits or head-to-tail linked Ub multimers. Cleavage from the C-terminal fusion, which reveals the final two glycine residues of Ub, is necessary for Ub maturation. 2) Deubiquitinate a ubiquitinated substrate and thus alter its fate: for example, rescue a protein from proteasomal degradation (e.g. USP14) [13]. 3) Function at the proteasome to remove Ub from substrates destined for degradation, thereby rescuing Ub (eg. PSMD14, UCHL5 and USP14) [19]. 4) Edit the length and/or type of Ub chain on substrates and assist with targeting to a specific pathway. In fact, several DUBs interact with E3 ligases [14] and may optimize substrate ubiquitination. 5) Disassemble un-anchored Ub chains, including ones recycled from the proteasome; Isopeptidase T (also known as USP5) has been implicated in this process [17].

B) By functioning in one or more catalytic processes outline above, DUBs regulate processes and pathways in essentially all major cellular compartments (blue arrows). As no subcellular compartment operates in isolation, DUB-dependent processes and pathways in one compartment can affect others (red arrows).



Box 2. Figure I.

Structural and functional domains of ataxin-3, a DUB that is expressed in the nervous system of invertebrates and mammals.

Table 1

DUBs implicated in nervous system development, function and/or disease[a]

UCH				
UCHL1		Brain-enriched DUB most likely involved in mono-Ub generation/ stabilization at the synapse. UCHL1 is important for synapse structure and function. Lack of UCHL1 causes gracile axonal dystrophy in mice, and mutations have been linked to PD.		Reviewed in [26]
UCHL3		Mutations cause spatial learning and working memory deficits in mice, but LTP seems unaffected. Mutant mice also show postnatal retinal and muscular degeneration. UCHL3 may protect against mitochondria-related oxidative stress in the retina.		[88-90]
UCHL5	19S-associated DUB necessary for brain development. Knockout mouse embryos have telencephalon, mesencephalon and metencephalon malformations. Pathways leading to these anomalies are unknown.			[91]
USP				
USP5		Co-purifies with 26S synaptic proteasomes isolated from rat cortex.		[92]
USP7	Prevents differentiation of neuronal progenitor cells by deubiquitinating and stabilizing the Repressor Element 1-Silencing Factor. Brain-restricted knockout leads to neonatal lethality and abnormal brain development in mice, in part through p53-dependent mechanisms.	Co-purifies with 26S synaptic proteasomes isolated from rat cortex.	Interacts with the polyQ disease protein ataxin-1, which causes SCA1. Interaction in cells and in mice may be weakened by polyQ expansion. Physiological significance is unknown.	[92-95]
USP9X	May be involved in neural fate determination. Its fly orthologue (Faf) regulates neural fate determination in the developing eye and is involved in synapse function. USP9X protein expression is altered in at least one mouse model of Parkinson's Disease.			[40, 44]
USP13		Co-purifies with 26S synaptic proteasomes isolated from rat cortex.		[92]
USP14	19S-associated DUB necessary for synapse development and function. Deficiency in USP14 underlies the *ax*^{J} mice, characterized by ataxia and premature death. USP14 appears mostly involved in synaptic function by maintaining monomeric Ub levels.			[10, 13, 32-35]
USP18		Knockout mice develop normally and are viable. Necrotic cell death of ependymal cells correlates with hydrocephalus in knockout mice, which show tremors, loss of balance, rolling, circling, convulsions and die prematurely. Molecular pathways may involve deconjugation of the Ub-like protein ISG15, though a deubiquitinating role for USP18 has not been excluded.		[96-97]
USP22	Its fruit fly orthologue, Nonstop, is necessary for correct axonal projections of photoreceptor cells, by participating in a chemokine gradient by glial cells.			Reviewed in [20]
USP24			Based on gene-linkage analysis, may play a role in susceptibility to PD.	[98]
USP25			Overexpressed in human Down Syndrome fetal brains.	[99]
USP33	Necessary for midline crossing of commissural neurons in response to the guidance cue Slit in mice and chicks. Appears to act by			[45]

	deubiquitinating and stabilizing the Slit receptor Robo1.			
USP40			Based on gene-linkage analysis, may play a role in susceptibility to PD.	[94]
USP46	Mutations in murine USP46 cause abnormal behavior in the tail suspension and forced swimming tests, as well as circadian rhythm irregularities. Phenotypes, which are rescued by re-introduction of USP46, could be due to USP46 involvement in pre- and postsynaptic GABAergic signaling. GABA is neurotrophic during development, thus USP46 may also be involved in NS development. Its orthologue in *C. elegans*, *usp-46*, protects glutamate receptors from lysosomal degradation, most likely by deubiquitinating them.			[100, 101]
MJD				
Ataxin-3		Ub chain editing DUB involved in protein quality control. Most likely necessary during proteotoxic stress in cell culture, mice and humans.	Expansion in polyQ region causes neurodegeneration in SCA3.	[48, 50, 51, 55, 102]
OTU				
OTUB1			Constituent of Lewy Bodies in human postmortem PD brain.	[103]
JAMM				
AMSH	Necessary for postnatal survival of specific neuronal populations. Knockout mice die between day 19-23 postnatally. Histopathology indicates neuronal loss in hippocampus and cerebral cortex. Molecular details are unknown.			[104]
PSMD14	RNAi-mediated knockdown in midbrain and cortical mouse primary cultures leads to neuronal mitotic re-entry and apoptosis. It may be that PSMD14, through the 26S, is necessary to maintain the post-mitotic status of neurons.			[105]
Partial List of Zebrafish DUBs Whose Knockdown Was Associated with CNS Necrosis or Abnormal NS Development				[87]
BAP1, UchL5, USP1, USP3, USP4, USP5, USP6, USP7, USP8, USP10, USP12, USP13, USP15, USP18, USP19, USP20, USP21, USP24, USP25, USP28, USP32, USP37, USP39, USP42, USP43, USP44, USP45, USP53, CYLD, ataxin-3, JosD1, JosD2, Otub1, Otud3, Otud4, Otud5, VCPIP1.				

DUBs are grouped by enzymatic class (see Figure 1 for details). The bottom-most cell lists zebrafish DUBs whose knockdown was determined to be associated with abnormal nervous system morphology [87]. The physiological significance of these knockdowns awaits confirmation.

[a]Abbreviations: UCHL3: Ubiquitin C-terminal Esterase L3; OTUB1: OTU domain, ubiquitin aldehyde binding 1; CYLD: Cylindromatosis; JosD1: Josephin domain containing 1; JosD2: Josephin domain containing 2; Otud3: OTU domain containing 3; Otud4: OTU domain containing 4; Otud5: OTU domain containing 5; VCPIP1: Valosin containing protein (p97)/p47 complex interacting protein 1.