



REVIEW

Protein monoubiquitylation: targets and diverse functions

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Ubiquitin is a 76-amino acid protein whose conjugation to protein targets is a form of post-translational modification. Protein ubiquitylation is characterized by the covalent attachment of the COOH-terminal carboxyl group of ubiquitin to an amino group of the substrate protein. Given that the NH₂-terminal amino group is usually masked, internal lysine residues are most often targeted for ubiquitylation. Polyubiquitylation refers to the formation of a polyubiquitin chain on the substrate as a result of the ubiquitylation of conjugated ubiquitin. The structures of such polyubiquitin chains depend on the specific lysine residues of ubiquitin targeted for ubiquitylation. Most of the polyubiquitin chains other than those linked via lysine-63 and methionine-1 of ubiquitin are recognized by the proteasome and serve as a trigger for substrate degradation. In contrast, polyubiquitin chains linked via lysine-63 and methionine-1 serve as a binding platform for proteins that function in immune signal transduction or DNA repair. With the exception of a few targets such as histones, the functions of protein monoubiquitylation have remained less clear. However, recent proteomics analysis has shown that monoubiquitylation occurs more frequently than polyubiquitylation, and studies are beginning to provide insight into its biologically important functions. Here, we summarize recent findings on protein monoubiquitylation to provide an overview of the targets and molecular functions of this modification.

Introduction

Ubiquitin is a highly conserved 76-amino acid protein that plays key roles in many aspects of eukaryotic cell function as a protein modifier. Ubiquitin is synthesized as an inactive precursor protein from four distinct genes (*UBA52*, *UBA80*, *UBB*, *UBC*), and the precursor is then cleaved by a deubiquitinase (DUB) enzyme to yield ubiquitin with active diglycine residues exposed at its COOH-terminus (Komander *et al.* 2009) (Fig. 1). In cultured mammalian cells, ~25% of ubiquitin molecules are estimated to exist in the free (unconjugated) form, with the remainder being covalently attached to other protein molecules, including ubiquitin itself (Kaiser *et al.* 2011). The formation of a peptide bond between ubiquitin and its target proteins requires the activation of ubiquitin by a ubiquitin-activating enzyme (E1) and its subsequent conjugation to a ubiquitin-conjugating enzyme (E2), with energy being stored

in a thioester bond between a cysteine residue in E1 or E2 and the carboxyl group at the COOH-terminus of ubiquitin. A ubiquitin ligase (E3) then catalyzes the transfer of ubiquitin to an amino group of the target protein and thereby determines target specificity (Nakayama & Nakayama 2006). Given that the amino group at the NH₂-terminus of most proteins is masked (Arnesen *et al.* 2009), internal lysine residues are targeted for ubiquitylation in most cases.

E3 ligases have been divided into two major classes on the basis of a domain structure that reflects the catalytic mechanism (Metzger *et al.* 2012). One class consists of enzymes that possess a RING (really interesting new gene) domain or a related PHD (plant homeodomain), LAP (leukemia-associated protein), or U-box domain and which bind E2 through these domains and promote the direct transfer of ubiquitin from E2 to the substrate protein (Deshaies & Joazeiro 2009). The second class includes E3 ligases that contain a HECT (homologous to E6-AP carboxyl terminus) domain and which receive ubiquitin from E2 at an active site cysteine residue in this domain before it

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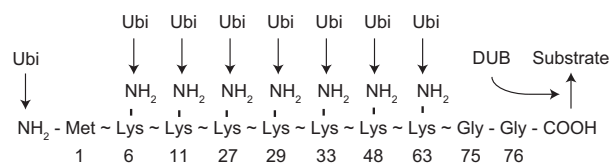


Figure 1 Schematic representation of amino (NH₂) and carboxyl (COOH) groups of ubiquitin that play a role in ubiquitylation. Numbers indicate amino acid position. A deubiquitinase (DUB) cleaves the ubiquitin (Ubi)–substrate linkage.

is linked to the substrate (Rotin & Kumar 2009). The domain–mechanism relationship of E3 ligases was recently broadened by the RING–IBR (in-between ring)–RING (RBR) domain which forms thioester bond with ubiquitin before transfer to the substrates although it has RING, but not HECT, domains (Smit & Sixma 2014).

E3 function requires simultaneous binding of both substrate and either E2–ubiquitin (for RING–type E3 ligases) or ubiquitin (for HECT–type E3 ligases). Whereas some E3 ligases function as a monomer, most, especially those with a RING domain, exist as a complex that includes an E2–ubiquitin binding module and a substrate-binding module. Cullin–RING E3 ligases (CRLs) are multisubunit E3 ligases that are capable of associating with various substrate-binding proteins. For example, Cullin1–based E3 ligases (CRL1) associate with proteins that contain an F–box domain and which function as substrate-binding subunits. Substrate-binding proteins in CRLs are usually indicated by superscript notation, as in CRL1^{SKP2}, in which SKP2 serves as the substrate-binding subunit. Eight CRLs (CRL1, CRL2, CRL3, CRL4A, CRL4B, CRL5, CRL7, CRL9) have been identified in mammals (Marin 2009), some of which function in monoubiquitylation, as described below.

Polyubiquitylation is a form of ubiquitylation in which a polyubiquitin chain becomes attached to the substrate protein as a result of the ubiquitylation of conjugated ubiquitin. It is thought to be achieved either as a consequence of the processive nature of E3 ligase action—the attachment of multiple ubiquitin molecules to the substrate during a single binding event—or by the en bloc transfer of polyubiquitin to the substrate (Hochstrasser 2006). Given that eight amino groups are available as ubiquitylation sites in ubiquitin (Fig. 1), many different patterns of ubiquitin–ubiquitin linkage are possible. The best-known function of polyubiquitylation is mediated by K6,

K11, K27, K29, K33, and K48 linkage and is to provide a mark for substrate degradation by the proteasome. In contrast, polyubiquitylation mediated by M1 or K63 linkage serves to generate a binding platform for the organization of immune signal transduction or DNA repair machinery.

Compared with polyubiquitylation, the functions of monoubiquitylation of substrate proteins are in general less clear. However, recent proteomics analysis has shown that monoubiquitylation is more prevalent than polyubiquitylation even in cells in which proteasome activity is blocked, suggesting the importance of this modification (Kaiser *et al.* 2011). Furthermore, the accumulation of polyubiquitylated proteins that occurs in response to proteasome inhibition results in a rapid reduction in the extent of monoubiquitylation, suggesting that monoubiquitylation is reversible in a manner that is sensitive to changes in cellular state (Mimnaugh *et al.* 1997; Kim *et al.* 2011). In this review, we present recent findings related to monoubiquitylation, with a focus on the best-studied mammalian systems, and we provide an overview of monoubiquitylated proteins and the effects of monoubiquitylation on substrates categorized according to their function.

Histone monoubiquitylation and transcriptional regulation

Eukaryotic DNA is packaged into chromatin, in which two copies each of the histones H2A, H2B, H3, and H4 form the nucleosome core particle around which DNA is wrapped. The histone tails are extruded from the nucleosome and are targeted for post-translational modification including monoubiquitylation. Initially considered an abundant nonhistone chromatin protein because of its differential response to hepatectomy and different amino acid composition compared with histones, A24 was the first identified monoubiquitylated protein and was subsequently found to comprise histone H2A with a ubiquitin molecule attached to its K119 residue (Table 1). Such monoubiquitylation generated a Y-shaped protein with one COOH-terminus and two NH₂-termini (Goldknopf & Busch 1977). At least six ubiquitin ligases that catalyze the ubiquitylation of H2A at K119 have since been identified: RNF2 (also known as RING1B) in polycomb repressive complex (PRC) 1 (Wang *et al.* 2004), TRIM37 in PRC2 (Bhatnagar *et al.* 2014), BRCA1 in the BRCA1–BARD1 complex (Chen *et al.* 2002), 2A–HUB (also known as hRUL138) in the 2A–HUB complex (Zhou *et al.* 2008), CRL4A/B^{DDB2} (Guerre-

Table 1 Monoubiquitylated substrates related to transcription

Substrate	Ubi site	E3 ligase	DUB	Effect of monoubiquitylation
H2A	K119	RNF2, TRIM37, BRCA1, 2A-HUB, CRL4A/B ^{DDDB2} , CRL4B ^{RBBP4/7}	USP16, USP21, BAP1, 2A-DUB	Transcriptional repression
H2B	K120	RNF20, RNF40	USP7, USP22	Transcriptional activation
H1	K46	TAF1	Not reported	Transcriptional activation
TET1	K1537	CRL4 ^{VprBP}	Not reported	Recruitment to chromatin
TET2	K1212	CRL4 ^{VprBP}	Not reported	Recruitment to chromatin
TET3	K983	CRL4 ^{VprBP}	Not reported	Recruitment to chromatin
IκBα	Not reported	Not reported	Not reported	Prevention of association with IKK
p105	Not reported	CRL1 ^{β-TrCP}	Not reported	Processing
NEMO	K277, K309	cIAP1	Not reported	Nuclear export
PKCε	K321	RINCK1	Not reported	Binding to NEMO
p53	K351, K357, K370, K372, K373, K381, K382, K386	MDM2, MSL2	USP7, USP10	Nuclear export
FOXO4	K199, K211	MDM2	USP7	Nuclear import
SMAD3	K33, K53, K81	Smurf2	USP15	Inhibition of DNA binding
SMAD4	K507, K519	Ectodermis	USP9X	Inhibition of R-SMAD binding
TLE3	Q domain	XIAP	Not reported	Inhibition of TCF binding
PAX3	Not reported	TAF1	Not reported	Degradation by proteasome

ro-Santoro *et al.* 2008) and CRL4B^{RBBP4/7} (Hu *et al.* 2012). Consistent with the function of these six ubiquitin ligase complexes as transcriptional repressors, monoubiquitylated H2A inhibits transcription by several mechanisms including down-regulation of methylation of histone H3 at lysine 4 (H3K4) (Nakagawa *et al.* 2008), a mark of active promoters, and recruitment of PRC2 (Blackledge *et al.* 2014). Multiple DUBs that remove ubiquitin from H2A have also been identified and shown to be transcriptional activators, including ubiquitin-specific peptidase (USP) 16 (Joo *et al.* 2007), USP21 (Nakagawa *et al.* 2008), BRCA1-associated protein 1 (BAP1) (Scheuermann *et al.* 2010) and 2A-DUB (Zhu *et al.* 2007). Although H2A is the most abundant ubiquitylated protein in multicellular organisms, ubiquitylated H2A has not been detected in yeast (Robzyk *et al.* 2000).

In contrast to H2A, monoubiquitylation of H2B is conserved from yeast to mammals. It occurs at conserved lysine residues (K123 in yeast, K120 in mammals) and is catalyzed by the homologous E3 ligases Bre1 in budding yeast and RNF20 and RNF40 in mammals (Espinosa 2008). Genetic analysis in yeast has indicated that H2B monoubiquitylation at K123 is required for H3K4 methylation and consequent transcriptional activation. Most studies in mammals,

however, support a function for H2B monoubiquitylation in transcriptional elongation. Monoubiquitylation of H2B is also dynamically regulated, with the ubiquitin moiety being removed by several DUBs including Ubp8 homologues in the SAGA complex (Ubp8 in yeast, USP22 in mammals) (Henry *et al.* 2003; Zhang *et al.* 2008), Ubp10 in budding yeast (Gardner *et al.* 2005) and USP7 in humans (Emre *et al.* 2005). Although histones H3 and H4 have been shown to undergo ubiquitylation, the function of their monoubiquitylated forms is unclear (Cao & Yan 2012), with the exception of H3 monoubiquitylated at K23 by UHRF1 as described below.

Histone H1 binds the linker region of DNA between nucleosome core particles and controls the dynamics of chromatin structure and gene activity as a result of its high mobility within the nucleus (Catez *et al.* 2006). *In vitro* studies have shown that the interaction of H1 with nucleosomes organizes the nucleosome arrays into a condensed 30-nm chromatin fiber that inhibits DNA-dependent activities such as transcription and replication (Li & Reinberg 2011). As with other histones, diverse post-translational modifications including monoubiquitylation are thought to regulate H1 function. TAF1 [TATA box binding protein (TBP)-associated factor 1] has been found to catalyze the monoubiquitylation of H1 (Pham &

Sauer 2000), presumably at K46 (Wisniewski *et al.* 2007), and thereby to activate transcription. TAF1 monoubiquitylates H1 in the absence of other E1, E2 and E3 enzymes *in vitro*, suggesting that it may function as a combined E1/E2 enzyme and that H1 ubiquitylation does not require an E3 ligase. TAF1 also monoubiquitylates the transcription factor PAX3, as described below.

Transcriptional regulation by monoubiquitylation of nonhistone proteins

DNA methylation at the C-5 position of cytosine regulates gene expression and plays pivotal roles in numerous biological processes. The TET dioxygenases catalyze iterative oxidation of 5-methylcytosine (5mC) that results in demethylation of 5mC, indicating that such DNA methylation is reversible (Wu & Zhang 2014). We recently found that CRL4A/B^{VprBP} catalyzes the monoubiquitylation of all three mouse TET family proteins at a conserved lysine residue (K1537 in TET1, K1212 in TET2, K983 in TET3) (Table 1). Monoubiquitylation of TET proteins promotes their binding to DNA, which is essential for catalytic function of TET in cells (Nakagawa *et al.* 2015). How monoubiquitylation enhances TET binding to DNA is unclear. The level of TET monoubiquitylation is also regulated by at least one DUB, given that a DUB inhibitor increased TET monoubiquitylation in cells, although the identity of the responsible enzyme (or enzymes) remains unknown.

In the process of DNA replication, the pattern of DNA methylation is also replicated by a monoubiquitylation-based mechanism. Immediately after DNA replication, the E3 ligase UHRF1 (ubiquitin-like with PHD and RING finger domains 1) binds to the hemimethylated DNA and monoubiquitylates histone H3 at K23 to generate a binding site for DNA methyltransferase 1, which is responsible for DNA methylation on the daughter strand (Nishiyama *et al.* 2013).

The activity of transcription factors is also regulated by monoubiquitylation. The nuclear factor (NF)- κ B family of transcription factors consists of five members—p50, p52, p65 (also known as RelA), c-Rel, and RelB—that share an NH₂-terminal Rel homology domain (RHD) responsible for DNA binding as well as for homo- or heterodimerization (Hayden & Ghosh 2008). The active NF- κ B dimers bind to κ B sites in DNA and thereby regulate transcription of target genes. In the absence of activating stimuli, NF- κ B is maintained inactive either by its association with a member of the I κ B family of inhibitor proteins (I κ B α ,

I κ B β , I κ B ϵ) or the precursor proteins p105 (precursor of p50) or p100 (precursor of p52). Cell stimulation results in the rapid phosphorylation of I κ B by the I κ B kinase (IKK) complex and its consequent polyubiquitylation by the CRL1 ^{β -TrCP} E3 ligase and degradation by the proteasome. The IKK complex, which comprises the kinase subunits IKK α (also known as IKK1) and IKK β (IKK2) as well as the regulatory subunit IKK γ (NF- κ B essential modulator, or NEMO), is activated by TRAF proteins. Monoubiquitylation regulates NF- κ B activity at several steps. First, I κ B α monoubiquitylation by an unidentified E3 ligase inhibits its association with and phosphorylation by IKK and thereby negatively regulates NF- κ B activity (Da Silva-Ferrada *et al.* 2011). Second, p105 is monoubiquitylated at multiple lysine residues by CRL1 ^{β -TrCP}, which is necessary for its processing to active p50 by the proteasome (Kravtsova-Ivantsiv *et al.* 2009). Third, monoubiquitylation of NEMO at K277 and K309 by the E3 ligase cIAP1 in response to genotoxic stress triggers its export from the nucleus and assembly into the IKK complex, resulting in NF- κ B activation (Jin *et al.* 2009). And fourth, monoubiquitylation of protein kinase C ϵ (PKC ϵ) at K321 by the E3 ligase RINCK1 in response to growth factor stimulation promotes its association with NEMO, resulting in IKK β phosphorylation and activation (Yang *et al.* 2012). Monoubiquitylation thus regulates the activity of NF- κ B in both positive and negative manners.

The tumor suppressor protein p53 is inactivated in more than half of all sporadic human cancers. It suppresses oncogenesis and cancer progression through transcriptional regulation of target genes related to the cell cycle, cellular senescence, apoptosis, metabolism, stem cell maintenance, metastasis and the interaction of tumor cells with their microenvironment (Bieging *et al.* 2014). Given that p53 hyperactivation as well as p53 loss is detrimental to cells, the amount and activity of this protein are tightly regulated by transcriptional and post-transcriptional mechanisms including monoubiquitylation-induced nuclear exclusion (Li *et al.* 2003; Kruse & Gu 2009). MDM2 and MSL2 each mediate monoubiquitylation of the COOH-terminal region of p53 (at K370, K372, K373, K381, K382, or K386 by MDM2, and at K351 or K357 by MSL2), which results in a conformational change of the protein that exposes its nuclear export signal (Nie *et al.* 2007). Interestingly, over-expression of MDM2 was shown to induce polyubiquitylation of p53, indicating that the amount of MDM2 determines whether p53 is monoubiquitylated or polyubiquitylated. Two DUBs influence the level of p53 monoubiquitylation: USP7

removes ubiquitin from p53 in the cytosol to allow p53 translocation to mitochondria (Marchenko *et al.* 2007), whereas USP10 does so in the nucleus to activate p53 (Yuan *et al.* 2010).

The FOX (Forkhead box) family of transcription factors is characterized by the presence of a DNA binding domain (known as the Forkhead domain) and contributes to several physiological processes including development, metabolism, and the immune response. The human genome encodes 50 FOX family proteins, which are divided into 19 subclasses (FOXA to FOXS) on the basis of sequence similarity of the Forkhead domain (Jackson *et al.* 2010). The FOXO family comprises four members (FOXO1, FOXO3, FOXO4, FOXO6), among which FOXO3 and FOXO4 are monoubiquitylated. The level of FOXO4 monoubiquitylation is determined by enzymes that also target p53 but with opposite consequences. MDM2 thus monoubiquitylates FOXO4 at K199 or K211 and thereby induces its nuclear translocation (rather than nuclear exclusion, as for p53) and activation in response to oxidative stress (Brenkman *et al.* 2008), and this monoubiquitylation is reversed by USP7-catalyzed deubiquitylation (van der Horst *et al.* 2006). Regulation of FOXO3 by MDM2 and USP7 has not been described to date.

SMAD (homologs of the *Caenorhabditis elegans* protein SMA [small body size] and the *Drosophila melanogaster* protein MAD [mothers against decapentaplegic]) proteins, mediators of transforming growth factor- β (TGF- β) signaling, are also monoubiquitylated (Dupont *et al.* 2012). On ligand activation, the TGF- β receptor phosphorylates receptor-regulated SMADs (R-SMADs: SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8), which then associate with SMAD4 to form an active transcriptional complex that regulates gene expression. Monoubiquitylation of lysine residues that are essential for DNA recognition by R-SMADs (K33, K53, and K81 in SMAD3) results in the obstruction of DNA binding through steric hindrance. Smurf2 was shown to catalyze monoubiquitylation of SMAD3 (Tang *et al.* 2011), whereas USP15 deubiquitylates and thereby activates R-SMADs (Inui *et al.* 2011). SMAD4 is also regulated by monoubiquitylation. Its E3 ligase, Ectodermis, was first identified in *Xenopus* embryos, in which this enzyme prevents ectodermal cells from differentiating into endodermal and mesodermal cells (Dupont *et al.* 2005). Subsequent studies showed that K507 or K519 of SMAD4 is monoubiquitylated by Ectodermis associated with chromatin and that monoubiquitylated SMAD4 dissociates from its active transcriptional complex with

R-SMAD, resulting in SMAD inactivation (Agricola *et al.* 2011). USP9X (also known as FAM) deubiquitylates SMAD4 and thereby sustains TGF- β signaling (Dupont *et al.* 2009).

WNT (Wingless-related integration site) signaling is also regulated by monoubiquitylation. Binding of WNT to its coreceptors Frizzled and LRP5/6 (LDL receptor-related protein 5 or 6) results in stabilization of β -catenin and its translocation to the nucleus, where it binds members of the TCF/LEF family of transcription factors to activate a WNT-specific transcriptional program (Clevers & Nusse 2012). In the absence of WNT ligand, TCF/LEF factors are maintained inactive by binding to Groucho/TLE transcriptional corepressors. WNT stimulation induces the monoubiquitylation of TLE3 by the E3 ligase XIAP and its consequent dissociation from TCF/LEF (Hanson *et al.* 2012). Ubiquitylation sites as well as DUBs for TLE3 remain to be elucidated.

Although it is well established that the proteasome recognizes polyubiquitin chains composed of at least four ubiquitin molecules (Thrower *et al.* 2000), it is also reported that monoubiquitylation of model substrates can stimulate proteasomal degradation if the size of substrate is small (less than 150 amino acids) (Shabek *et al.* 2012). Consistently, in cells, evidence suggests that monoubiquitylated proteins can also be targeted by the proteasome, with one example being monoubiquitylated p105 as described above. Another example is monoubiquitylated PAX3 (Boutet *et al.* 2007). Nine PAX proteins (PAX1 to PAX9), which are characterized by the presence of a DNA binding domain known as the paired domain, play important roles in the development of various organs and tissues in mammals (Blake & Ziman 2014). PAX3 is transiently expressed in highly proliferative intermediate progenitor cells during postnatal myogenesis, with such expression declining at the differentiation stage (Conboy & Rando 2002) coincident with PAX3 monoubiquitylation by TAF1 and its delivery to the proteasome for degradation (Boutet *et al.* 2010). As with the case of histone H1 monoubiquitylation, PAX3 is monoubiquitylated by TAF1 alone *in vitro*, indicating that TAF1 can directly bind ubiquitin and transfer it to PAX3. DUBs and ubiquitylation sites for PAX3 remain to be identified.

Regulation of DNA damage repair and DNA replication by monoubiquitylation

The maintenance of genetic information is critical for proper execution of cellular function. Given that

DNA is repeatedly exposed to genotoxic stressors such as ionizing radiation and ultraviolet light in sunlight, the detection and repair of DNA damage are central to such maintenance. Although K63-linked polyubiquitylated forms of histones H2A and H2AX have been found to provide a signaling scaffold, RING1B and RNF20/RNF40 catalyze the monoubiquitylation of H2A/H2AX and H2B, respectively, at sites of DNA damage (Cao & Yan 2012). Monoubiquitylation of H2A and H2B is thought to interfere with chromatin compaction (as observed in transcriptional regulation) and thereby to facilitate assembly of the repair machinery at foci of DNA damage (Moyal *et al.* 2011), but the underlying mechanisms remain elusive.

Interstrand cross-linking (ICL) occurs when chemotherapeutic drugs or certain by-products of lipid peroxidation mediate the covalent joining of the two DNA strands of genomic DNA (Deans & West 2011). Central components of the ICL repair pathway include the products of 15 genes that are mutated in Fanconi anemia (FA), a rare recessive genetic disease (Kim & D'Andrea 2012). Eight of these 15 proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM) form the FA core complex which functions as an E3 ubiquitin ligase. Another two of these proteins, FANCD2 (at K561) and FANCI (at K523), collectively referred to as ID2, are monoubiquitylated in response to ICL (Table 2), which is detected on the basis of the association of FANCM with a stalled replication fork in S phase (Kim *et al.* 2008). FANCL contains a RING finger domain and is the catalytic subunit of the FA core complex. Monoubiquitylation induces ID2 translocation to chromatin foci containing ICL lesions (Moldovan & D'Andrea 2009). FAN1 (FANCD2/FANCI-associated nuclease 1) is then recruited to monoubiquitylated ID2 via its

UBZ4-type ubiquitin binding domain (UBD) and cleaves the DNA, thereby initiating ICL repair. After the damage is fixed, monoubiquitylated ID2 is inactivated through deubiquitylation by USP1. Given that USP1 knockout mice show an FA-like phenotype, the essential function of USP1 might be deubiquitylation of ID2 (Kim *et al.* 2009).

Replication forks can bypass DNA damage by translesion synthesis (TLS), which allows DNA replication through a lesion with the use of specialized DNA polymerases. The recruitment of TLS polymerases to DNA is precisely regulated to prevent mutations caused by the low fidelity of these enzymes. Fifteen genes for DNA polymerases have been identified in mammals, among which three replicative polymerases (Pol α , Pol δ , Pol ϵ) are responsible for accurate replication. Seven polymerases (Pol η , Pol ι , Pol κ , REV1, Pol ζ , Pol θ , Pol ν) have been shown to possess TLS activity (Lange *et al.* 2011). Proliferating cell nuclear antigen (PCNA) functions as a homotrimeric 'clamp' that surrounds double-stranded DNA and tethers replicative polymerases to the DNA template during replication. Stalling of the replication fork at a site of DNA damage results in the monoubiquitylation of PCNA at K164 by RAD18 or CRL4A/B^{CDT2} ubiquitin ligases and thereby leads to the recruitment of members of the Y-family of TLS polymerases (Pol η , Pol ι , Pol κ , REV1), which harbor a PIP (PCNA-interacting peptide) box and UBD (Bergink & Jentsch 2009; Terai *et al.* 2010). In the absence of DNA damage, monoubiquitylation of PCNA is counteracted by USP1, the same DUB involved in ICL repair, and the switch from replicative to TLS polymerases is inhibited (Huang *et al.* 2006).

In unstressed cells, Pol η is monoubiquitylated at its COOH-terminus by Pirh2, resulting in inhibition of the interaction of Pol η with PCNA (Bienko *et al.*

Table 2 Monoubiquitylated substrates related to DNA repair, replication and segregation

Substrate	Ubi site	E3 ligase	DUB	Effect of monoubiquitylation
FANCD2	K561	FANCL	USP1	Recruitment to chromatin
FANCI	K523	FANCL	USP1	Recruitment to chromatin
PCNA	K164	RAD18, CRL4A/B ^{CDT2}	USP1	Recruitment to TLS polymerase
Pol η	C-terminus	Pirh2	Not reported	Inhibition of PCNA binding
PAF15	K15, K24	CRL4A/B ^{CDT2} ?	Not reported	Basis of polyubiquitylation
RFC2/4	Not reported	RAD18	Not reported	Not reported
ORC1	Not reported	Not reported	Not reported	Nuclear export
CENP-A	K124	CRL4A ^{COP8}	Not reported	Binding to HJURP

2010; Jung *et al.* 2011). Monoubiquitylation thus has both positive and negative effects on DNA repair. The level of Pol η ubiquitylation declines during the repair of DNA damage, suggesting Pol η is deubiquitylated, but the DUB responsible for this process has not been identified.

PCNA-associated factor 15 (PAF15) also prevents inopportune binding of Pol η to PCNA and is monoubiquitylated at K15 and K24 in a PCNA-dependent manner (Povlsen *et al.* 2012), possibly by CRL4A/B^{CDT2} (Havens & Walter 2011). Monoubiquitylation of PAF15 is not directly related to its function, however, instead serving as the basis for polyubiquitylation and consequent degradation at the site of DNA damage. A DUB for PAF15 also remains to be identified.

The doughnut-like structure of PCNA must be opened by the clamp loader complex RFC (comprised of RFC1 to RFC5) in order for PCNA to be able to encircle DNA and initiate DNA replication (Indiani & O'Donnell 2006). At sites of DNA damage, the PCNA-like complex 9-1-1 (RAD9-HUS1-RAD1) is loaded by another clamp loader complex (comprising RAD17 and RFC2 to RFC5) to initiate the DNA damage response (Cimprich & Cortez 2008). The shared clamp loader proteins RFC2 and RFC4 are monoubiquitylated on chromatin by RAD18 in response to DNA damage (Tomida *et al.* 2008). The monoubiquitylation of these proteins has been suggested to be related to the switching of clamp loader complexes, but the corresponding ubiquitylation sites and DUBs have not been identified.

Potential replication initiation sites are marked by the formation of a prereplication complex that begins with loading of the proteins ORC1 to ORC6. ORC1 was shown to be exported from the nucleus in response to its monoubiquitylation to prevent the re-formation of the prereplication complex during S phase, although the corresponding ubiquitylation sites, E3 ligases, and DUBs remain unknown (Saha *et al.* 2006).

From the various findings presented so far, it is clear that monoubiquitylation serves to fine-tune DNA-based processes including replication, transcription and repair. Emerging evidence also suggests that epigenetic regulators such as TET proteins are among the ranks of monoubiquitylated proteins. The histone H3 variant CENP-A, which is specifically loaded onto the centromere to build the kinetochore, was thus recently shown to be monoubiquitylated at K124 by CRL4A^{COP58} (Niikura *et al.* 2015). Monoubiquitylation of CENP-A promotes its association

with the CENP-A-specific chromatin assembly factor HJURP and is required for CENP-A loading. This finding thus also implicates monoubiquitylation in regulation of mitotic events.

Regulation of membrane-associated processes by monoubiquitylation

Protein monoubiquitylation also contributes to the regulation of membrane-associated processes, among which its role in endocytosis is the most well characterized (Table 3). The abundance of cell surface receptors and channels is strictly controlled to ensure the appropriate timing and duration of ligand-induced signaling—with activated receptors having been shown to induce their own internalization and subsequent lysosomal degradation, for example. The activation of receptor tyrosine kinases (RTKs) such as the epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (MET), and stem cell factor receptor (c-KIT) by cognate growth factors results in receptor autophosphorylation as well as the receptor-mediated phosphorylation of tyrosine residues in other cytoplasmic proteins. The phosphorylated tyrosine residues in RTKs are recognized by the E3 ligase Cbl, which then mediates receptor monoubiquitylation on multiple lysine residues. The multiple monoubiquitin moieties on RTKs recruit endocytic adaptor proteins that possess a UBD and are necessary for endocytosis (Haglund & Dikic 2012). Of note, many of these adaptor proteins [EPS15, EPS15R, epsin 1, epsin 2, HRS, STAM, RabGEF1 (also known as Rabex5), STS1, STS2, ZFYVE28] are themselves negatively regulated by monoubiquitylation, which induces an intramolecular UBD–monoubiquitin interaction (Hoeller *et al.* 2006). Two E3 ligases, NEDD4 and Parkin, have been implicated in the monoubiquitylation of EPS15 (Fallon *et al.* 2006; Woelk *et al.* 2006). The E3 ligases responsible for the monoubiquitylation of other adaptor proteins remain to be identified, however.

Ion channels also undergo endocytosis in response to the monoubiquitylation of multiple lysine residues, although this process is less well characterized. TRPC4 and TRPV4, two members of the transient receptor potential (TRP) family of Ca²⁺ channels, as well as the epithelial Na⁺ channel (ENaC) and the K⁺ channel Kir1.1 (also known as ROMK), are thus internalized as a result of monoubiquitylation by the E3 ligases ITCH (also known as AIP4) (Wegierski *et al.* 2006), NEDD4-2 (Eaton *et al.* 2010) and SH3RF1 (also known as POSH) (Lin *et al.* 2005), respectively.

Table 3 Monoubiquitylated substrates related to membrane-associated processes

Substrate	Ubi site	E3 ligase	DUB	Effect of monoubiquitylation
RTKs	Not reported	Cbl	Not reported	Endocytosis
Endocytic adaptors	Not reported	NEDD4, Parkin	Not reported	Self-inactivation
TRPC4	Not reported	ITCH	Not reported	Endocytosis
TRPV4	Not reported	ITCH	Not reported	Endocytosis
ENaC	Not reported	NEDD4-2	Not reported	Endocytosis
Kir1.1	Not reported	SH3RF1	Not reported	Endocytosis
Cx43	Not reported	NEDD4	Not reported	Endocytosis
CXCR4	Not reported	ITCH	Not reported	Endocytosis
TfR	Not reported	Not reported	Not reported	Endocytosis
NOTCH1	K1749	Not reported	Not reported	Endocytosis
SEC31	Not reported	CRL3 ^{KLHL12}	Not reported	Enlargement of COPII
H-Ras	K117, K147, K170, K185	Rabex5	Not reported	Endosomal sorting
N-Ras	Not reported	Rabex5	Not reported	Endosomal sorting
K-Ras	K104, K147	Not reported	Not reported	Activation
PLD1	Not reported	Not reported	Not reported	Localization to near Golgi
Spartin	Not reported	WWP1	Not reported	Dissociation from lipid droplets
PEX5	C11	PEX12	USP9X	Extraction from peroxisomes

Regulation of endocytosis by monoubiquitylation also extends beyond RTKs and channels, with other examples including that of the gap-junction protein connexin 43 (Cx43) by NEDD4 (Girao *et al.* 2009), the chemokine receptor CXCR4 by ITCH (Marchese *et al.* 2003), the transferrin receptor (TfR) by an unknown E3 ligase (Raiborg *et al.* 2002) and NOTCH1 at K1749 also by an unidentified E3 ligase (Gupta-Rossi *et al.* 2004).

Monoubiquitylation also participates in the regulation of protein secretion. Most secreted proteins are not directly released from the cytosol but instead enter the endoplasmic reticulum cotranslationally and then move to the Golgi complex before their delivery to the cell surface. Transport of cargo proteins from the endoplasmic reticulum to the Golgi is mediated by COPII vesicles, which consist of five cytosolic proteins of the SAR1 GTPase, SEC23-SEC24 adaptors, and SEC13-SEC31 outer-layer heterotetramers (Jensen & Schekman 2011), among which SEC31 is monoubiquitylated by the E3 ligase CRL3^{KLHL12} (Jin *et al.* 2012). SEC31 monoubiquitylation is necessary for the enlargement of COPII vesicles by an unknown mechanism that is required to accommodate large secretory cargos such as procollagen fibers and chylomicrons.

Monoubiquitylation plays an important role in the function of membrane-associated proteins. The Ras superfamily of proteins comprises >150 members in humans and has been divided into five major

branches: Ras, Rho, Rab, Ran and Arf. The activity of these proteins is regulated by membrane association dependent on a COOH-terminal lipid modification and by the binding of GTP or GDP (Wennerberg *et al.* 2005). The founding member of this superfamily, Ras itself, is frequently mutated in human cancers and transmits proliferation, differentiation and survival signals in response to diverse extracellular stimuli. Mammalian cells express three closely related Ras isoforms designated H-, K- and N-Ras. At the steady state, H-Ras and N-Ras are localized at the plasma membrane, endosomes and Golgi membrane, and their activity is regulated differentially depending on their location, whereas K-Ras is mostly present at the plasma membrane. Monoubiquitylation of Ras has been shown to influence its subcellular localization and activity. The endosomal E3 ligase RabGEF1 (also known as Rabex5) catalyzes monoubiquitylation of H-Ras (at K117, K147, K170, or K185) and N-Ras, resulting in their anchoring to the endosomal membrane (Jura *et al.* 2006; Xu *et al.* 2010), whereas monoubiquitylation of K-Ras at K104 or K147 by an unknown E3 ligase does not affect its intracellular localization but rather promotes the binding and activation of the downstream effector proteins RAF and phosphoinositide 3-kinase by an unknown mechanism (Sasaki *et al.* 2011).

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to yield phosphatidic acid and free choline, with the former product and its

derivative diacylglycerol both serving as signaling molecules. Mammalian cells contain two PLD isoforms, PLD1 and PLD2. PLD1 localizes to a perinuclear structure, the Golgi complex, endosomes and the plasma membrane, whereas PLD2 is present at the plasma membrane (Jenkins & Frohman 2005). Intriguingly, PLD1, but not PLD2, has been found to be monoubiquitylated in a manner dependent on its own lipase activity. Forced monoubiquitylation of PLD1 at its NH₂-terminus resulted in the formation of enlarged vesicles near the Golgi complex and its localization there (Yin *et al.* 2010). Such forced monoubiquitylation of a catalytically inactive mutant of PLD1 also resulted in its localization near the Golgi, but the formation of enlarged vesicles was less pronounced. These observations suggested that the production of phosphatidic acid by PLD near vesicles might induce vesicle enlargement, whereas the localization of PLD1 is dependent on its monoubiquitylation. Of note, PLD1, phosphatidic acid and the Golgi complex are all thought to play an important role in the formation of lipid droplets (Hesse *et al.*, 2013), suggesting that PLD1 monoubiquitylation might be involved in this process.

Lipid droplets are dynamic structures that store fat in the form of neutral lipids such as triacylglycerol and cholesteryl esters. They are surrounded by a monolayer of amphipathic lipids (phospholipids and cholesterol) that is coated with PAT proteins. Dissociation of PAT proteins from lipid droplets is induced by competition with Spartin (also known as SPG20) for binding to the droplets and results in droplet turnover. The E3 ligase WWP1 monoubiquitylates Spartin and thereby reduces its abundance both at the droplet surface and overall (Eastman *et al.* 2009), although the mechanism of this effect remains unknown. Given that knockdown of Spartin induces the accumulation of lipid droplets, WWP1 might be expected to regulate droplet size through Spartin monoubiquitylation, although such regulation has not yet been shown. Interestingly, a recent study showed that Spartin directly binds to mono- and diubiquitin as well as localizes to another subcellular compartment, DALIS (dendritic aggresome-like induced structures), in lipopolysaccharide-stimulated macrophages (Karlsson *et al.* 2014). It was not examined whether monoubiquitylation inhibits the ubiquitin binding function of Spartin or its ability to localize to DALIS in a manner similar to that operative for endocytic adaptor proteins.

Peroxisomes are eukaryotic intracellular organelles with essential functions in lipid metabolism and

reduction in reactive oxygen species (Ma *et al.* 2011). Given that these organelles are bounded by a single membrane, peroxisomal matrix proteins must be actively imported from the cytosol. The peroxisomal shuttling receptor PEX5 recognizes newly synthesized proteins with a peroxisomal targeting sequence and delivers them to the translocation machinery on the peroxisomal membrane. The PEX5–cargo complex is then imported into peroxisomes, the cargo is released, and PEX5 is recycled back to the cytosol. This process is dependent on monoubiquitylation of PEX5, which occurs at a conserved cysteine residue (C11), rather than at a lysine, and is mediated by the ubiquitin-conjugating enzyme PEX4 and the ligase PEX12 in peroxisomes. The monoubiquitylated PEX5 is extracted by the receptor export module comprising the ATPases PEX1 and PEX6 and the peroxisomal membrane protein PEX26. Monoubiquitylated PEX5 is then deubiquitylated in the cytosol by USP9X, regenerating unmodified PEX5, which initiates another round of peroxisomal matrix protein import (Grou *et al.* 2012). The monoubiquitylation of PEX5 in peroxisomes suggests that other intra-organellar ubiquitylation systems might exist, although no evidence to support this possibility has been obtained to date. Monoubiquitylation of PEX5 in peroxisomes is also unusual in that the target residue is a cysteine.

Regulation of neuronal proteins by monoubiquitylation

Several proteins mutated in neurological disorders are subject to regulation by monoubiquitylation (Table 4). Parkinson's disease is the second most prevalent neurodegenerative disorder, after Alzheimer's disease. It is usually diagnosed after the sixth decade of life and is characterized by motor dysfunction and cognitive decline due to progressive degeneration of dopaminergic neurons in the substantia nigra and other monoaminergic neurons in the brainstem. Genetic analysis has identified >10 genes or loci that are mutated in familial as well as sporadic forms of Parkinson's disease (Corti *et al.* 2011).

Among the genes associated with Parkinson's disease, *PARK2* encodes the E3 ligase Parkin, which mediates polyubiquitylation or monoubiquitylation depending on the substrate. At least five proteins have been shown to be monoubiquitylated by Parkin. Two of these substrates are protein chaperones of the heat-shock protein (Hsp) 70 family—Hsp70 and

Table 4 Monoubiquitylated substrates related to neuronal, metabolic and apoptotic processes

Substrate	Ubi site	E3 ligase	DUB	Effect of monoubiquitylation
Hsp70	Not reported	Parkin	Not reported	Not reported
Hsc70	Not reported	Parkin	Not reported	Not reported
Bcl-2	Not reported	Parkin	Not reported	Stabilization
PICK1	Not reported	Parkin	Not reported	Inactivation
α -Synuclein	K10, K12, K21, K23, K34, K43, K96	SIAH	USP9X	Aggregation
UCH-L1	K4, K65, K71, K157	Not reported	UCH-L1	Self-inactivation
α 4	Not reported	MID1	Not reported	Cleavage
DLG3	SH3 domain	NEDD4, MEDD4-2	Not reported	Localization to apical membrane
CPEB3	Not reported	Neuralized1	Not reported	Activation
PGM-B	Not reported	Not reported	Not reported	Not reported
LDH-A	Not reported	Not reported	Not reported	Lysosomal degradation
Ferritin	Not reported	Not reported	Not reported	Disassembly
Caspase-3/7	Not reported	cIAP2	Not reported	Not reported
DEDD	Not reported	cIAP1, cIAP2	Not reported	Nuclear export
PTEN	K289	NEDD4, XIAP	USP7	Nuclear import

Hsc70—that facilitate the folding of newly synthesized proteins as well as the refolding of misfolded and aggregated proteins (Mayer & Bukau 2005; Moore *et al.* 2008). Although the effects of monoubiquitylation of these proteins are unclear, the observation that the solubility of Hsp70 is reduced in Parkin-deficient brain tissue suggests that monoubiquitylation may be a determinant of protein solubility. Parkin has also been found to have anti-apoptotic effects, which are mediated in part by Parkin-dependent monoubiquitylation and consequent stabilization of the anti-apoptotic protein Bcl-2 (Chen *et al.* 2010). Parkin also attenuates autophagy through Bcl-2 stabilization, consistent with the role of Bcl-2 as an inhibitor not only of apoptosis but also of autophagy (Pattingre *et al.* 2005). Another mechanism by which Parkin inhibits apoptosis was shown by the finding that Parkin monoubiquitylates and thereby inactivates the endocytic adaptor protein EPS15 (Fallon *et al.* 2006). EPS15 is an important regulator of growth factor receptor internalization, as described above, and its inactivation by Parkin therefore enhances survival signaling emanating from cell surface receptors—enhancement that is compromised by Parkin mutations. Excessive stimulation of neurons can lead to cell death as a result of Na⁺ and Ca²⁺ overload. The ion channel-associated protein PICK1 (protein interacting with C-kinase 1) is another substrate for Parkin-mediated monoubiquitylation (Joch *et al.* 2007).

The channel ASIC2a (acid-sensing ion channel 2a) is activated by PICK1, whereas this function of PICK1 is lost as a result of Parkin-catalyzed monoubiquitylation. Given that PICK1 also interacts with several ion channels implicated in neurological diseases (Focant & Hermans 2013), whether Parkin-mediated monoubiquitylation of PICK1 also affects the activity of these channels warrants further investigation.

α -Synuclein, which is encoded by the Parkinson's disease-associated loci *PARK1* and *PARK4*, is a component of the Lewy bodies characteristic of brain tissue in affected individuals. The processes of α -synuclein oligomerization and fibril growth play central roles in the pathogenesis of Parkinson's disease (Lashuel *et al.* 2013) and are influenced by monoubiquitylation. Monoubiquitylation by the E3 ligase SIAH (at K10, K12, K21, K23, K34, K43 or K96) promotes α -synuclein aggregation (Rott *et al.* 2008), whereas deubiquitylation by USP9X attenuates it (Rott *et al.* 2011). Although the mechanism underlying this effect of α -synuclein monoubiquitylation remains unclear, these observations suggest that manipulation of such monoubiquitylation is a potential therapeutic approach to Parkinson's disease.

Another Parkinson's disease-associated protein, UCH-L1, which is encoded by the *PARK5* locus, is regulated by monoubiquitylation. UCH-L1 is one of four members of the UCH (ubiquitin COOH-terminal hydrolase) family of DUB proteins that hydrolyze

small ubiquitin chains or possibly short COOH-terminal extensions of polymeric ubiquitin precursors, with this specificity being due to the confined structure of the active site (Komander *et al.* 2009). The DUB activity of UCH-L1 is negatively regulated by monoubiquitylation at K4, K65, K71 or K157, all of which residues are located near the active site (Meray & Lansbury 2007). Monoubiquitin attached to these lysine residues binds to the ubiquitin binding region of UCH-L1 itself and thereby prevents its association with ubiquitylated substrates. Although not confirmed, the E3 ligase for UCH-L1 monoubiquitylation is likely to be UCH-L1 itself, given that dimerized UCH-L1 shows E3 ligase activity (Liu *et al.* 2002). The DUB for UCH-L1 is also UCH-L1. These observations indicate that UCH-L1 regulates its own activity by cycles of intramolecular monoubiquitylation and deubiquitylation, but the role of such autoregulation remains unclear. Importantly, the physiological substrates of UCH-L1 are still unidentified, with the result that the mechanism of UCH-L1-related Parkinson's disease pathogenesis is also unknown. Possible insight into the pathological function of UCH-L1 has been provided by *Gad* (gracile axonal degeneration) mice, which do not express UCH-L1 as a result of a corresponding gene mutation (Saigoh *et al.* 1999). The axonal degeneration apparent in these mice suggests that UCH-L1 is essential for functional maintenance of axons.

Intellectual disability is a developmental brain disorder characterized by limitations in both intellectual functioning and adaptive behavior. The skewed male-to-female ratio of affected individuals and the identification of families showing X-linked disease segregation are suggestive of the presence of causative genes on the X chromosome. Indeed, >100 such genes have been identified to date (Piton *et al.* 2013). The products of at least two validated X-linked intellectual disability genes are involved in monoubiquitylation. Loss-of-function mutation of the E3 ligase MID1 thus causes Opitz syndrome, one form of X-linked intellectual disability. MID1 forms a ternary complex with the protein $\alpha 4$ and the catalytic subunit of protein phosphatase 2 (PP2c) on microtubules, with $\alpha 4$ being necessary for PP2c stabilization. However, MID1-catalyzed monoubiquitylation of $\alpha 4$ at unidentified lysine residues induces its cleavage at Phe²⁵⁵-Gly²⁵⁶ by calpain, negating its effect on PP2c stabilization (Watkins *et al.* 2012). Interestingly, MID1 was also shown to polyubiquitylate PP2c and thereby to mark it for degradation (Troddenbacher *et al.* 2001). However,

the cleavage of $\alpha 4$ induced by MID1 releases MID1 from the ternary complex, suggesting that other E3 ligases are responsible for PP2c polyubiquitylation in the absence of associated MID1.

Mammalian cells contain four DLG (disks large) family proteins that play an important role in the establishment of epithelial cell polarity at the basolateral membrane below the apical junction (Roberts *et al.* 2012). The *DLG3* gene is located on the X chromosome and is mutated in some individuals with X-linked intellectual disability. Monoubiquitylation of DLG3 by the E3 ligases NEDD4 and NEDD4-2 at unidentified lysine residues in its Src homology 3 (SH3) domain was shown to be required for the binding to motor proteins that recruit DLG3 to the apical membrane at the site of tight-junction formation (Van Campenhout *et al.* 2011). Interestingly, other DLG proteins (DLG1, DLG2, DLG4) do not bind to NEDD4 or NEDD4-2, indicating that regulation of localization by monoubiquitylation is specific to DLG3. Whether monoubiquitylation of DLG3 is relevant to the pathogenesis of intellectual disability remains unknown.

Efficient neurotransmission is dependent on specialized neuronal structures including the cell body (soma), dendrites and axon. In general, neurons receive signals from other neurons at dendrites and then transmit this information through the soma to the axon, the terminal of which makes contact with dendrites of other neurons. Formation of an appropriate structure is thus of utmost importance for neuronal function. Although no mutations in the corresponding gene have been associated with neurological diseases, the E3 ligase Neuralized1 has been shown to regulate the development of dendrites by monoubiquitylation. Neuralized1 monoubiquitylates CPEB3, which binds mRNA and therefore regulates translation. This monoubiquitylation of CPEB3 results in its stabilization and activation and the consequent induction of CPEB target proteins such as the GluA1 and GluA2 subunits of AMPA-type glutamate receptors as well as the formation of new dendritic spines (Pavlopoulos *et al.* 2011). Overexpression of Neuralized1 in mouse brain enhances memory formation and synaptic plasticity in parallel with up-regulation of GluA1 and GluA2 expression and formation of functional synapses. Neuralized1 is induced by neuronal activity and thereby promotes the function of active neurons by monoubiquitylation of CPEB3. The ubiquitylation sites and DUBs for CPEB3 remain to be identified.

Regulation of metabolism by monoubiquitylation

In a screen for cancer-specific ubiquitylated proteins, monoubiquitylated phosphoglycerate mutase-B (PGM-B) was detected in colorectal cancer specimens but not in normal colon tissue, suggesting that PGM-B monoubiquitylation may play a role in colorectal carcinogenesis (Usuba *et al.* 2001). PGM is an enzyme of the glycolytic pathway and catalyzes the reversible conversion of 3-phosphoglycerate to 2-phosphoglycerate. It is a dimeric protein with two different subunits, PGM-B and PGM-M, and the combination of subunits is tissue dependent—with PGM-BB being present in colorectal tissue and PGM-MM in skeletal muscle, for example. Although PGM enzymatic activity is higher in colorectal cancer than in adjacent normal tissue (Durany *et al.* 1997), the possible role of monoubiquitylation in this difference has not been reported (Table 4). Another glycolytic enzyme, lactate dehydrogenase (LDH), supplies NADH by reducing NAD⁺ in parallel with oxidation of pyruvate, the final product of glycolysis, under conditions of high energy demand associated with anaerobic metabolism. LDH forms a tetramer from combinations of three different subunits—LDH-A, LDH-B, and LDH-C—giving rise to six different isoforms: LDH-B4 (LDH1), LDH-A1B3 (LDH2), LDH-A2B2 (LDH3), LDH-A3B1 (LDH4), LDH-A4 (LDH5) and LDH-C4. As with PGM, the expression of these three subunits is tissue dependent, with LDH-A being expressed in skeletal muscle and LDH-B in cardiac muscle, for example. A screen for proteins ubiquitylated in response to oxidative stress in skeletal muscle identified monoubiquitylated LDH-A (Onishi *et al.* 2005). Oxidative stress induced a transient increase in LDH-A monoubiquitylation, and this effect was prolonged by treatment with a lysosomal protease inhibitor, suggesting that LDH-A monoubiquitylation induces lysosomal sorting and consequent degradation of LDH-A. Whether other isoforms of LDH are also regulated by monoubiquitylation remains unknown.

Iron plays essential roles in various biochemical activities such as oxygen transport, energy metabolism and demethylation of DNA and histones. Given that iron is also a hazardous biometal, its metabolism is tightly regulated. After its delivery to cells by transferrin, iron is transported into mitochondria for the synthesis of heme or iron-sulfur clusters as well as to the nucleus for DNA and histone demethylation. Excess iron is stored and detoxified in cytosolic nanocages

consisting of 24 heavy and light ferritin subunits (Wang & Pantopoulos 2011). In response to iron deficiency, ferritin nanocages are disassembled and degraded by the proteasome and lysosomes. This disassembly process was reported to be promoted by ferritin monoubiquitylation (De Domenico *et al.* 2006).

Collectively, these various observations implicate monoubiquitylation in the regulation of metabolic processes. However, it seems premature to conclude that monoubiquitylation plays a prominent role in the control of metabolism on the basis of these few examples uncovered to date.

Regulation of apoptosis by monoubiquitylation

Apoptosis is a form of programmed cell death by which multicellular organisms eliminate excess or damaged cells. The caspase family of proteases plays an essential role in the execution of apoptosis. Apoptotic caspases have been categorized as initiators (caspase-2, caspase-8, caspase-9, and caspase-10) and effectors (caspase-3, caspase-6, and caspase-7). Two effector caspases, caspase-3 and caspase-7, were shown to be monoubiquitylated by the E3 ligase cIAP2 *in vitro* (Huang *et al.* 2000) (Table 4). Although XIAP, a paralog of cIAP2, directly binds to and inhibits the activation of caspase-3/7, neither cIAP1 nor cIAP2 appear to share this function (Eckelman *et al.* 2006). However, cIAP2-deficient macrophages were found to be more susceptible to apoptosis than wild-type cells (Conte *et al.* 2006), suggesting that cIAP2 might inhibit caspase-3/7 activation by monoubiquitylation in these cells. Curiously, cIAP1 was shown to polyubiquitylate and thereby to promote the proteasomal degradation of caspase-3/7, whereas the polyubiquitylation activity of cIAP2 toward caspase-3/7 in cells was much weaker (Choi *et al.* 2009). Both cIAP1 and cIAP2 monoubiquitylate DEDD (death effector domain-containing DNA binding protein) in the nucleolus and thereby regulate apoptosis. Monoubiquitylation of DEDD induces its translocation to the cytosol, where it facilitates caspase-3/7 activation (Lee *et al.* 2005). These findings indicate that cIAP1/2 exert both anti-apoptotic and proapoptotic functions through monoubiquitylation. The ubiquitylation sites and DUBs for caspases and DEDD have not been identified. As for its role in metabolic control, there is currently too little evidence to judge the importance of monoubiquitylation in the regulation of

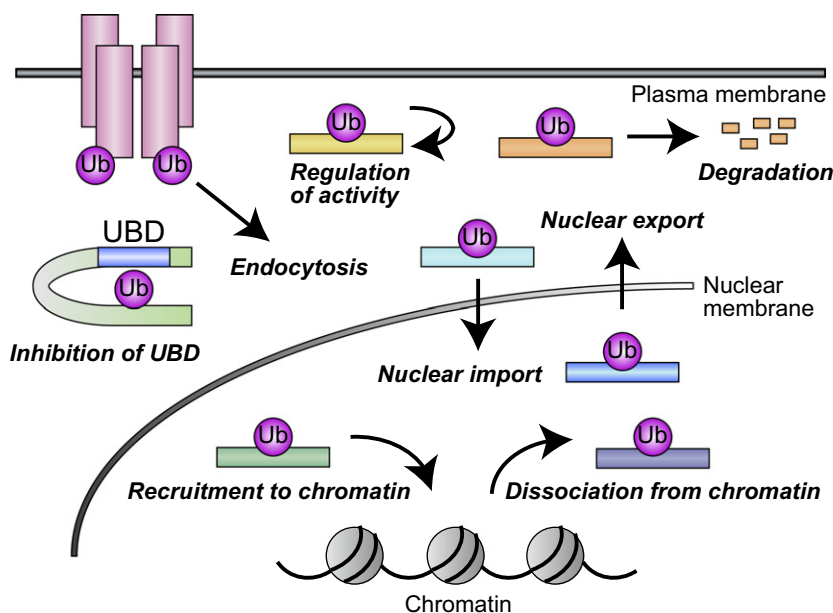


Figure 2 Representative functions of monoubiquitylation. Ub, ubiquitin.

apoptosis. Both cIAP1 and cIAP2 were recently shown to inhibit another form of programmed cell death, necroptosis, by an unknown mechanism (McComb *et al.* 2012), suggesting that monoubiquitylation might also contribute to the regulation of this death pathway.

Regulation of PTEN by monoubiquitylation

Phosphatase and tensin homologue (PTEN) is a tumor suppressor that is frequently lost or mutated in a variety of human tumors. The primary substrate of PTEN phosphatase activity is phosphatidylinositol 3,4,5-trisphosphate localized in the plasma membrane, which is required for membrane recruitment and activation of the protein kinase AKT. PTEN thus antagonizes AKT-dependent cellular activities such as survival, growth and proliferation (Hopkins *et al.* 2014). However, PTEN also has nuclear functions that are thought to be regulated by monoubiquitylation.

There are at least four E3 ligases for PTEN [NEDD4, XIAP, WWP2, TRIM27 (also known as RFP)], among which NEDD4 and XIAP were shown to monoubiquitylate PTEN at K289 and to induce its nuclear translocation (Trotman *et al.* 2007; Van Themsche *et al.* 2009) (Table 4). Ubiquitin fusion rescued the nuclear import defect of the K289E mutant of PTEN, indicative of a key role for monoubiquitylation in nuclear import. NEDD4

knockout did not substantially affect the nuclear localization of PTEN (Fouladkou *et al.* 2008), whereas XIAP-deficient cells show reduced amounts of PTEN in the nucleus, suggesting that XIAP is the major E3 ligase for regulation of the nuclear localization of PTEN by monoubiquitylation. Importantly, these E3 ligases also polyubiquitylate PTEN and thereby mark it for degradation. Although the mechanisms underlying the regulation of monoubiquitylation vs polyubiquitylation of PTEN remain to be determined, the expression level of E3 ligases might play an important role, analogous to regulation of p53 by MDM2. Two DUBs have been found to regulate the ubiquitylation of PTEN: USP7 targets monoubiquitylated PTEN to counteract its nuclear translocation (Song *et al.* 2008), whereas USP13 removes polyubiquitin from and thereby stabilizes PTEN (Zhang *et al.* 2013). The different subcellular localizations of these DUBs—USP7 in the nucleus and USP13 in the cytosol—may allow the targeting of different populations of ubiquitylated PTEN, a scenario that is again reminiscent of p53 regulation by DUBs.

Concluding remarks

We have here provided an overview of studies addressing the substrates and functions of monoubiquitylation. These studies have uncovered several characteristic functions of monoubiquitylation such as

regulation of the subcellular localization, activity and stability of protein targets (Fig. 2). Future work should allow many of the 'Not reported' entries in Tables 1–4 to be replaced with the relevant information.

As a post-translational protein modification, monoubiquitylation is similar to acetylation, which is also reversible, targets lysine residues as well as the NH₂-terminal methionine, and regulates the subcellular localization, activity and stability of substrate proteins (Xiong & Guan 2012). Studies that address the relation between monoubiquitylation and acetylation might be expected to shed light on the biological and functional differences between these modifications.

One of the biggest unsolved problems in the ubiquitin field is how the ubiquitin conjugation machinery distinctively conjugates monoubiquitin or ubiquitin chains onto the substrate proteins. We consider several potential strategies, based on the characters of substrates, E3 ligases, DUBs or trans-acting factors such as UBD proteins. First, only monoubiquitylation might be allowed due to the structural restriction of substrate. Second, E3 ligases might only conjugate single ubiquitin molecule due to its low processivity. Third, monoubiquitylation could be the most preferred form in the dynamic equilibrium between ubiquitylation and deubiquitylation. Fourth, several DUBs might only deubiquitylate ubiquitin–ubiquitin linkage but not be able to remove ubiquitin directly conjugated to the substrate. Fifth, monoubiquitin on the substrate might be immediately recognized by UBD protein which prevents further ubiquitin from being attached to the monoubiquitin moiety. In some cases, the choice of E2s might also contribute to mono-, but not poly-, ubiquitylation (Ye & Rape 2009; Ramanathan & Ye 2012).

Another important issue to be resolved is how monoubiquitin conjugated to a protein target is interpreted for subsequent functional changes. For some UBD-containing proteins, such monoubiquitin moieties engage in an intramolecular interaction with the UBD and thereby prevent it from binding to other monoubiquitylated proteins. Monoubiquitin recognition by UBD proteins clearly contributes to regulation of protein function (Husnjak & Dikic 2012). Characterization of the mechanisms by which different UBD proteins recognize their cognate monoubiquitylated proteins will be key to gaining further insight into such regulation. The diverse outcomes of monoubiquitylation indicate that the surrounding structure of the monoubiquitin moiety is also recog-

nized. Another biochemical consequence of monoubiquitylation is structural interference, as exemplified by inhibition of the binding of SMAD3 to DNA. In this instance, no UBD protein is required, and this mechanism of action might be more prevalent than is currently appreciated.

Compared with the study of polyubiquitylation, whose function in most cases is to mark a protein for degradation, research on monoubiquitylation has progressed more slowly, which is due in part to the more diverse functions of this modification as well as to methodological challenges. Knowledge of the functions of monoubiquitylation uncovered to date, as surveyed in this review, may serve as the basis for hypothesis generation regarding the role of novel instances of protein monoubiquitylation. Forced ubiquitin fusion has provided key insights into the function of monoubiquitylation for some proteins but not others, the latter probably as a result of structural differences between artificially fused and native monoubiquitylated conjugates. New methodological approaches that allow specific modification of target lysine residues with monoubiquitin may circumvent such problems. Manipulation of E3 ligases or DUBs as a means to uncover the functions of monoubiquitylation might lead to changes in the ubiquitylation level of unrelated proteins, whereas lysine mutation might affect not only ubiquitylation but also other modifications such as acetylation, stressing the necessity of caution in practicing these methods.

Given the large number of monoubiquitylated proteins estimated by proteomics data, many such proteins remain to be identified and characterized. The identification of novel targets of monoubiquitylation should be facilitated by large-scale proteomics studies of ubiquitylated sites and proteins based on mass spectrometry. One such study, based on the fact that the abundance of monoubiquitylated histones is decreased in cells treated with a proteasome inhibitor, identified >20 monoubiquitylated nonhistone proteins that were also down-regulated in response to such treatment (Kim *et al.* 2011). With such technical advances in hand, more monoubiquitylated proteins and their functions are certain to be identified, and such knowledge will lead to an increased understanding of the monoubiquitin world.

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