

“Ubiquitylation: mechanism and functions” Review series

Dynamic survey of mitochondria by ubiquitin

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Abstract

Ubiquitin is a post-translational modifier with proteolytic and non-proteolytic roles in many biological processes. At mitochondria, it performs regulatory homeostatic functions and contributes to mitochondrial quality control. Ubiquitin is essential for mitochondrial fusion, regulates mitochondria-ER contacts, and participates in maternal mtDNA inheritance. Under stress, mitochondrial dysfunction induces ubiquitin-dependent responses that involve mitochondrial proteome remodeling and culminate in organelle removal by mitophagy. In addition, many ubiquitin-dependent mechanisms have been shown to regulate innate immune responses and xenophagy. Here, we review the emerging roles of ubiquitin at mitochondria.

Keywords biogenesis; dynamics; mitochondria; mitophagy; ubiquitin

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See the Glossary for abbreviations used in this article.

Introduction

The 76-amino acid protein ubiquitin is the founding member of the ubiquitin-like (UBL) protein family that is known for its regulatory functions in a large variety of different cellular pathways in the cytoplasm and nucleus [1,2]. Ubiquitin can be covalently attached to target proteins as a single moiety, but can also form chains through internal lysine residues [3]. As several lysines in ubiquitin can be used, many different chain types are possible, which have distinct cellular functions [4]. Ubiquitin chains linked by its lysine 48 (K48) are best known as a signal for degradation by the ubiquitin proteasome system (UPS), whereas regulatory functions have been attributed to other types of chains [5]. Deubiquitylases (DUBs), which remove ubiquitin chains, render ubiquitylation reversible and offer possibilities for regulation [6]. Ubiquitylation begins with the activation of the modifier by E1 enzymes, followed by its transfer to E2-conjugating enzymes, and its ligation to the target substrate by E3 ubiquitin ligases [3]. Three major types of E3 ligases have been described, the HECT, the RING, and the RING-between-RING (RBR)

ligases [4,7]. HECT ligases covalently accept ubiquitin before transferring it to the substrate, whereas RING ligases promote substrate ubiquitylation by bringing together the E2 and the substrate and RBR ligases have a RING/HECT hybrid mechanism of action.

More recent studies have demonstrated that ubiquitin orchestrates mitochondrial functions [8,9]. Mitochondria are unique organelles, bound by two membranes, and harboring their own DNA (mtDNA), which encodes essential respiratory chain subunits and therefore enables energy production. Several subcompartments can be distinguished within mitochondria, the outer membrane (OM), intermembrane space (IMS), inner membrane (IM), and matrix. The electron flow through the mitochondrial electron transport chain in the IM builds up an electric potential difference between the matrix and the IMS sides. This difference in proton concentration is then converted into ATP production by the ATP synthase. Besides being the ATP powerhouse, mitochondria are essential for a number of other metabolic pathways, including the synthesis of iron-sulfur clusters and phospholipids. Moreover, mitochondria participate in many cellular processes, such as apoptosis, calcium buffering, aging, cellular differentiation, and antiviral immune responses [10–12].

Here, we review our current knowledge on how ubiquitylation influences mitochondrial activities. On the one hand, ubiquitylation maintains mitochondrial homeostasis and regulates interorganelle communications and developmental programs. On the other hand, ubiquitylation is essential for pathogen defense and, under stress, for mitochondrial quality control (QC) and mitophagy.

Ubiquitin is a master regulator of mitochondrial dynamics

Mitochondria are highly dynamic organelles, the morphology of which is dictated by balanced fusion and fission events [10–14] (Fig 1A). The predominance of one over the other leads to a range of morphologies, from an interconnected mitochondrial network to the presence of a multitude of small, dispersed mitochondria. Mitochondrial fission is driven by Drp1 (Dnm1 in yeast), whereas fusion requires mitofusins—Mfn1 and Mfn2 in mammals and Fzo1 in yeast—in the OM and OPA1 (Mgm1 in yeast) in the IM. All three belong to a special class of GTPases, the dynamin-related proteins (DRPs), which provide the mechanical forces necessary for membrane remodeling [15,16]. Drp1/Dnm1 is a cytosolic protein that is recruited to mitochondria when fission is initiated and forms spirals around mitochondrial tubules

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Glossary

AIP4	atrophin-1-interacting protein 4	Mfb1	mitochondria-associated F-box protein 1
APC/C	Anaphase-promoting complex/cyclosome	Mfn	mammalian mitofusin
ATP	adenosine triphosphate	Mgm1	mitochondrial genome maintenance 1
BAG4	BCL2-associated athanogene 4	MIA	mitochondrial intermembrane space import and assembly
CARD	caspase activation and recruitment domains	Miro1,2	mitochondrial Rho GTPase 1,2
Cdc48	cell division cycle 48	MITOL	mitochondrial ubiquitin ligase
Cdh1	cadherin 1	Mmm1	maintenance of mitochondrial morphology
CMTA	Charcot–Marie–Tooth type 2A	mtDNA	mitochondrial DNA
CUL4	cullin 4	NEMO	NF- κ B essential modulator
Dnm1	yeast dynamin-related protein 1	Opa1	optic atrophy 1
DRP	dynamin-related proteins	PARIS	Parkin interacting substrate
Drp1	mammalian dynamin-related protein 1	Park2	autosomal recessive juvenile Parkinson disease-2
DUB	deubiquitylating enzyme	PARL	presenilin-associated rhomboid-like
ER	endoplasmic reticulum	PCBP2	poly(rC) binding protein 2
ERAD	endoplasmic reticulum-associated degradation	PGC1-α	peroxisome proliferator-activated receptor γ coactivator 1- α
ERMES	ER-mitochondria encounter structure	Pink1	PTEN-induced putative kinase 1
Fis1	mitochondrial fission 1	RHOT1,2	ras homolog family member T1,2
Fzo1	yeast mitofusin, fuzzy onions homolog 1	RIG-1	retinoic acid-inducible gene 1
Gp78	glycoprotein 78	RING	really interesting new gene
GTPase	guanosine triphosphate hydrolase	RNF5	RING finger protein 5
HBX	hepatitis B virus protein X	SCF	Skp1/Cullin/F-box protein
HECT	homologous to the E6-AP carboxyl terminus	siRNA	small-interfering RNA
HIV-1	human immunodeficiency virus 1	SMURF1	SMAD-specific E3 ubiquitin protein ligase 1
HR1	heptad repeat 1	SUMO	small ubiquitin-related modifier
HSP70	heat shock 70-kDa protein	TOM	translocase of the outer mitochondrial membrane
HSPA1L	heat shock 70-kDa protein 1-like	TRAF	TNF receptor-associated factor
Huwe1	HECT, UBA, and WWE domain containing 1	UBL	ubiquitin-like
IRF3	interferon regulatory transcription factor 3	Ubp	ubiquitin-specific protease
JNK	Jun N-terminal kinase	UBX	ubiquitin regulatory X domain
LUBAC	linear ubiquitin chain assembly complex	USP30	ubiquitin-specific protease 30
MAPL	mitochondrial-anchored protein ligase	VCP	valosin-containing peptide
March5	membrane-associated ring finger (C3HC4) 5	Vms1	VCP/Cdc48-associated mitochondrial stress-responsive
MAVS	mitochondrial antiviral signaling protein	Vpr	viral protein R
Mcl1	myeloid cell leukemia sequence 1		
Mdm	mitochondrial distribution and morphology (10,12,30,34)		

(Fig 1B). Interestingly, endoplasmic reticulum (ER) tubules were found to wrap around mitochondria, marking the sites for mitochondrial division by Drp1/Dnm1 [17]. Mitofusins are exposed to the cytoplasm but anchored to the OM by two transmembrane domains [18] (Fig 1B). In contrast, Opa1/Mgm1 is present in the IM and IMS and therefore does not contact the cytoplasm [18].

A number of regulatory mechanisms that fine-tune opposing fusion and fission events have been described. Phosphorylation and ubiquitylation of mitofusins modulate the fusion of mitochondrial membranes [13]. In turn, mitochondrial fission is regulated by various post-translational modifications of Drp1, including phosphorylation, S-nitrosylation, SUMOylation, glycosylation, and ubiquitylation [14]. Recent groundbreaking discoveries identified proteins and protein complexes that link mitochondria to the cytoskeleton and to both the cell cortex and the ER, also contributing to the proper segregation of mtDNA [19–23] (Fig 1B). Interestingly, increasing evidence suggests that ubiquitylation by the same E3 ubiquitin ligases regulates fusion, fission, motility, and ER contacts of mitochondria. This interdependent regulation of dynamic morphological changes allows an efficient response to different physiological challenges.

Mitochondrial fusion

Mitofusins show a distinct ubiquitylation pattern that is conserved throughout evolution from yeast to mammals [24–26]. Mutations in

Mfn2 cause the Charcot–Marie–Tooth type 2A peripheral neuropathy, and ubiquitylation and degradation of mitofusin was suggested to be implicated in the disease [27]. Once it was recognized that mitofusins are ubiquitylated and degraded [24–26,28–34], several E3 ligases and DUBs acting on yeast, fly, and mammalian mitofusins were identified [13]. The analysis of these players revealed that ubiquitin is a double-faced regulator of mitochondrial fusion (Fig 1C). On the one hand, ubiquitylation triggers the conserved, proteasome-dependent degradation of mitofusins [24–26,28–37] (Fig 1C, lower panel), leading to the inhibition of mitochondrial fusion, which fragment due to ongoing fission events. On the other hand, the ubiquitylation of mitofusins plays a positive, critical role for mitochondrial fusion [38–41] (Fig 1C, upper panel) as it can also activate them [40–44] (Sidebar A).

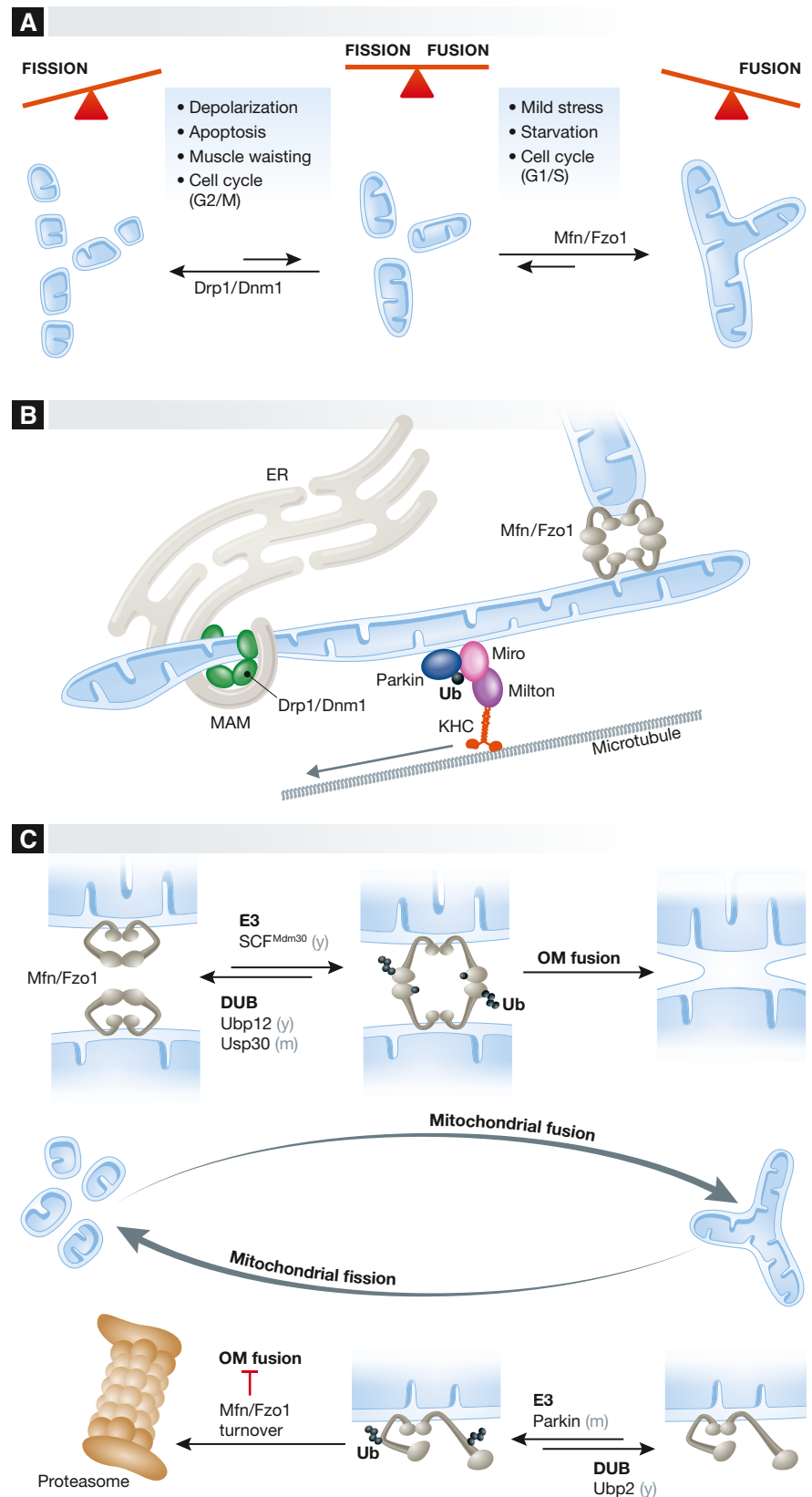
The most detailed mechanistic insight into how ubiquitylation of mitofusins promotes fusion is currently available for yeast. Fzo1 is ubiquitylated by the E3 ligase SCF^{Mdm30} and deubiquitylated by Ubp12, which are soluble proteins that also associate with mitochondria [24,40,45,46] (Fig 1C, upper panel). The positive role of Fzo1 ubiquitylation in fusing mitochondria appears to be linked to Fzo1 oligomerization [40] (Fig 1C, upper panel). In analogy to other DRP-mediated membrane remodeling events [15,16], mitochondrial fusion requires the self-assembly of yeast and mammalian mitofusins [38,39,47]. In addition, OM fusion depends on the inter-

Figure 1. Mitochondrial dynamics and interconnectivity in the cell.

(A) Balanced fusion and fission events establish a large variety of mitochondrial network topologies in different cells and tissues and in response to various stimuli. (B) Mitochondria are physically tethered: to each other via mitofusins during fusion, to the mitochondrial transport machinery via Miro proteins, and to the ER via various protein complexes to allow the exchange of metabolites and signaling molecules and to mark sites for mitochondrial fission by Drp1. (C) Ubiquitylation of mitofusins has a dual function in yeast and mammals. Constitutive ubiquitylation, depending on the E3 ligase SCF^{Mdm30} and the DUB Ubp12 in yeast and USP30 in mammals, promotes mitochondrial fusion. In contrast, ubiquitylation of mitofusins inhibits mitochondrial fusion upon growth arrest in yeast or in response to mitochondrial depolarization in mammals. The E3 ubiquitin ligase Parkin and other ligases ubiquitylate Mfn1 and Mfn2, marking them for proteasomal turnover. The DUB Ubp2 was identified in yeast to reverse Fzo1 ubiquitylation resulting in its stabilization. See Glossary for the other definitions and text for details.

molecular cross-talk between Fzo1 monomers: The conserved lysine 464 is initially ubiquitylated and K48-linked ubiquitin chains are subsequently formed on lysine 398 of a different Fzo1 monomer [40,43] (Fig 1C, upper panel). In mammals, ubiquitylated forms of Mfn1 and Mfn2 that promote mitochondrial fusion have been recently shown to be deubiquitylated by USP30 [41]. USP30 was known to localize to mitochondrial OM and regulate mitochondrial elongation [48]. Now, inhibition of USP30 was shown to efficiently revert the mitochondrial fusion defects of the single Mfn1- and Mfn2-knockout cell lines [41]. This strongly suggests that in mammals, both mitofusins are ubiquitylated in order to allow for fusion, in line with the absolute requirement of Fzo1 ubiquitylation for mitochondrial fusion in yeast [40]. Importantly, oxidized glutathione promotes disulfide-bond-mediated oligomerization of mammalian Mfn1 and Mfn2 and thereby directly activates mitochondrial fusion [49]. This suggests that cysteine- and lysine-mediated covalent modifications of mitofusin together orchestrate mitochondrial fusion (Sidebar A).

The pathway that triggers mitochondrial fragmentation by ubiquitylating and marking mitofusin for degradation by the proteasome has been longer known (Fig 1C, lower panel). In yeast, proliferation arrest by cell cycle blockage at the G1 phase



induces Fzo1 proteolytic breakdown and organelle fragmentation, independently of the SCF^{Mdm30} [28,29]. In mammals, ubiquitylation and turnover of Mfn1 and/or Mfn2 occurs under various stress conditions—such as apoptosis, depolarization of mammalian mitochondria, and muscle atrophy—but also during progression through the cell cycle [32,34,36,50]. Parkin and Gp78—an ER membrane-anchored ubiquitin E3 ligase involved in the ERAD pathway—ubiquitylates both Mfn1 and Mfn2 after mitochondrial depolarization or upon apoptosis induction [26,31,32,51] (Fig 1C, lower panel). Parkin is encoded by *Park2*, whose loss-of-function mutations are associated with early onset of Parkinson's disease (PD), the most common neurodegenerative movement disorder [52]. Therefore, Parkin—a cytosolic RBR E3 ubiquitin ligase [53–55]—has been subject to extensive research. Notably, once ubiquitylated by Parkin, mammalian and fly mitofusins were shown to be recognized and extracted from the membrane by the p97/VCP/Cdc48 AAA-ATPase, and subsequently degraded by the proteasome [32,48,56]. Cdc48 and its co-factor Vms1 play a role in the QC of OM proteins in worms and yeast, which lack Parkin [57]. Upon oxidative stress, Vms1 translocates to mitochondria and appears to contribute to the degradation of mitochondrial OM proteins [57]. However, the involvement of Vms1 in the turnover of Fzo1 is controversial [57,58] (Sidebar A).

Specific ubiquitylation and turnover of one of the two mammalian mitofusins has also been described [34,36,50] (Sidebar A). Genotoxic and other stresses were shown to induce an apoptotic response that requires JNK-dependent phosphorylation and subsequent ubiquitylation of Mfn2 by Huwe1—a HECT family ubiquitin ligase also termed Mule/ARF-BP1/HectH9/E3Histone/Lasu1. This was the first demonstration of the relevance of mitofusin phosphorylation [34]. MAPL/Mul1 also leads to the specific ubiquitylation of Mfn2 in response to muscle-wasting stimuli, targeting it to the proteasome [50]. MAPL is a SUMO E3 ligase integral to the OM, with two TM domains and a carboxyl terminal RING domain facing the cytosol [59,60]. MAPL sumoylates Drp1 and regulates mitochondrial fission [60]. Although suggested to exert ubiquitin ligase activities as well [50,59,61], *in vitro* studies demonstrated a clear preference of MAPL for SUMO instead of ubiquitin [60]. Equally unknown is why the E3 ligase March5/MITOL specifically ubiquitylates Mfn1 but not Mfn2, in this case during the G2/M phase of the cell cycle [36,62]. March5, a RING-type E3 ubiquitin ligase, harbors four transmembrane domains embedded in the OM and an amino terminal RING domain facing the cytosol [63,64]. March5-mediated ubiquitylation and degradation of Mfn1 leads to mitochondrial fragmentation, perhaps to facilitate equal partitioning of cellular material to the two daughter cells, overcoming the hyperfused giant network formed during G1/S phase [36]. The control of mitochondrial morphology by the degradation of mitofusins could thus play an important role in the regulation of cell proliferation and differentiation [65].

Mitochondrial fission

Drp1/Dnm1 is a major hub for the regulation of mitochondrial fission. Several physiological and pathological stimuli lead to post-translational modifications of Drp1, which allow the coupling of mitochondrial division to mitosis and orchestrate the response to hypoxia, apoptosis, and mitophagy [14,66]. The best-studied Drp1 modifications are SUMOylation and multiple phosphorylation events

mediated by several kinases [14], but Drp1 ubiquitylation has also been observed. Interestingly, the hyperfused giant mitochondrial network formed during G1/S phase discussed above also seems to involve Drp1 ubiquitylation by the APC/C^{Cdh1} E3 ubiquitin ligase complex, a central regulator of the M to G1 phase transition [67]. In addition to APC/C^{Cdh1}, the ubiquitin E3 ligases Parkin and March5/MITOL have been shown to modify Drp1 and one of its membrane anchors, Fis1.

Parkin induces the proteasomal degradation of Fis1 and Drp1 [68,69], which can be co-immunoprecipitated with ubiquitin [68], suggesting that Parkin ubiquitylates Drp1 and Fis1. These observations, combined with the inverse correlation between Parkin expression levels and Drp1-dependent mitochondrial fragmentation [70], indicated that ubiquitin and Parkin are inhibitory for mitochondrial fission. Interestingly, a similar role was attributed to March5/MITOL, which interacts with Drp1 and human Fis1, leading to their ubiquitylation and proteasomal-dependent turnover [63,64]. These observations, combined with an elongation of mitochondrial tubules upon March5 overexpression and a fragmentation of mitochondria upon March5 inactivation, led to the initial proposal that March5 is an inhibitor of fission [63,64]. However, the model that March5 ubiquitylates Drp1 to induce its proteolytic turnover, thus elongating mitochondria due to unopposed fusion events, is controversial. In fact, the opposite mitochondrial phenotypes were observed in a latter study, including the formation of long and interconnected mitochondria upon March5 inactivation [71]. In this study, mutations in the RING domain of March5 decreased the cellular mobility of Drp1, suggesting that March5 promotes the ubiquitin-dependent recruitment of Drp1 to mitochondria [71]. Consistently, the expression of a March5 variant with a mutant RING domain restored tubular mitochondria in *Mfn1*^{-/-} and *Mfn2*^{-/-} cells, as expected from the simultaneous inhibition of mitochondrial fusion and fission [71]. In conclusion, although mitochondrial fission is clearly regulated by ubiquitylation of Drp1 and Fis1, further studies are required to dissect the roles and mechanisms involved.

Mitochondrial transport

Mitochondrial dynamics are particularly important in highly polarized cells, such as neurons, which depend on the long-range transport of mitochondria to ensure energy supply [72–74]. Mitochondria must be transported from the cell body to neurites, properly sustained, and removed if damaged. In fact, more than one quarter of the total mitochondria are actively moving, leading to constant changes in mitochondrial density in different synapses [72–74].

Mitochondrial transport occurs along microtubule tracks that are linked to mitochondria by the Miro, Milton, and kinesin heavy chain motor complex [72–74]. Miro1/RHOT1 and Miro2/RHOT2 are integral Rho-like GTPase proteins in the OM, each containing two GTPase motifs and a pair of EF hands involved in calcium binding [75,76]. Milton is an adaptor protein that binds to both Miro and the kinesin heavy chain motor complex, and thereby loads mitochondria onto microtubules for anterograde axonal transport [75,77]. Mitochondrial motility is regulated by the stability of the adaptor protein Miro. Mitochondrial depolarization results in the ubiquitylation of Miro by Parkin, which triggers its proteolytic breakdown [78–80]. Loss of Miro releases the mitochondria–motor complex bridge, arresting mitochondrial movement. Mfn2 was found to inter-

act with both Miro and Milton proteins [81], possibly explaining the correlation of mitochondrial movement with fusion frequency [82]. Consistently, Mfn2 knockdown in neuronal culture cells impairs mitochondrial mobility. Similarly, mutations in Mfn2 associated with CMT2A impair the axonal transport of mitochondria [83] (Sidebar A).

Mitochondria-ER interactions

The existence of a special domain of the ER that is in contact with mitochondria—termed mitochondria-associated membranes (MAM)—is long known [84]. MAMs are enriched in enzymes of the lipid metabolism, as well as in proteins involved in calcium buffering, but also Mfn2 and Drp1 were found at the mitochondrial-ER contact sites [85]. MAMs physically associate with mitochondria via protein tethers, such as the yeast ER-mitochondria encounter structure (ERMES), which is composed of the ER protein Mmm1 and the mitochondrial OM proteins Mdm34, Mdm10 and Mdm12 [19]. Mdm34 is ubiquitylated by the SCF^{Mdm30} [86]. Both *MDM30* and *MDM34* were originally found in a screen for genes implicated in mitochondrial morphology defects [45,87]. However, how ubiquitylation affects the function of Mdm34 is not yet known. Interestingly, in addition to SCF^{Mdm30}, mitochondrial morphology is also regulated by the E3 ubiquitin ligase SCF^{Mfb1} by an unknown mechanism [46,88].

In addition to the ERMES complex, a number of other proteins have been proposed to tether ER to mitochondria in mammalian cells, including Mfn2 [89–92]. Moreover, March5/MITOL was shown to interact with the mitofusins Mfn1 and Mfn2 [62,64]. A potential link between these findings stems from the observation that Mfn2 ubiquitylation by March5/MITOL contributes to interdependent functions of MAM and mitochondria [93]. March5/MITOL specifically catalyzes the formation of K63-linked ubiquitin chains

on Mfn2 K129, which is not conserved in Mfn1 [93]. Notably, the ubiquitylation-deficient mutant Mfn2^{K129R} still interacts with March5/MITOL [93], suggesting that there might be other roles for March5/MITOL in Mfn2 regulation. Mfn2 ubiquitylation by March5/MITOL does not affect its turnover, but rather affects ER-mitochondrial contacts and calcium exchange. Interestingly, the most frequent mutation in Mfn2 found in CMT2A patients, R94Q, is incompetent in promoting ER-mitochondria interaction [92]. These results suggest that March5/MITOL could be involved in CMT2A pathogenesis. The relevance of Mfn2 for ER-mitochondrial interactions is highlighted by the finding that the HIV-1 Vpr protein participates in the ubiquitylation and turnover of Mfn2 by the CUL4 E3 ligase, consequently leading to reduced ER-mitochondrial contacts [94]. These studies demonstrate that ubiquitin regulates mitochondria-ER contacts in different ways and that the E3 ubiquitin ligases that participate in cell cycle progression also modulate organelle contacts (Sidebar A).

Ubiquitin and mitochondrial quality control

Ubiquitin contributes to mitochondrial QC in various ways. The UPS mediates the proteolytic breakdown of nuclear-encoded pre-proteins before their import into mitochondria [95,96], whereas mitochondrial proteases degrade damaged proteins present within the organelle [97] (Fig 2A). Severe damage or depolarization of mitochondria triggers the recruitment of Parkin to the mitochondrial surface [98], where it ubiquitylates OM proteins initiating their proteasomal degradation [99–101] and culminating in mitophagy, the selective autophagic removal of the whole organelle [102–104] (Fig 2B). Finally, ubiquitin participates in apoptosis via the regulation of the integral OM protein Mcl1, as reviewed recently [105].

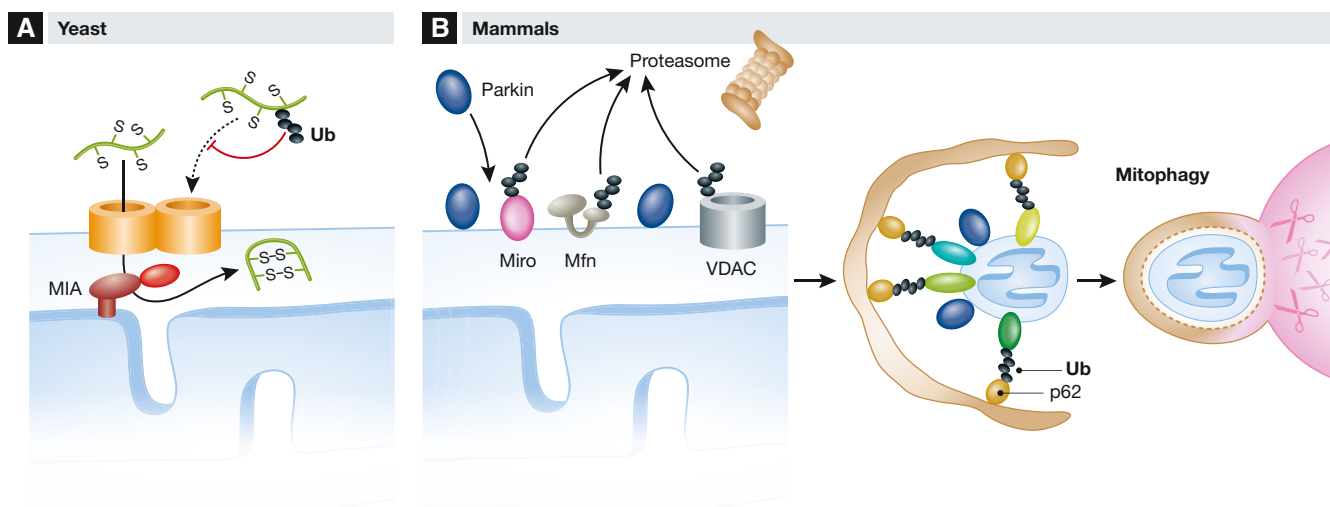


Figure 2. Role of ubiquitin in the quality control of mitochondrial proteins.

(A) Regulation of mitochondrial proteostasis by the UPS in yeast. Nuclear-encoded mitochondrial precursor proteins, such as substrates of the MIA pathway, are ubiquitylated and degraded by the UPS in the cytosol, limiting their accumulation in mitochondria. (B) Ubiquitin-dependent mitophagy of dysfunctional mitochondria in mammals. The recruitment of the E3 ubiquitin ligase Parkin to the OM of depolarized organelles leads to the ubiquitylation and proteasomal degradation of OM proteins, such as Miro, Mfns, and VDAC, inhibiting various processes, including mitochondrial transport or fusion. It also triggers mitophagy through the recruitment of adaptor factors for the autophagy machinery, such as p62. Ub, ubiquitin. Mfns, mitofusins. See Glossary for the other definitions and text for details.

Mitochondrial biogenesis

The vast majority of mitochondrial proteins are encoded by the nucleus and synthesized in the cytosol prior to their import into mitochondria. Dedicated machineries are present in each mitochondrial subcompartment that recognize, import, and sort newly imported proteins to their final destination within the organelle [106]. Several cytosolic kinases phosphorylate the protein import machinery in the OM, demonstrating that post-translational modifications regulate mitochondrial biogenesis, at least in yeast [107]. Ubiquitylation and proteasomal degradation, on the other hand, appear to control the influx of precursor proteins from the cytosol to mitochondria, as has been recently demonstrated for IMS proteins in yeast [96]. IMS proteins are imported by the MIA pathway through a folding-trap mechanism. The UPS regulates the mitochondrial proteome by constitutively ubiquitylating and degrading MIA substrates, suggesting that it can act as a negative regulator of mitochondrial biogenesis (Fig 2A). This is also consistent with the ubiquitylation of many nuclear-encoded mitochondrial proteins observed in proteome-wide studies [108–111] (Sidebar A).

Removal of damaged mitochondria through mitophagy

Pioneering work from the Youle laboratory has established a central role for the Ser/Thr kinase Pink1 and Parkin in the selective removal of damaged mitochondria [98]. Pink1 is an unstable mitochondrial IM protein that is rapidly degraded by the rhomboid protease PARL after import and maturation in healthy mitochondria [112]. A C-terminal proteolytic fragment is released from the IMS to the cytoplasm and degraded by the UPS through the N-end rule pathway [113]. Loss of membrane potential prevents the import of Pink1 into mitochondria and results in its accumulation at the OM and Parkin recruitment [98]. Pink1 activates Parkin, promoting the formation of a Parkin-ubiquitin thioester intermediate [114], perhaps by directly phosphorylating Parkin [115]. Parkin then ubiquitylates OM proteins, triggering their degradation by the UPS and thereby reshaping the protein composition of the mitochondria [99–101] (Fig 2B). Notably, if stabilized by proteasomal inhibitors, mature Pink1 can directly regulate cytosolic Parkin by binding to its RING1 domain, inhibiting Parkin activity [116,117]. This suggests a new regulatory layer of the Pink1–Parkin complex that awaits the elucidation of whether at least some processed Pink1 is spared from degradation in healthy cells.

A recent genome-wide siRNA screen for factors affecting Parkin recruitment to mitochondria revealed an essential role of TOMM7, a subunit of the TOM complex, the protein translocase in the OM [118]. Binding of Pink1 to the TOM complex stabilizes it at the OM and promotes the recruitment of Parkin to mitochondria [118,119]. Other factors involved in Parkin recruitment are HSPA1L, a widely distributed but low abundant member of the HSP70 family, and BAG4, a putative nucleotide-exchange factor of HSPA1L [118]. Both proteins interact physically with Parkin but have opposing roles in Parkin translocation to the OM [118].

Parkin accumulation at the surface of depolarized mitochondria allows the ubiquitylation of a variety of OM proteins with K48- and K63-linked chains, triggering their proteasomal degradation [99,100] (Fig 2B). A systematic approach—using antibody capture of the diglypeptide combined with SILAC to monitor depolarization-dependent changes in abundance of the targets—recently expanded the knowledge of how Parkin acts on its substrates [101]. Parkin

specificity was shown to be driven primarily by substrate recruitment or proximity, rather than by binding to specific target sequences [101]. In addition, Parkin dramatically altered the ubiquitylation status of the cytosolic domains of OM proteins, dependent on its active site residue C431, which is mutated in some PD patients.

The currently favored view is that general ubiquitylation at the mitochondrial surface of the damaged organelles recruits ubiquitin-binding adaptors, such as p62, which interact with the autophagic machinery [98,99,120,121] (Fig 2B). However, a subset of OM proteins—such as VDAC—have also been suggested to have a specific role in mitophagy [99,122]. An analysis of Parkin-mediated autophagy substrates in flies highlights how specific autophagy can be for individual proteins/complexes [123]. Selective mitophagy was also observed in yeast, which lack Parkin [124], but a role for ubiquitin has not yet been shown. Moreover, different pathways for selective mitophagy exist in mammalian cells [102], and two mitophagy-inducing conditions—oxygen or iron depletion—have been recently described. Hypoxia-induced mitophagy is regulated by FUNDC1 and phosphorylation and occurs in a Parkin- and seemingly ubiquitin-independent manner [125]. In turn, the Parkin-independent iron depletion-induced mitophagy pathway involves a metabolic switch from oxidative phosphorylation to glycolysis without depolarization of the engulfed mitochondria [117,126] (Sidebar A).

Mitochondrial dynamics and mitophagy

The processes of fusion, fission, and movement of mitochondria are intimately linked to mitophagy. Starvation and mild oxidative stress induce hyperfusion of mitochondria and spare them from mitophagy [35,49,127,128] (Fig 1A). In contrast, mitochondrial depolarization triggers mitochondrial fragmentation and mitophagy of the damaged organelles (Fig 1A). It is noteworthy that fission events, independently of stress insults, generate a mitochondrial population with uneven membrane potential, which affects the probability for subsequent fusion events [129,130]. The impaired fusion of depolarized mitochondria allows their segregation from the mitochondrial network as fragments and therefore facilitates their removal by mitophagy. Similarly, damage leads to an arrest of mitochondrial movement in highly differentiated cells, such as neurons (see above) [30–32,79] (Fig 2B). Interestingly, Parkin overexpression led to an increase in life span in flies and this could reflect the beneficial effects of an increased rate of mitophagy [37]. Consistently, it led to a decrease in Mfn2 levels that contributed to clearance of damaged mitochondria and increased mitochondrial activity [37].

Gp78 also activates mitophagy upon mitochondrial depolarization [51]. Although recognizing both Mfn1 and Mfn2 as substrates, Gp78-induced mitophagy depends only on ubiquitylation of Mfn1 [51]. In addition to Parkin and Gp78, the E3 ligase MAPL/Mul1 was recently shown to participate in mitophagy [50]. MAPL-dependent UPS turnover of Mfn2 was shown to facilitate mitophagy during skeletal muscle wasting, a process that is essential for recycling amino acids from proteins of the skeletal muscle [50,131].

Recent evidence suggests that Mfn2 is involved in the QC surveillance of cardiac mitochondria [132]. In the heart, damaged-induced phosphorylation of Mfn2 at Thr111 and Ser442 was found to recruit Parkin to mitochondria. Consistently, mitochondrial dysfunction over time contributes to age-related heart failure, and heart-specific *Mfn2*^{-/-} mice developed cardiomyopathies. However, earlier studies had demonstrated that Parkin can still induce mitophagy in

Mfn1^{-/-} *Mfn2*^{-/-} mouse embryonic fibroblasts [98]. Although it is well established that activation of Parkin triggers mitophagy, whether the turnover of specific proteins such as Mfn2 is necessary remains to be clarified (Sidebar A).

Parkin and Parkinson's disease

PD is an important neurodegenerative disease that affects 1% of the population over 55 years old [133]. Genetic mutations are responsible for about 10% of all PD cases [134]. Mutations in *Park2* cause autosomal recessive forms of PD and account for about 50% of the familial cases and 20% of the early-onset cases of PD [52]. Therefore, impaired mitophagy was suggested to contribute to neurodegeneration [135]. However, it should be noted that the majority of the findings indicating a role of Parkin in mitophagy were obtained in tumor cells upon depolarization of mitochondria and overexpression of Parkin. Parkin translocation to mitochondria was not observed in primary neurons or in mouse models accumulating mitochondria marked with ubiquitin, suggesting that alternative pathways do exist [103,136]. Moreover, even in cases where Parkin recruitment to mitochondria was observed, it did not induce mitophagy [137]. Neither the analysis of PD mice models nor of *park2* KO mice provided supportive evidence for Parkin-mediated mitophagy *in vivo* [138–140]. However, a progressive degeneration of dopaminergic neurons was observed after ablating *park2* in adult mice [141], suggesting that the lack of PD phenotypes in PD mouse models might be explained by developmental compensation [141]. Similarly, the differences in Parkin recruitment and mitophagy induction observed between immortalized cells and neuronal cultures could arise from variations in protocols [142]. For example, Parkin was robustly

recruited to neuronal cells cultured in the absence of antioxidants, which may counteract the action of chemical uncouplers; whether this can cause mitophagy requires further investigation [143].

Many Parkin substrates have already been identified, affecting a wide range of signaling and stress metabolic pathways, which is consistent with the broad neuroprotective capacity of Parkin. For instance, Parkin ubiquitylates and triggers proteolytic breakdown of PARIS, thus releasing the repression of the transcription factor PGC1- α that induces mitochondrial biogenesis [141]. Cellular stress recruits Parkin to the LUBAC complex [144], leading to the formation of linear ubiquitin chains on NEMO. Consequently, OPA1 transcription is upregulated, inhibiting apoptosis [144]. Thus, the relative contribution of various pathways modulated by Parkin to the pathogenesis of PD remains to be clarified (Sidebar A).

Maternal inheritance of mtDNA

In contrast to the nuclear DNA, mtDNA is maternally inherited [145,146]. Several species-dependent mechanisms ensuring selective removal of sperm-derived mtDNA have been described. Sperm-derived mammalian mitochondria are marked with ubiquitin during spermatogenesis, and this was proposed to constitute the specific sorting signal for proteasomal-dependent elimination of male-derived mammalian mitochondria [147]. However, the lysosome has also been suggested to play a role [148]. Indeed, paternal mitochondria are actively disposed of by mitophagy after fertilization at early stages of *C. elegans* embryogenesis [149,150]. Nevertheless, the relevance of these findings for mammals was recently challenged, because elimination of sperm mitochondria in mice was found not to be dependent on autophagy [151]. Although ubiquity-

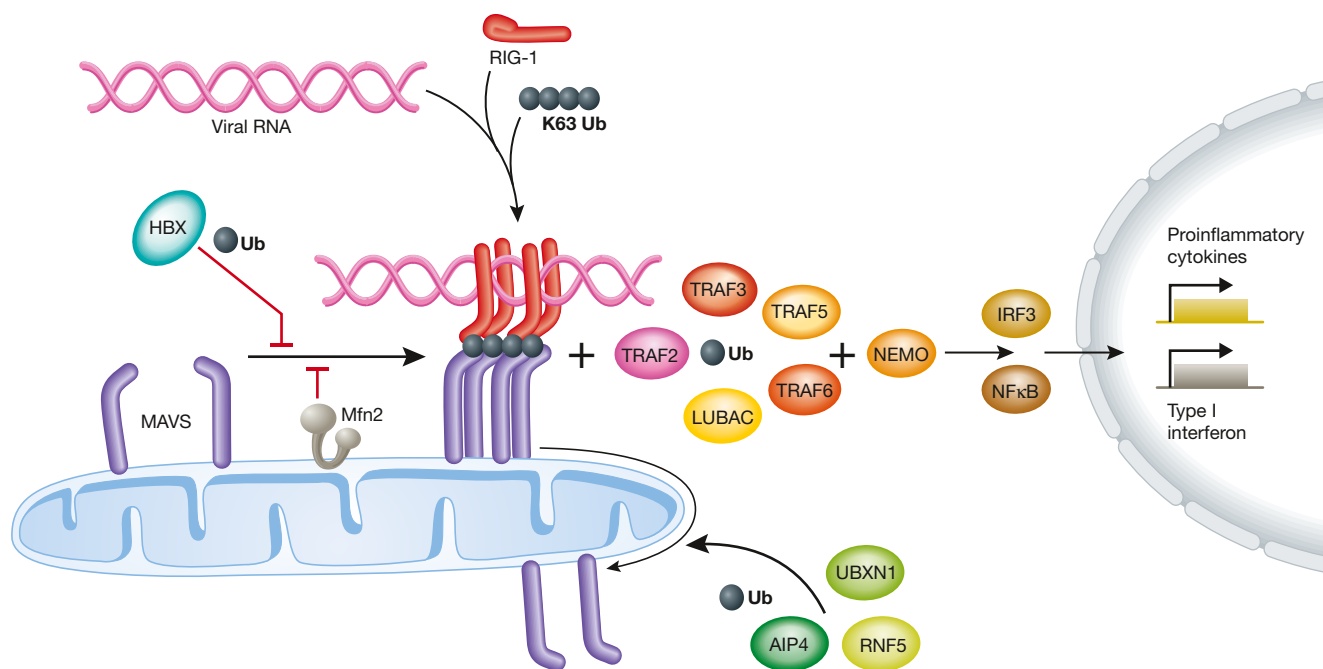


Figure 3. Ubiquitin modulates the antiviral response.

K63-linked ubiquitin chains, viral RNA, and the helicase RIG-1 participate in the assembly of a signal platform on mitochondria that contains MAVS. This allows several E3 ubiquitin ligases to activate LUBAC and TRAF transcription factors, which induce innate immunity genes. Moreover, ubiquitin is required for disassembly of the MAVS complex, terminating signal transduction. On the other hand, ubiquitin and Mfn2 impair MAVS assembly before signaling initiation. Ub, ubiquitin. See Glossary for the other definitions and text for details.

lation of the sperm tail was observed, murine sperm mitochondria were not degraded post-fertilization. Rather, the most motile sperm that reached the oviduct were already depleted of mtDNA. Therefore, although a role of ubiquitin has been established, further studies are required to understand whether mitophagy is generally required to ensure maternal inheritance of mtDNA.

Mitochondria, ubiquitin, and antiviral defense

The innate immune system responds to pathogens such as bacteria and viruses using receptors from two different families, culminating in the activation of the NF- κ B and type I interferon signaling pathways [152] (Fig 3). Double-stranded viral RNA is recognized in the cytoplasm by the RNA helicase RIG-1 [153]. Upon binding to viral RNA, RIG-1 is activated and exposes its CARD signaling domains, which associate with unanchored K63-linked ubiquitin chains. This leads to the formation of heterotetramers that interact with and activate the mitochondrial OM protein MAVS, a central player of the defense response (Fig 3). MAVS dimers or higher-order multimers recruit several downstream signaling effectors, such as the TRAF family members, which trigger the production of type I interferons and other cytokines [154–156]. MAVS polymers were recently shown to bind the E3 ubiquitin ligases TRAF2, TRAF5, TRAF6, and LUBAC, which collectively promote ubiquitylation reactions that recruit NEMO to the MAVS signaling complex, leading to the activation of the transcription factors IRF3 and NF- κ B [157] (Fig 3).

The majority of the mechanisms that terminate the antiviral response involve the proteolytic breakdown of MAVS. For example, the RNA-binding protein PCBP2 negatively regulates the immune response by recruiting the E3 HECT ligase AIP4 to MAVS, leading to its ubiquitylation and UPS-dependent turnover [158]. Similarly, the E3 RING ligase RNF5 attaches K48-linked ubiquitin chains at K362 and K461 of MAVS, targeting it for degradation after viral infection [159]. Interestingly, several viruses suppress the innate immune response, for example, by expressing specific proteases or—in the case of hepatitis B virus—by interaction of its protein X (HBX) with MAVS, thus targeting it for UPS-dependent degradation [160] (Fig 3).

The disruption of MAVS self-interactions precludes MAVS interaction with the TRAF proteins and represents another mechanism of curtailing MAVS activation [161]. The UBXN1 protein, a member of the ubiquitin-binding UBX protein family, binds to MAVS via its ubiquitin-associated domain and impairs antiviral responses [161]. Mfn2 has also been proposed to inhibit MAVS oligomerization by binding to it via its HR1 region [156,162], which is in line with the fact that *Mfn2*^{-/-} mouse embryonic fibroblasts have an increased antiviral response [162] (Fig 3).

Interestingly, most of the genes involved in the immune response to Sindbis virus are required for Parkin-mediated mitophagy, pointing to an intimate relationship between mitophagy and xenophagy, which is the autophagic degradation of incoming pathogens [163]. The E3 ubiquitin ligase SMURF1, for example, was identified as a mediator in both xenophagy and mitophagy [163]. Mitophagy is also related to the autophagic eradication of bacteria, because the innate immune response to bacterial infection involves Parkin [164]. Thus, multiple functions of ubiquitylation at the surface of mitochondria are emerging, acting upstream and downstream of MAVS during the immune response (Sidebar A).

Sidebar A: In need of answers

- (i) How are ubiquitylated mitofusins diverted from proteasomal turnover to promote mitochondrial fusion? Is ubiquitylation of Mfn1 and Mfn2 critical for embryonic viability?
- (ii) How are ubiquitylated OM proteins degraded by 26S proteasomes? Which additional components are involved and how are they regulated?
- (iii) Which properties distinguish Mfn1 from Mfn2, making them specific targets for selective ubiquitylation in apoptosis, muscle wasting, and cell cycle progression?
- (iv) How are mitochondrial fusion and fission coordinated by ubiquitin and other post-translational modifications, for example, during the cell cycle? How does ubiquitin regulate cell proliferation and differentiation?
- (v) Is mitochondrial fusion controlled by the range of post-translational modifications that regulate mitochondrial fission? Conversely, how does ubiquitylation regulate mitochondrial fission?
- (vi) How does ubiquitin regulate interorganellar contacts of mitochondria?
- (vii) To which extent does the UPS-dependent turnover of mitochondrial precursor proteins in the cytosol contribute to the regulation of mitochondrial biogenesis?
- (viii) What is the precise role of ubiquitylation and OM protein turnover for mitophagy? How does it affect aging and neurodegeneration?
- (ix) What is the contribution of Pink1/Parkin-mediated mitophagy to mitochondrial quality control *in vivo*? How is Parkin activity regulated? What are the pathophysiological implications of the modification of its different targets?
- (x) How critical is ubiquitylation of mitochondrial proteins for the cellular immune response?

Conclusions

Ubiquitin serves as an important regulator of mitochondrial dynamics, surveys mitochondrial damage, and regulates innate immune responses in many ways. The ubiquitylation and turnover of several OM proteins contributes to maintaining mitochondrial homeostasis. We are only beginning to understand the emerging regulatory functions of ubiquitin, such as its essential role in promoting mitochondrial fusion or its wide participation in the initiation or termination of the innate immune response. The considerable research efforts in

“Ubiquitylation: mechanism and functions” Review series

Previous issues of EMBO reports include:

- Building and remodeling Cullin-RING E3 ubiquitin ligases, by Wade Harper *et al*
- Ubiquitin in the immune system, by Henning Walczak *et al*
- RBR E3 ligases at work, by Judith Smit and Titia Sixma

Other reviews in this series, which will be published in consecutive issues of EMBO reports, will cover:

- Regulation of stem cell function by protein ubiquitylation, by Iannis Aifantis *et al*
- Understanding ubiquitylation one structure at a time, by Ronald Hay *et al*

this active field hold the promise for more surprises to come, completing our picture of this master regulator of mitochondria. Future studies will shed light on the pathophysiological mechanisms of mitochondria-related diseases and thus define the contribution of the different processes regulated by ubiquitin, possibly identifying new therapeutic targets.

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Conflict of interest

The authors declare that they have no conflict of interest.

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