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REVIEW

The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier

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ABSTRACT

The ubiquitin-related modifier Urm1 can be covalently conjugated to lysine residues of other proteins, such as yeast Ahp1 and human MOCS3, through a mechanism involving the E1-like protein Uba4 (MOCS3 in humans). Similar to ubiquitination, urmylation requires a thioester intermediate and forms isopeptide bonds between Urm1 and its substrates. In addition, the urmylation process can be significantly enhanced by oxidative stress. Recent findings have demonstrated that Urm1 also acts as a sulfur carrier in the thiolation of eukaryotic tRNA via a mechanism that requires the formation of a thiocarboxylated Urm1. This role is very similar to that of prokaryotic sulfur carriers such as MoaD and ThiS. Evidence strongly supports the hypothesis that Urm1 is the molecular fossil in the evolutionary link between prokaryotic sulfur carriers and eukaryotic ubiquitin-like proteins. In the present review, we discuss the dual role of Urm1 in protein and tRNA modification.

KEYWORDS Urm1 system, tRNA modification, Ub-like protein modification

INTRODUCTION

Ubiquitin and ubiquitin-like proteins (UBLs) are small post-translational modifiers that regulate a multitude of cellular processes by covalently conjugating to target substrates (Ulrich, 2002; Pickart and Fushman, 2004; Sun and Chen, 2004). UBLs are characterized by a conserved C-terminal diglycine and a core β-grasp-fold structural motif (Furukawa et al., 2000). Known and putative UBLs are listed in Table 1 (Hochstrasser, 2009; Humbard et al., 2010; Miranda et al., 2011). Although not all UBLs have distinct sequence identities with ubiquitin, they seem to share a similar cascaded enzyme reaction pathway. In the presence of ATP, the C-terminal diglycine motif of UBLs is activated by adenylation, which is catalyzed by the activating enzyme E1 and subsequently forms a high-energy thioester bond with the E1 catalytic cysteine (Ciechanover et al., 1981; Haas et al., 1982). Afterward, activated UBLs are transferred to the conjugating enzyme E2 via transthioesterification, and finally form a covalent isopeptide bond with the ε-amine of the lysine residues of their respective protein substrates, with the help of the ligase E3 (Fig. 1) (Hershko et al., 2000; Pedrioli et al., 2008).

The eukaryotic ubiquitin-related modifier (Urm1) was discovered in 2000 via BLAST analysis of several prokaryotic sulfur carrier proteins such as MoaD and ThiS (Furukawa et al., 2000). Compared with other UBLs, Urm1 shows significantly higher sequence identities with sulfur carriers. In *Escherichia coli*, MoaD and ThiS (Fig. 1) play crucial roles in biosynthetic pathways, with MoaD being involved in molybdenum cofactor (Moco) biosynthesis (Lake et al., 2001) and ThiS in thiamin biosynthesis (Wang et al., 2001). In contrast to the pathways of eukaryotic UBLs, prokaryotic sulfur carriers diverge after the initial ATP-dependent adenylation activation by forming an acyl disulfide bond with their E1s. As a consequence, a thiocarboxylate is formed to provide sulfur for subsequent reactions (Xi et al., 2001; Iyer et al., 2006). Since 2008, several independent groups have described the novel function of Urm1 as a sulfur carrier in the 2-thiolation modification of 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) (Fig. 2B) (Huang et al., 2008; Nakai et al., 2008; Schlieker et al., 2008; Leidel et al., 2009; Noma et al., 2009). Results in these studies strongly suggest that Urm1 activation is highly similar to those of prokaryotic sulfur carrier proteins. Urm1 forms an acyl disulfide bond with its E1 (Uba4 in budding yeast, MOCS3 in humans), leading to the formation of a thiocarboxylated Urm1 (Fig. 1) (Schmitz et al., 2008).

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Table 1 Known and putative UBLs and their activating and conjugating enzymes

UBL*	Identity with Ub (%)	E1*	E2*	Comments on UBL
Known UBLs				
Ubiquitin	100	Uba1 (UBA6)	Many	Precursors encoded by multiple genes
Rub1 (NEDD8)	55	Uba3–Ula1	Ubc12	Substrates are cullins and p53
FUBI	38	NI	NI	Derived from a ribosomal-protein precursor
FAT10	32 and 40†	UBA6	NI	Contains a β-grasp fold, substrates unknown
ISG15	32 and 37†	UBE1L	UBCH8	Production induced by type I interferons
Smt3 (SUMO1, SUMO2, SUMO3)	18	Uba2–Aos1	Ubc9	SUMO encoded by 3–4 genes in vertebrates, depending on the species
Atg8	ND	Atg7	Atg3	Three known isoforms in humans Contains a β-grasp fold
Atg12	ND	Atg7	Atg10	~20% identical to Atg8
Urm1	ND	Uba4	NI	Involved in tRNA modification Contains a β-grasp fold
UFM1	ND	UBA5	UFC1	Contains a β-grasp fold
SAMP1, SAMP2	ND	UbaA	NI	Archaeal modifier proteins Contains a β-grasp fold
Putative UBLs				
BUBL1, BUBL2	Variable (up to 80%)	NI	NI	Putative autoprocessed proteins in ciliates
UBL-1	40	NI	NI	A precursor to ribosomal proteins in nematodes
SF3A120	30	NI	NI	UBL domain at C terminus No data about conjugation
Oligoadenylate synthetase	30 and 42†	NI	NI	UBL domain at C terminus No data about conjugation

ND, not detectable by standard BLAST searches. NI, not identified.

* UBLs are listed as the yeast (Saccharomyces cerevisiae) symbol if the UBL is present in yeast, otherwise vertebrate symbols are listed. Known vertebrate orthologues with symbols that differ from yeast proteins are listed in parentheses. SAMP1 and SAMP2 are proteins in archaeal. For E1s and E2s, yeast symbols are listed if the protein is found in yeast.

† The identities listed are for each of two ubiquitin-related domains.

Data in this table is adapted from the article published by Mark Hochstrasser (2009).

Urm1 is the most ancient UBL and plays a number of important roles in yeast bioprocesses (Table 1) such as budding (Goehring et al., 2003b), nutrient sensing (Rubio-Texeira, 2007), high temperature sensitivity (Furukawa et al., 2000), antioxidant stress response (Goehring et al., 2003a), and post-translational modification of the elongator subunit (Fichtner et al., 2003). Like UBLs, Urm1 also contains the C-terminal diglycine and β-grasp-fold structural motif. Previous studies have demonstrated that it can be covalently conjugated to a single substrate, Ahp1p, in budding yeast, mediated by the E1-like protein Uba4 (Furukawa et al., 2000; Goehring et al., 2003a). Recently, Van der Veen et al. (Van der Veen et al., 2011) significantly expanded this conclusion by revealing the specific oxidative stress-induced process of Urm1-protein conjugation in both yeast and human cells, and detected the 21 human proteins modified by Urm1 in response to oxidative stress. These include proteins involved in the Urm1/Ub pathway, nuclear transport, RNA binding and processing, oxidative stress, and tRNA modification (Table 2). Through a series of biochemical experiments, they confirmed a covalent linkage between Urm1 and the lysine residues of its protein substrates. These linkages are largely resistant to the reducing agents DTT and hydroxylamine, indicating the high probability that they are isopeptide bonds. Based on these experimental results, the function of Urm1 as a sulfur carrier is directly coupled to its functions as a ubiquitin-like modifier. Urm1 seems to occupy an important place in the evolutionary link between prokaryotic sulfur carrier proteins and eukaryotic UBLs (Fig. 1) and is regarded as a molecular fossil (Xu et al., 2006; Pedrioli et al., 2008; Petroski et al., 2011; Van der Veen et al., 2011).

URM1 SYSTEM COMPONENTS

To date, three independent yeast genome-wide screens have identified the five components responsible for the 2-thiolation modification of mcm^5s^2U and protein urmylation, and these are Urm1, Uba4, Tum1, Ncs6, and Ncs2 (Lu et al., 2005, 2008; Nakai et al., 2008; Leidel et al., 2009). The cysteine desulfurase Nfs1 is believed to be the upstream component in the system and acts as a sulfur donor (Nakai et al., 2004; Marelja et al., 2008), and its essential functions in cell survival

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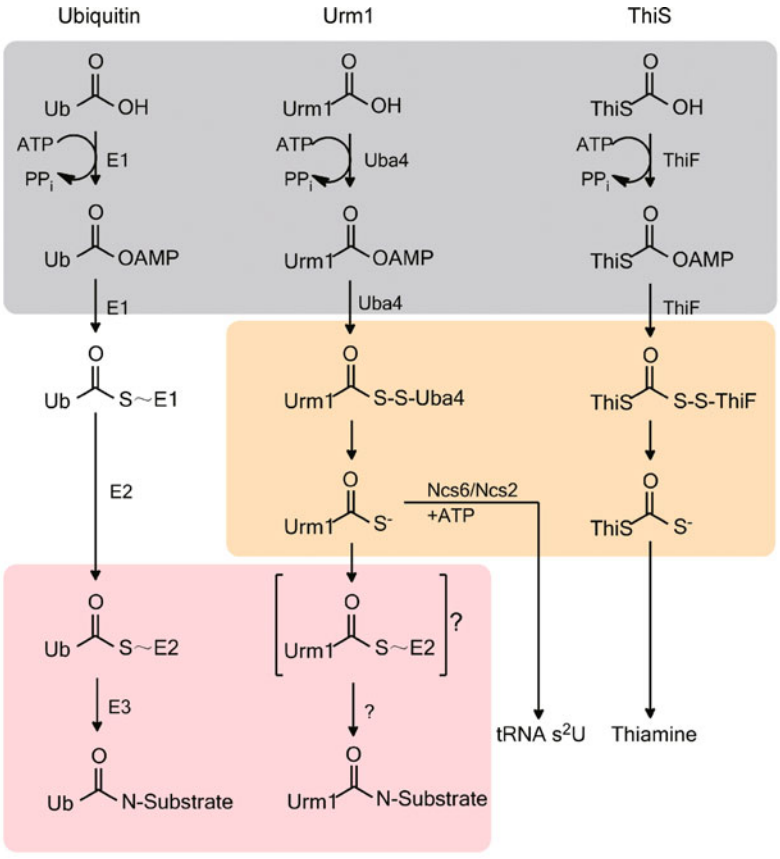

Figure 1. Similarities and differences between the ubiquitin, Urm1, and ThiS systems. The shaded areas show the overlapping parts of these pathways, revealing that Urm1 has features similar to ubiquitin and ThiS in the activation and conjugation process. This figure is a combination of the excellent work done by Petroski et al. (2011) and Pedrioli et al. (2008). No E2 or E3 for Urm1 has been detected, so the existence of an Urm1-E2 intermediate is still hypothetical. However, the isopeptides formed between Urm1 and the substrates have been proven by Van der Veen (2011).

may explain its nondetection in the screens (Nakai et al., 2001).

Among these proteins, Uba4 was discovered via a yeast two-hybrid screen using Urm1 as bait. Uba4 has a distinct sequence similarity to Uba1, an ubiquitin-activating enzyme. Furukawa et al. (Furukawa et al., 2000) demonstrated that Urm1 conjugation depends on the function of Uba4 as its E1-like protein. They found that a DTT-sensitive bond is formed between Uba4 and Urm1, and deduced that this bond may also be a thioester, similar to other UBLs. This conclusion has been recently challenged by the discovery of the presence of the Urm1 C-terminal thiocarboxylate, which suggests that the Urm1-Uba4 complex is bound by a disulfide bridge (Pedrioli et al., 2008; Schmitz et al., 2008; Van der Veen et al., 2011). Uba4 has two conserved cysteines: Cys397 in the C-terminal rhodanese-like domain (RLD) and Cys225 in the MoeB-like domain. Rhodaneses are widespread enzymes that catalyze the transfer of sulfane sulfur atoms from thiosulfate to cyanide and serve as versatile sulfur carriers (Bordo and Bork et al., 2002; Mueller, 2006). Several groups have demonstrated that the two Uba4 cysteines are essential for Urm1 thiocarboxylate formation and tRNA thiolation, but only Cys397 is essential for thiosulfate sulfurtransferase activity (Fig. 2A) (Schmitz et al., 2008; Hochstrasser, 2009; Noma et al., 2009).

Before activating Urm1, Uba4 needs to receive sulfur atoms from Nfs1 and Tum1. Noma et al. (2009) used a series of clever *in vitro* sulfur transfer assays and revealed that by using pyridoxal-5-phosphate (PLP) as a co-factor, Nfs1 accepts sulfur from cysteine to form a persulfide and subsequently transfers this bond to the rhodanese-like domain in Tum1 or Uba4. Sequence alignment has detected two rhodanese-like domains in Tum1, but site-directed mutagenesis demonstrates that only the Cys259 in the C-terminal RLD is essential for persulfide formation. The N-terminal RLD seems inactive, with no conserved cysteines. Thus, Tum1 is believed to be a crucial Nfs1 activator as well as an optional persulfide carrier. Nfs1 can directly transfer persulfide sulfurs to Uba4, and Tum1 can significantly enhance this transfer activity (Fig. 2A) (Bordo and Bork et al., 2002; Noma et al., 2009).

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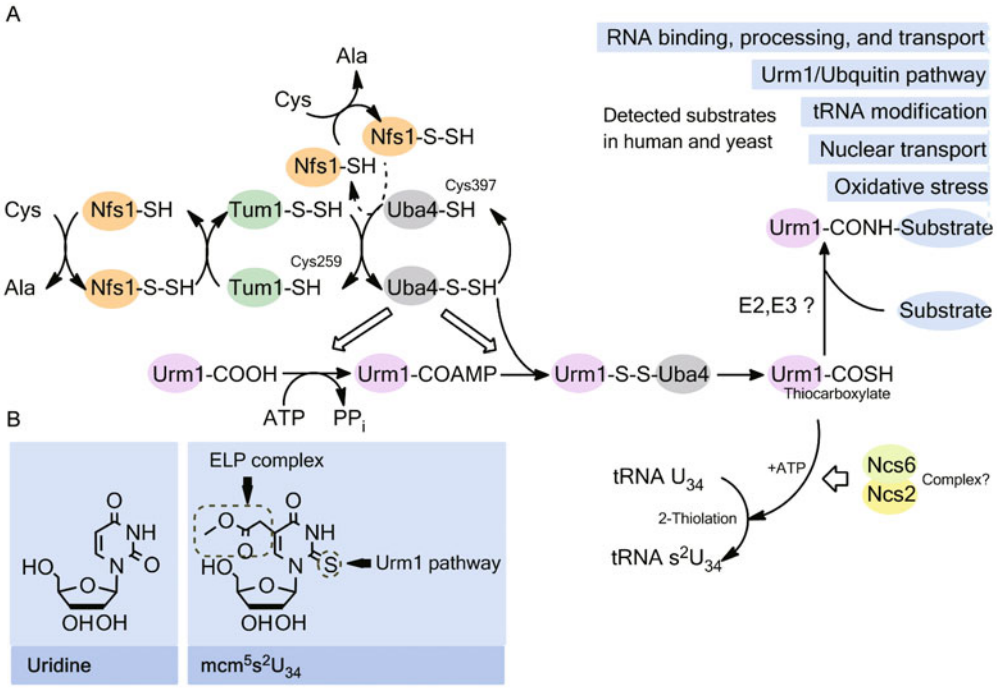

Figure 2. Urm1 pathway involved in the tRNA modification and protein conjugation processes. (A) Overview of the sulfur flow process between the Urm1 system components for 2-thiouridine formation of $mcm^5s^2U_{34}$ and the Urm1 conjugation process with its detected substrates. (B) Structure of uridine and $mcm^5s^2U_{34}$. The Urm1 pathway is responsible for 2-thiolation, whereas the ELP complex is responsible for mcm^5U_{34} modification. These pictures are adapted from the articles published by Pedrioli et al. (2008), Noma et al. (2009), and Van der Veen et al. (2011).

After receiving a sulfur atom from Nfs1 or Tum1, Uba4 activates Urm1 by forming an acyl adenylate intermediate at the Urm1 C-terminal, and then forms an acyl disulfide bond between Uba4 Cys397 and the Urm1 C-terminal glycine (Leidel et al., 2009; Noma et al., 2009; Van der Veen et al., 2011). Afterward, a thiocarboxylated Urm1 is released, which provides the necessary sulfur for subsequent tRNA modification or protein conjugation (Fig. 2A). The Urm1 C-terminal glycine is essential for thiocarboxylate formation, and this has been proven by analyzing Urm1 WT and Urm1 ΔG through a polyacrylamide gel supplemented with [(N-acryloylamino)-phenyl]mercuric chloride (APM-modified gels) (Van der Veen et al., 2011).

Ncs6 and Ncs2 belong to the PP-loop ATPases and are essential for the *in vivo* 2-thiolation modification of mcm^5s^2U, using Urm1-COSH as the substrate. Several articles have reported the essential role of Ncs6 and its homologues in the 2-thiolation of tRNA uridine residues of yeast, worms, and *Thermus thermophilus* (Shigi et al., 2006; Björk et al., 2007; Dewez et al., 2008). *In vitro* binding assay also reveals that recombinant MBP-Ncs6 can directly bind yeast tRNA, whereas MBP-Uba4 cannot (Leidel et al., 2009). The function of Nfs2 in this process remains unclear, because recombinant Ncs2 is insoluble and difficult to test *in vitro*. Dewez, Leidel, and their co-workers (Dewez et al., 2008; Leidel et al., 2009) proposed that the two ATPases may form a complex (Fig. 2A), but this has been challenged by Nakai and his co-workers (Nakai et al., 2008). To date, the specific protein that transfers sulfur atoms from the thiocarboxylated Urm1 has not been identified, and the mechanism behind Ncs6 and Ncs2 recognition of the specific tRNA and catalysis of 2-thiouridine formation has not been established (Noma et al., 2009).

URM1 AS A PROTEIN MODIFIER

As previously mentioned, the function of Uba4 as E1 in the Urm1 conjugation process has been clearly revealed. To date, no Urm1-specific Urm1-conjugating E2 enzyme, Urm1-lignase enzyme E3, and deurmylation enzyme have been detected. Therefore, Hochstrasser (Hochstrasser, 2000) proposed that Uba4 may also function as an E2-like enzyme. However, this hypothesis is challenged by the recent discovery of an acyl-disulfide bond between Urm1 and the conserved cysteine of Uba4 (Schmitz et al., 2008), because a thioester linkage is typically formed in UBL E2 enzymes. Van der Veen et al. (2011) have proven that the C-terminal thiocarboxylated Urm1 is necessary for the conjugation process. No conjugation was detected when Urm1-COSH was replaced with EGFP-COSH, indicating that a thiocarboxylate intermediate is not enough for conjugation formation and

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Table 2 Human protein substrates uniquely modified by Urm1 in response to oxidative stress

Reference	Protein name	Architecture	Diamide	H_2O_2
Urm1/Ub pathway				
NP_660275	ATP binding domain 3	AANH-like II	+	+
NP_055299	Molybdenum cofactor synthesis protein 3	E1-like NBD, RHD	+	+
NP_006304	Ubiquitin specific protease 15	DUSP domain	−	+
NP_060414	Ubiquitin specific protease 47	UCH	+	+
NP_062538	E3 ubiquitin-protein ligase BRE1A	RING-finger	−	+
tRNA modification				
NP_060225	5-methycytoisine methyltransferase	NOL1/NOP2/sun family	+	+
NP_003631	Elongator complex protein 1	IKI3 family	−	+
Nuclear transport				
NP_001307	Chromosome segregation 1-like	CAS_CSE1	−	+
NP_002874	Ran GTPase-activating protein 1	LRR_RI	−	+
NP_006353	Nuclear RNA export factor 1 isoform 1	NTF2	−	+
NP_056046	Nuclear pore complex protein Nup160	Nucleoporin Nup160	−	+
RNA binding, processing, and transport				
NP_056455	Serpine 1 mRNA-binding protein 1	HABP4_PAI-RBP1 family	−	+
NP_001410	ELAV-like protein 1	RNA recognition motif	+	−
NP_002902	Regulator of nonsense transcripts 1	UPF1_Zn_bind family	+	−
NP_006436	Splicing factor Prp8	MPN_PRP8	+	−
NP_004388	ATP-dependent RNA helicase p54	DEAD-box helicases	−	+
Oxidative stress				
NP_000604	Hemopexin precursor	Hemopexin-like repeats	+	−
NP_001748	Carbonyl reductase [NADPH] 1	NADB_Rossmann	−	+
Miscellaneous				
NP_858059	O-GlcNAc transferase p110 subunit	TPR	+	−
NP_001054	Serotransferrin precursor	Transferrin super family	+	−
NP_005555	Migration-stimulating factor inhibitor	Lipocalin super family	+	−

These experiments were conducted by Van der Veen et al. (2011). They identified these urmylation substrates by large-scale affinity purification of Urm1 adducts from HA-Urm1 cells, which were exposed to either diamide or H_2O_2 *in vivo*.

that the recognition of protein substrates by Urm1 is specific; however, the mechanism for this remains unclear at present.

In yeast, the thioredoxin peroxidase protein Ahp1, which plays an essential role in cellular response to oxidative stress (Jeong et al., 1999; Lee et al., 1999), is the only known Urm1 substrate (Goehring et al., 2003a). Recently, Van der Veen et al. (2011) found that oxidative stress can induce Urm1 substrate conjugation in both yeast and human cells (Table 2). Interestingly, both H_2O_2 and diamide can stimulate the conjugation *in vivo*, but their substrates are different. In the present article, 21 proteins have been identified as Urm1 uniquely modified substrates induced by either H_2O_2 or diamide (Fig. 2A; Table 2). Site-directed mutagenesis assay clearly reveals that Urm1 is attached to the specific lysine residues of the substrates. However, the mechanism of this process remains to be determined. Polyurmylation has not been observed among these linkages, and all tested substrates appear to be modified by a single Urm1 molecule, despite the several exposed lysine residues on Urm1 itself (Singh et al., 2005; Xu et al., 2006; Yu and Zhou, 2008).

The known urmylation substrates include two members of the Urm1 pathway, namely, MOCS3 (Uba4 homologue in *Homo sapiens*) and ATPD3 (Ncs6 homologue in *Homo sapiens*). Why Urm1 modifies the components in its own pathway in response to oxidative stress is still unclear (Table 2). Furthermore, two deubiquitinating enzymes, USP15 and USP47, are modified by Urm1; however, immunoblotting assay did not detect their deurmylation activity. Furthermore, several proteins related to nuclear transport, tRNA modification, and RNA processing have been identified, such as the cellular apoptosis susceptibility protein (Behrens et al., 2003), but the functions of these Urm1 modifications remain unknown (Van der Veen et al., 2011). Observation reveals that, unlike ubiquitin, Urm1 does not appear to have a role in targeting proteins for degradation. Although Urm1 may regulate post-translational modification by modifying

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deubiquitinating enzymes, or prevent the translocation of CAS to the nucleus, evidence for this is still lacking (Petroski et al., 2011).

URM1 AS A SULFUR CARRIER IN tRNA MODIFICATION

The Urm1 pathway plays an essential role in the 2-thiolation modification of certain cytosolic tRNA. The sulfur transfer process in the pathway has already been described in detail. tRNA has four canonical bases—adenosine, guanosine, cytidine, and uridine—and also has more than 70 kinds of known post-transcriptional modifications (Huang et al., 2008). These modifications can stabilize tRNA, enhance the accuracy of codon binding, reduce the conformational dynamics (Wang et al., 2007; Agris, 2008), and slightly regulate tRNA interactions with mRNA and the ribosome (Sen and Ghosh, 1976; Björk et al., 2007; Johansson et al., 2008). Among these, the wobble modification of uridine, mcm^5s^2U, is required for the proper decoding of NNR codons in eukaryotes (Noma et al., 2009). This modification is formed via two steps: the oxygen atom at position 2 of the wobble uridine is substituted by a sulfur atom provided by the Urm1 pathway; and position 5 of the uridine residue is modified with a methoxy-carbonyl-methyl by the six-subunit elongator protein (ELP) complex (Fig. 2B) (Svejstrup, 2007; Pedrioli et al., 2008).

Thiocarboxylated Urm1 not only serves as a sulfur donor in the tRNA thiolation process, but also functions as a protein modifier in response to oxidative stress. So what are the relationships between these two functions? Urm1-mediated tRNA modification may be regulated by urmylation of the effective substrate (Huang et al., 2008). Interestingly, an essential part of the ELP complex, the elongator complex protein 1, is among the recently identified urmylation substrates in the presence of H_2O_2 (Van der Veen et al., 2011). This observation suggests a link between tRNA modification and protein urmylation, which needs further evidence.

FUTURE PERSPECTIVES

The past years have witnessed rapid progress in Urm1 pathway research, such as revealing its function as a sulfur carrier and detecting new substrates for Urm1 conjugation. However, several important questions still need to be answered.

First, questions concern the components involved in the tRNA- and protein-modifying branches of the Urm1 system. To date, no Urm1-specific conjugating and ligating enzymes (E2, E3) have been found. How does substrate recognition take place in the absence of a ligase? (Pedrioli et al., 2008) Biochemical experiments have demonstrated that the C-terminal thiocarboxylated EGFP is not sufficient to induce conjugation formation. In addition, given that Urm1 modifies the specific sites in a limited number of proteins, what determines the substrate recognition mechanism of Urm1 remains unclear. Furthermore, we still do not know whether the deurmylation process exists. Although two deubiquitinating enzymes, USP15 and USP47, are modified by Urm1, the author cannot find their activities in reversing urmylation (Van der Veen et al., 2011). Recent findings have revealed the necessary roles of Ncs2 and Ncs6 in the tRNA modification process, but we still do not know how they recognize specific tRNA and catalyze the reaction, and whether they can influence the urmylation level of proteins (Björk et al., 2007). Structural approaches may solve these questions by providing molecular models for these enzymes.

Questions have also been raised on the role of tRNA thiomodification: we still do not know the effect of the Urm1 pathway on protein translation. Translational efficiency and fidelity can be reduced by oxidative stress, which causes tRNA misacylation and impairs the editing activity of threonyl-tRNA synthetase, the crucial enzymes in genetic code translation (Netzer et al., 2009; Ling and Söll, 2010). Thus, Urm1 may act as an important post-translational modifier that alters the balance between tRNA thiolation and protein modification under oxidative stress.

In addition, components in the Urm1 system may be potential drug candidates, because they are essential for tRNA thiolation but are not crucial genes in yeast. Some retroviruses, such as the human immunodeficiency virus type 1 (HIV-1), use the cellular tRNA (Lys 3) as a primer for reverse transcription (Abbink and Berkhout, 2008). *In vitro* assay reveals that the AAAA/$Umcm^5s^2UUU$ loop-loop interaction is essential and conserved in the reverse transcription of HIV-1 isolates, and that dethiolation of the modified nucleotide mcm^5s^2U at position 34 of tRNA3Lys strongly destabilizes this interaction (Isel et al., 1993, 1996). Therefore, further studies on the relevance between the Urm1 pathway and such diseases are needed.

The third question focuses on the function of Urm1 modification during oxidative stress. Based on observations, Urm1 does not appear to have a role in targeting proteins for degradation, and no polyurmylation has been detected. Why its E1 MOCS3, two deubiquitinating enzymes, CAS, and some other important enzymes are modified still remains unclear, because no change was found in the activity of these enzymes after Urm1 conjugation (Petroski et al., 2011; Van der Veen et al., 2011). Although these questions remain unanswered, we now clearly know that Urm1 is an important protein modifier that shares similar features with both sulfur carriers and UBLs.

ABBREVIATIONS

Ahp1, alkyl hydroperoxide reductase 1; ATPD3, ATP-binding domain 3; CAS, chromosome segregation 1-like protein; DTT, dithiothreitol; EGFP, enhanced green fluorescence protein; ELP, elongator protein;

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MBP, maltose-binding protein; mcm^5s^2U, 5-methoxycarbonylmethyl-2-thiouridine; MoaD, molybdopterin synthase subunit; MOCS3, molybdenum cofactor synthesis protein 3; Ncs6/Ncs2, needs cla4 to survive protein 6/2; Nfs1, NIFS-like protein 1; PLP, pyridoxal-5-phosphate; RLD, rhodanese-like domain; ThiS, thiamin biosynthesis; Tum1, thiouridine modification 1; Uba4, ubiquitin-like protein activating protein 4; UBLs, ubiquitin-like proteins; Urm1, ubiquitin-related modifier 1; USP15/USP47, ubiquitin-specific protease 15/47

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