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Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier

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The ubiquitin (Ub)-related modifier Urm1 functions as a sulfur carrier in tRNA thiolation by means of a mechanism that requires the formation of a thiocarboxylate at the C-terminal glycine residue of Urm1. However, whether Urm1 plays an additional role as a Ub-like protein modifier remains unclear. Here, we show that Urm1 is conjugated to lysine residues of target proteins and that oxidative stress enhances protein urmylation in both *Saccharomyces cerevisiae* and mammalian cells. Similar to ubiquitylation, urmylation involves a thioester intermediate and results in the formation of a covalent peptide bond between Urm1 and its substrates. In contrast to modification by canonical Ub-like modifiers, however, conjugation of Urm1 involves a C-terminal thiocarboxylate of the modifier. We have confirmed that the peroxiredoxin Ahp1 is such a substrate in *S. cerevisiae* and found that Urm1 targets a specific lysine residue of Ahp1 in vivo. In addition, we have identified several unique substrates in mammalian cells and show that Urm1 targets at least two pathways on oxidant treatment. First, Urm1 is appended to lysine residues of three components that function in its own pathway (i.e., MOCS3, ATPBD3, and CTU2). Second, Urm1 is conjugated to the nucleocytoplasmic shuttling factor cellular apoptosis susceptibility protein. Thus, Urm1 has a conserved dual role by integrating the functions of prokaryotic sulfur carriers with those of eukaryotic protein modifiers of the Ub family.

posttranslational modification | hydrogen peroxide | nuclear transport | Uba4 | USP15

The ubiquitin (Ub)-related modifier Urm1 is a conserved ubiquitously expressed member of the Ub family (1). Similar to Ub, it comprises a β -grasp fold and terminates with a diglycine motif (2, 3). Ub is a well-known protein modifier involved in a multitude of processes. Ubiquitylation starts with the adenylation of the C-terminal glycine of Ub, followed by transfer of Ub, via a series of thioester intermediates with E1 and E2 (and E3) enzymes, to a lysine residue within a target protein (4–6). Although prokaryotes do not possess a Ub homolog, several prokaryotic proteins adopt a β -grasp fold, including Moad and ThiS (7). There are several mechanistic parallels between the ATP-dependent activation of Ub and Moad/ThiS, although they differ broadly in function (7, 8). Moad and ThiS are thiocarboxylated at their C terminus and serve as sulfur donors in molybdopterin and thiamine synthesis, respectively (8). Urm1 is also thiocarboxylated and functions as a sulfur donor in tRNA thiolation in *Saccharomyces cerevisiae* and mammalian cells, thus resembling prokaryotic sulfur carriers (9–13).

The carboxyl group of the C-terminal glycine in Urm1 is derivatized to a thiocarboxylate by the addition of sulfur. The sulfur atom is mobilized from cysteine and transferred by a series of enzymatic reactions to the sulfurtransferase MOCS3 (Uba4p in *S. cerevisiae*) in the form of a persulfide (14). Next, MOCS3 adenylates the C terminus of Urm1, followed by the transfer of sulfur to the terminal glycine of Urm1 (15). Urm1 associates with the thiouridylases ATPBD3 (also known as CTU1 in *Homo sapiens*

and Ncs6p in *S. cerevisiae*) and CTU2 (Ncs2p in *S. cerevisiae*), which mediate the thiolation of wobble uridines in tRNA^{Lys(UUU)}, tRNA^{Gln(UUG)}, and tRNA^{Glu(UUC)} (9–13, 16–18).

Can Urm1 function as a protein modifier in addition to its role in tRNA thiolation? Early work in *S. cerevisiae* suggested the existence of a few low-abundant proteinaceous adducts under steady-state conditions, but the identity of all but one of these substrates remained unknown (1, 19, 20), as was the nature of the linkage involved. In addition, it is not clear whether adduct formation requires Urm1 in its thiocarboxylated or unmodified form or whether urmylation results in an amide-, thioester-, or acyl disulfide-linked Urm1 conjugate.

Deletion of *URM1* in *S. cerevisiae* results in hypersensitivity toward a variety of stressors, including nutrient deprivation, elevated temperature, and oxidant [diazenedicarboxylic acid bis(*N,N*-dimethylamide) (diamide)] treatment (1, 19–21). The only urmylation substrate identified to date is the peroxiredoxin Ahp1, suggesting a link between the Urm1 pathway and defense mechanisms activated by alterations in redox status of the cell (i.e., by oxidative stress) (20). Oxidative stress occurs when levels of oxidizing radicals exceed the capacity of the cell to reduce and detoxify them. Oxidizing radicals [e.g., hydrogen peroxide (H₂O₂)] are generated during normal intracellular metabolism, but their generation can be triggered by external sources as well. To protect proteins and other molecules against damage from oxidation, cells have developed several antioxidant defense mechanisms (22, 23). Key to these processes is the low-molecular-weight tripeptide reduced glutathione (GSH), which scavenges oxidants by forming disulfide-linked oxidized glutathione (GSSG). Failure to counteract a rise in cellular oxidant levels results in cell damage, ultimately contributing to senescence and age-related diseases (22).

Here, we show that oxidative stress induces conjugation of Urm1 to target proteins in both *S. cerevisiae* and mammalian cells. This reaction requires the C-terminal thiocarboxylate of Urm1. We demonstrate that urmylation resembles ubiquitylation because it likely involves a thioester intermediate and results in the formation of a covalent lysine-linked Urm1 adduct. Using an in

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vitro urmylation assay, we explored the conditions and specificity of oxidant-induced Urm1 conjugation. Proteomic analysis revealed several previously undescribed substrates for urmylation *in vivo* and showed that Urm1 is conjugated to lysine residues in these substrates. Urm1 targets at least two pathways on oxidant treatment; in addition to several components of the urmylation pathway itself, we identified a protein involved in nucleocytoplasmic transport among the most abundant substrates.

Results

Oxidative Stress Induces Conjugation of Urm1 *in Vivo*. To investigate whether Urm1 is conjugated to proteins in mammalian cells, we stably transduced HeLa cells with HA epitope-tagged human WT Urm1 (HA-Urm1 WT) or Urm1 lacking its C-terminal glycine (HA-Urm1 Δ G) (Fig. 1A). We predicted that deletion of the C-terminal glycine of Urm1 would prevent thiocarboxylate formation. To test this, we analyzed the electrophoretic mobility of HA-Urm1 WT and HA-Urm1 Δ G on a polyacrylamide gel supplemented with *N*-acryloylamino phenyl mercuric chloride (APM). APM-modified gels have been used to distinguish electrophoretically between unmodified and sulfur-modified tRNA molecules (24). This compound should be equally capable of differentiating between thiolated and nonthiolated cysteine-free proteins (e.g., HA-Urm1 WT and HA-Urm1 Δ G, respectively). Indeed, immunoprecipitation of Urm1 via its HA epitope, followed by electrophoresis and anti-HA immunodetection, showed that >50% of HA-Urm1 WT is thiocarboxylated, as shown by its slower migration on an APM-containing acrylamide gel (Fig. 1B). In contrast, the electrophoretic mobility of HA-Urm1 Δ G was not

affected by inclusion of APM in the gel, indicating that this mutant indeed lacks a thiocarboxylate. Consequently, thiolation of tRNA^{Lys(UUU)} was reduced >50% in HA-Urm1 Δ G cells, confirming that this mutant competes with endogenous WT Urm1 in a dominant negative manner (Fig. S1A and B). The endogenous copy of Urm1 is present and remains functional.

Analysis of HA-Urm1 WT cells by anti-HA immunoblotting did not show any obvious signs of conjugation under normal conditions, unlike what has been reported in yeast (1, 19, 20). Given the reported sensitivity of Δ urm1 yeast strains toward several stress conditions, however, we hypothesized that urmylation might be a stress-dependent process (1, 19–21). We therefore treated HA-Urm1 WT cells with a variety of cellular stressors. SDS lysates prepared from these cells were analyzed by anti-HA immunoblotting. In addition to free Urm1, we noticed the appearance of a distinct pattern of higher molecular-weight polypeptides in cells treated with the oxidative stressor diamide (Fig. 1C). To confirm that oxidative stress induces urmylation, we treated cells with H₂O₂, a naturally occurring source of oxygen radicals. Urm1 was immunoprecipitated through its HA epitope to enrich for Urm1 adducts. Similar to diamide, H₂O₂ stimulated formation of an Urm1 ladder, although to a lesser degree (Fig. 1D). The appearance of a distinct banding pattern rather than a smear suggests that Urm1 is conjugated to specific sites of a limited number of target proteins. No urmylation was observed when HA-Urm1 Δ G cells were treated with diamide or H₂O₂ (Fig. 1D). Exposure of HA-Urm1 WT or Δ G HeLa cells to diamide or H₂O₂ for a short duration did not affect thiolation of tRNA^{Lys(UUU)} (Fig. S1A and B). In addition, shRNA-mediated reduction of ATPBD3 protein

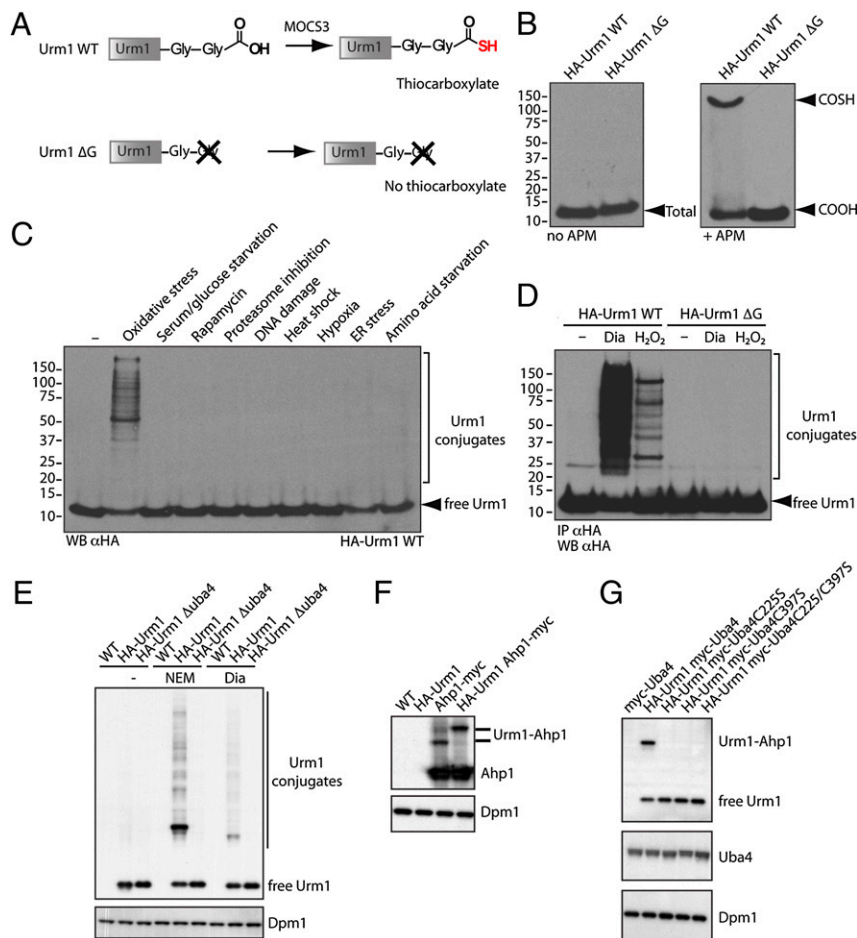


Fig. 1. Urm1 is conjugated on oxidative stress in a thiocarboxylate-dependent manner. (A) Schematic illustration of Urm1. The C-terminal glycine of WT Urm1 (WT) is modified by MOC53 to a thiocarboxylate. Deletion of the C-terminal glycine of Urm1 (Δ G) prevents thiocarboxylate formation. (B) HA-tagged Urm1 WT or Δ G was immunoprecipitated from stably transduced HeLa cells and separated on a polyacrylamide gel (Left) or a polyacrylamide gel supplemented with APM (Right). Transient sulfur-mercury interactions between thiol-containing proteins and APM result in reduced electrophoretic mobility. (C) HA-Urm1 WT-expressing HeLa cells were treated with different stressors (described in *SI Materials and Methods*) and lysed in 1% SDS. Total cell lysates were resolved by SDS/PAGE and subjected to anti-HA immunoblotting. (D) HA-Urm1 WT and HA-Urm1 Δ G HeLa cells were treated with 400 μ M diamide (Dia) or 5 mM H₂O₂ for 10 min and lysed in 1% SDS. HA-Urm1 was immunoprecipitated to enrich for adducts, resolved by SDS/PAGE, and subjected to anti-HA immunoblotting. (E) Immunoblot of HA-tagged Urm1 protein conjugates after treatment (1 h) of yeast cells with NEM (10 mM) or Dia (20 mM). HA-Urm1 is expressed from its chromosomal locus in WT or Δ uba4 cells. (Lower) Dpm1 was used as a loading control. (F) Urmylation of myc-tagged Ahp1. Cells expressing either Ahp1-myc or HA-Urm1 or both in combination were grown to an OD₆₀₀ of 1.0, and 10 mM NEM was added for 1 h. Blots were probed with myc (Upper) or Dpm1-specific (Lower) antibodies. (G) Ahp1 urmylation *in vivo* depends on both catalytically active cysteine residues in Uba4. The Δ uba4 strains were transformed with integrative plasmids expressing myc-tagged Uba4 variants. The blots were probed with antibodies specific for HA (Top, HA-Urm1), myc (Middle, myc-Uba4), and Dpm1 (Bottom, loading control).

levels did not inhibit urmylation in response to oxidant treatment, suggesting that oxidant-induced Urm1 protein conjugation is distinct from its role in tRNA modification (Fig. S1 C and D).

Previous work in *S. cerevisiae* described the existence of a few low-abundant Urm1 adducts under steady-state conditions (20), but neither the underlying enzymology nor the mode of linkage to these adducts was explored. To test whether oxidative stress also enhances urmylation in yeast, we expressed HA-tagged Urm1 from its chromosomal locus in either a WT or Uba4-deficient (Δ Uba4) yeast strain. In the absence of any oxidant, we only observed very low levels of Urm1 adducts. However, treatment of these strains with either *N*-ethylmaleimide (NEM) or diamide stimulated Urm1 conjugate formation in yeast expressing HA-Urm1, as determined by anti-HA immunoblotting (Fig. 1E). No urmylation was observed in a Δ Uba4 yeast strain. Treatment with NEM results in alkylation of free thiols, thereby altering the redox status of the cell as well as inactivating potential deurmylases, which would be expected to be thiol proteases by analogy with the proteases that resolve Ub, SUMO, or Nedd8 adducts. Because NEM and diamide both induce adduct formation (albeit yielding distinct banding patterns), these effects are likely attributable to an alteration in redox balance. To confirm that oxidant treatment leads to Urm1 conjugation, we investigated the urmylation status of Ahp1 in the presence of NEM. Yeast strains expressing HA-Urm1, Ahp1-myc, or a combination of these two were exposed to NEM. In addition to unmodified Ahp1, we detected the appearance of another polypeptide by anti-myc immunoblotting. This material corresponds to the fraction of Ahp1 that is modified by either WT Urm1 or HA-Urm1 (Fig. 1F and Fig. S1E). To determine whether the C-terminal thiocarboxylate of Urm1 is required for oxidant-induced urmylation not only in mammalian cells but in *S. cerevisiae*, we mutated either or both active site cysteines of myc-Uba4 and introduced these plasmids in a Δ Uba4 yeast strain. Mutation of C225 and/or C397, both of which are required for Urm1-thiocarboxylate formation (4, 10, 15), abrogated urmylation of Ahp1, indicating that thiocarboxylation of Urm1 is necessary for its conjugation to target proteins in *S. cerevisiae* (Fig. 1G).

Thus, oxidative stress enhances conjugation of Urm1 to target proteins in both yeast and mammalian cells, demonstrating that this is a conserved modification in eukaryotes. In addition, oxidant-induced urmylation is dependent on the thiocarboxylated C-terminal glycine of Urm1 in both organisms.

Urmylation Involves a Thioester Intermediate and Results in Covalent Adduct Formation. All immunoprecipitations were performed under fully denaturing conditions (SDS lysis), pointing to a covalent linkage between thiocarboxylated Urm1 and its target proteins. To explore the nature of this bond further, we investigated the effects of the reducing agent DTT on the observed Urm1 ladder. Urm1 conjugates were immunoprecipitated and boiled in the presence or absence of DTT. Inclusion of DTT had no effect on Urm1 ladder formation; therefore, the linkage between Urm1 and its targets is not an acyl disulfide bond (Fig. 2A).

We next investigated whether urmylation involves a thioester intermediate. Before treatment with diamide or H_2O_2 , we incubated cells with hydroxylamine (NH_2OH), a cell-permeable nucleophile that cleaves thioesters, without causing a morphological change in the cells for the duration of the treatment. Pretreatment with NH_2OH abrogated conjugation of Urm1 to target proteins (Fig. 2B). In contrast, addition of NH_2OH to the immunoprecipitates, before boiling the samples, did not affect our ability to detect conjugates (Fig. 2C). Together, these data suggest that urmylation requires a thioester intermediate. To explore the nature of this intermediate further, we coincubated recombinant MOCS3, recombinant Urm1, and an ATP-regenerating system. This results in the formation of a DTT-sensitive and NH_2OH -sensitive linkage between MOCS3 and Urm1 (Fig. S2). Thus,

conjugation of Urm1 to its substrates likely involves an intracellular thioester intermediate and eventually gives rise to a covalently coupled adduct that is neither acyl disulfide-linked nor NH_2OH -sensitive, and therefore likely a peptide bond.

Diamide-Induced Conjugation Is Specific to Thiocarboxylated Urm1 in Vitro. Diamide and H_2O_2 are both strong oxidants that convert GSH into GSSG, thereby modulating the redox balance of the cell and inducing oxidative stress. H_2O_2 requires an enzymatic activity, glutathione peroxidase, for this reaction (23) (Fig. S1F). In contrast, diamide directly reacts with GSH and converts it to GSSG in the absence of any enzyme (25). Indeed, postlysis addition of diamide to HA-Urm1 WT lysates induces Urm1 conjugation almost as efficiently as does treatment of intact cells, whereas postlysis addition of H_2O_2 has no effect (Fig. S1G). We exploited this observation to perform urmylation in vitro. We expressed HA-tagged human Urm1 fused to an intein and a chitin-binding domain in *Escherichia coli*. The recombinant protein was purified on a chitin column and released by induction of intein self-cleavage through addition of DTT or ammonium sulfide (26). DTT or ammonium sulfide treatment yields a product with a C-terminal

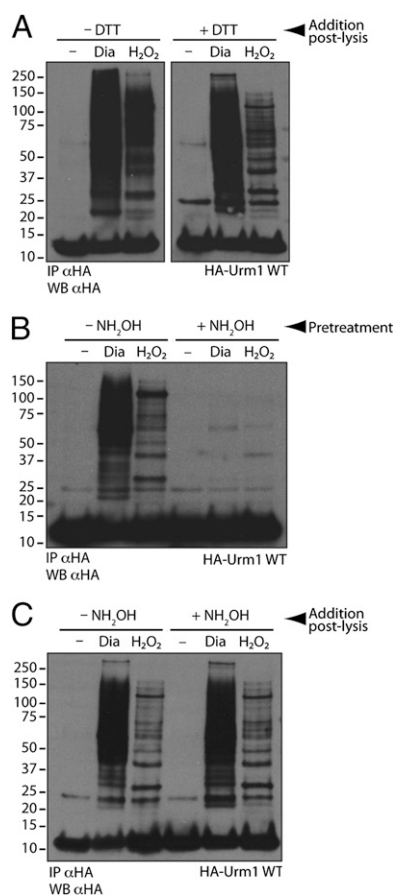


Fig. 2. Urmylation requires a thioester intermediate and gives rise to a covalently linked product that is not acyl disulfide-linked. (A) HA-Urm1 WT cells were treated with diamide (Dia) or H_2O_2 , and HA-Urm1 was immunoprecipitated as described in Fig. 1D. Immunoprecipitates were boiled in sample buffer in the absence or presence of DTT and analyzed by anti-HA immunoblotting. (B) NH_2OH pretreatment prevents urmylation. HA-Urm1 WT cells were pretreated for 10 min in PBS with or without 50 mM NH_2OH before administration of Dia or H_2O_2 . (C) Postlysis addition of NH_2OH does not affect Urm1 adducts. HA-Urm1 WT cells were treated with Dia or H_2O_2 , and HA-Urm1 was immunoprecipitated. Immunoprecipitates were incubated with or without 50 mM NH_2OH before boiling in DTT-containing sample buffer.

carboxylate or thiocarboxylate, respectively, thus generating HA-Urm1-carboxylate (COOH) or HA-Urm1-thiocarboxylate (COSH) (Fig. 3A). The masses of both species were verified by electrospray ionization (ESI) MS (Fig. S3A). In addition, analysis of both products on a polyacrylamide gel supplemented with APM revealed that $\approx 60\%$ of HA-Urm1-COSH was modified and carried a thiocarboxylate (Fig. 3B).

To test whether recombinant HA-Urm1-COSH is conjugated to target proteins in response to diamide, we immobilized HA-Urm1 on anti-HA agarose and added either a HeLa lysate prepared in SDS lysis buffer or SDS lysis buffer alone. We chose stringent SDS lysis conditions to select for substrates covalently interacting with Urm1. These samples were incubated with or without diamide for 1 h at 4 °C, after which the resin was washed several times and the immunoprecipitated material was eluted by boiling in sample buffer containing DTT. SDS/PAGE analysis showed that only HA-Urm1-COSH treated with both HeLa lysate and diamide is able to form conjugates (Fig. 3C and Fig. S4A). This inarguably demonstrates that the C-terminal thiocarboxylate of Urm1 is required for conjugate formation. The presence of DTT did not affect the stability of Urm1 adducts (Fig. S4B).

To exclude the possibility that diamide itself directly modifies the thiocarboxylate of HA-Urm1-COSH and forms an activated thioester, we incubated immobilized HA-Urm1-COSH with diamide and, after three wash steps, subsequently with HeLa lysate. No urmylation was observed on such sequential treatment (Fig. S4C). In addition, when HA-Urm1-COSH treated with diamide is analyzed by ESI-MS, no change in mass is detected (Fig. S3B). Thus, diamide does not form a stable intermediate with HA-Urm1-COSH, and only simultaneous addition of diamide and cell extract leads to urmylation *in vitro*.

To investigate the nature of the linkage formed between Urm1 and substrates during this *in vitro* reaction, we treated HA-Urm1-COSH with NH_2OH either during the reaction or after completion of the reaction. Inclusion of NH_2OH during the reaction abrogates urmylation, whereas addition of NH_2OH to the immunoprecipitate has no effect (Fig. S4D and E). These data are consistent with our observations for HA-Urm1 WT cells (Fig. 2B and C) and suggest that urmylation involves a thioester intermediate and yields an isopeptide-linked Urm1 adduct.

To test whether diamide-induced conjugation is specific to Urm1, or a more general phenomenon of proteins bearing a thi-

ocarboxylate at their C terminus, we generated carboxylated EGFP (HA-EGFP-COOH) and thiocarboxylated EGFP (HA-EGFP-COSH) in a manner similar to that described above for Urm1. The X-ray structure of EGFP (PDB ID code 1GFL) shows that its C terminus is fully solvent-exposed. Both GFP variants were immobilized onto anti-HA agarose and treated with HeLa lysate and/or diamide. Although a faint increase in background is observed, HA-EGFP-COSH is not efficiently conjugated to proteins when compared with HA-Urm1-COSH (Fig. 3D). Thus, conjugation of thiocarboxylated proteins to substrates appears to be specific to Urm1 and not a general characteristic of any protein engineered to incorporate an exposed thiocarboxylate at its C terminus. Because diamide-induced urmylation does not obviously involve an enzymatic activity, other amino acids or tertiary structures may contribute to the specificity of the reaction.

Proteomic Identification of Urm1 Substrates in Mammalian Cells. To identify urmylated substrates, we proceeded with large-scale affinity purification of Urm1 adducts from HA-Urm1 WT and HA-Urm1 ΔG cells either left untreated or exposed to diamide or H_2O_2 *in vivo*. We analyzed 5% of the immunoprecipitates by anti-HA immunoblotting to confirm that Urm1 was efficiently conjugated (Fig. S5A). We resolved the remainder of the polypeptides by SDS/PAGE and visualized them by silver staining. Individual polypeptides were analyzed by trypsinolysis and liquid chromatography coupled with tandem MS (MS/MS). We considered only those proteins represented by at least three peptides and altogether absent from control samples as valid hits.

We identified 21 proteins that were uniquely present in samples from HA-Urm1 WT cells treated with diamide or H_2O_2 and absent from untreated cells or HA-Urm1 ΔG cells exposed to diamide or H_2O_2 (Table S1). We found two members of the Urm1 pathway itself (i.e., MOCS3, ATPBD3) that were modified by Urm1 on administration of either oxidant. In addition, we identified two deubiquitylating enzymes, USP15 and USP47, that have not been previously linked to the Urm1 pathway. Furthermore, several components of the nuclear import/export pathway were among the identified substrates. A few proteins involved in RNA processing were present, which is perhaps not unexpected, given the role of Urm1 in thiolation of tRNA and possibly other types of RNA. Although the peroxiredoxin Ahp1 is the most abundant substrate for Urm1 in yeast, we did not find a homolog of Ahp1 or any other thioredoxin or peroxiredoxin among the identified proteins.

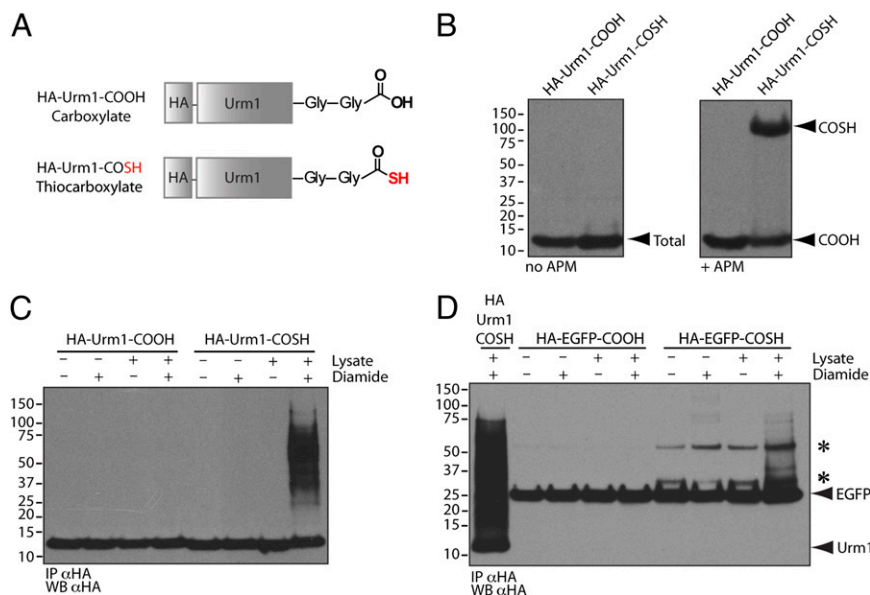


Fig. 3. Diamide induces conjugation of recombinant thiocarboxylated Urm1 (but not thiocarboxylated EGFP) to target proteins *in vitro*. (A) Schematic illustration of recombinant HA-tagged Urm1 with either a carboxylate (COOH) or thiocarboxylate (COSH) at its C terminus. (B) Analysis of recombinant HA-Urm1-COOH and HA-Urm1-COSH on a polyacrylamide gel in the absence (Left) or presence (Right) of APM. (C) HA-Urm1 was immobilized on anti-HA agarose and treated with either SDS lysis buffer or a HeLa cell extract in the presence or absence of diamide for 1 h at 4 °C. Immunoprecipitates were boiled in DTT-containing sample buffer, resolved by SDS/PAGE, and detected by anti-HA immunoblotting. (D) Recombinant HA-EGFP-COOH and HA-EGFP-COSH were immunoprecipitated and treated as described in C and compared with HA-Urm1-COSH treated with both HeLa extract and diamide. Note that half of the HA-Urm1-COSH reaction was analyzed, whereas the entire HA-EGFP reaction was loaded onto the gel. *Contaminant in the purified EGFP fraction.

Urm1 Is Conjugated to Lysine Residues of MOCS3, ATPBD3, CTU2, and USP15 in Vivo. Because MOCS3 was urmylated on addition of either oxidant, we decided to validate this protein as a bona fide target for urmylation in vivo. Untransduced HA-Urm1 WT or HA-Urm1 ΔG HeLa cells were transiently transfected with MOCS3-FLAG and treated with diamide or H₂O₂. Because MOCS3 nonspecifically adheres to agarose, we immunoprecipitated MOCS3 via its FLAG tag and examined modification by Urm1 through anti-HA immunoblotting. Urmylated MOCS3 was detectable in HA-Urm1 WT cells exposed to diamide or H₂O₂ but not in untransduced or HA-Urm1 ΔG cells (Fig. 4A). Total lysate from HeLa cells transfected with HA-MOCS3 was included on the gel to visualize the position of nonurmylated MOCS3 on a polyacrylamide gel. Urmylation of MOCS3-FLAG results in a shift from 52 to 65 kDa, suggesting that MOCS3 is urmylated by a single molecule of Urm1 (i.e., monourmylated). Although MOCS3 contains 15 lysine residues, multiurmylation was not detected.

To test whether other components of the Urm1 pathway can be urmylated, we carried out a similar experiment with both FLAG-ATPBD3 and CTU2-FLAG. ATPBD3, the human homolog of Ncs6p, was identified in our MS-based screen. In our screen, we did not recover CTU2, the human homolog of Ncs2p, which is a thiouridylase also associated with the Urm1 pathway (9–12, 16). FLAG immunoprecipitation followed by HA immunoblotting confirmed that both proteins are targeted by Urm1 during oxidative stress (Fig. 4B and C). Despite the presence of several solvent-exposed lysine residues in Urm1 itself (2, 3), polyurmylation is not observed and all substrates examined are modified by what appears to be a single Urm1 molecule. Thus, oxidative stress induces conjugation of Urm1 to three members of its own pathway.

Several proteins that are part of the Ub machinery were present in our MS-based screen, including the deubiquitylating enzyme USP15 that was uniquely urmylated in response to H₂O₂ treatment. We verified that WT Urm1 is indeed appended to endogenous USP15 on addition of H₂O₂ (Fig. 4D). By analogy with MOCS3, ATPBD3, and CTU2, USP15 could represent a component of the Urm1 pathway that is urmylated in response to oxidant exposure. To test this idea, we determined whether USP15 has activity as a deurmylase. Recombinant human USP15 was incubated with immunopurified HA-Urm1 adducts, and deconjugation was measured by anti-HA immunoblotting (Fig. S5B). Although USP15 is reactive toward Ub adducts, as determined by Ub-AMC hydrolysis (Fig. S5B, Left), we did not detect reactivity toward urmylated substrates (Fig. S5B, Right). In addition, shRNA-mediated depletion of USP15 from HeLa cells did not affect steady-state or oxidant-induced levels of urmylation (Fig. S5C).

To investigate which substrate residues are targeted by Urm1, we expressed a mutant of MOCS3 in which all lysines were replaced by arginines (MOCS3 KtoR). This mutant is not modified by Urm1 on oxidant treatment, indicating that urmylation must occur on the ϵ -amino group of lysine residues (Fig. 4E). Mutation of only the active site lysine of MOCS3 (K413R) did not interfere with urmylation (Fig. S6A). To confirm that Urm1 functions as a lysine-directed modifier in vivo, we used the *S. cerevisiae* system and focused on the NEM-induced Urm1 conjugation to Ahp1. We generated several Ahp1 variants that possess lysine-to-arginine alterations and expressed them as myc epitope-tagged variants in a $\Delta urm1$ yeast strain that additionally expressed HA-tagged Urm1. We found that urmylation of Ahp1 was absent only in the variant that harbors an arginine replacement of K32, indicating that Urm1 targets indeed substrate lysine residues and in a very specific manner (Fig. 4F and Fig. S6B). Notably, Ahp1 with an alteration of the neighboring cysteine C31 to a serine residue was urmylated as the WT protein, indicating that this cysteine residue is not relevant for the conjugation reaction.

Taken together, these results indicate that on oxidant treatment, thiocarboxylated Urm1 is specifically conjugated to a lim-

ited set of proteins both in yeast and mammalian cells and that Urm1 is not attached randomly but to specific lysine residues.

Urm1 Is Appended to the Nucleocytoplasmic Transport Factor Cellular Apoptosis Susceptibility Protein. Several proteins involved in nuclear transport were identified in our MS-based screen, of which cellular apoptosis susceptibility protein [CAS, also known as chromosome segregation 1-like (CSE1L)] was among the most abundant hits. Proteins >40 kDa destined for nuclear translocation associate with importin β or its adaptor importin α via a nuclear localization signal (27). This ternary complex is transported into the nucleus, where the high concentration of GTP-bound Ran dissociates the complex. CAS binds cargo-free importin α in the nucleus and, with the help of Ran-GTP, shuttles it back to the cytosol (27, 28). In addition, CAS has been implicated in apoptosis and proliferation and has been identified as a component of p53 transcriptional complexes (29–31).

Our MS-based screen suggested that CAS is urmylated in response to H₂O₂. To confirm this, we treated HA-Urm1 WT and HA-Urm1 ΔG cells with H₂O₂ and retrieved urmylated targets by anti-HA immunoprecipitation. Anti-CAS immunoblotting showed that endogenous CAS is urmylated in response to H₂O₂ in a thiocarboxylate-dependent manner (Fig. 5A). Urmylation results in an estimated increase in molecular weight of 10–25 kDa, suggesting that CAS is modified by either one or two molecules of Urm1. Titration of H₂O₂ revealed that urmylation of CAS is detectable at low concentrations of H₂O₂ (200 μ M) and significantly increases at higher concentrations (Fig. 5B).

Because CAS shuttles between the cytosol and nucleus, we determined the localization of urmylated CAS. Untreated or H₂O₂-treated HA-Urm1 WT cells were lysed in a Nonidet P-40-based lysis buffer, which disrupts cellular membranes and protein complexes but leaves the nuclei largely intact. The nuclear pellet was then disrupted in an SDS-based lysis buffer. Anti-HA immunoprecipitation from these fractions showed that urmylated substrates, including CAS, are exclusively present in the cytosol (Fig. 5C). To determine the stability of urmylated CAS, we allowed H₂O₂-treated cells to recover for 1–4 h posttreatment. Anti-HA immunoprecipitation showed that urmylated substrates, including CAS, persist over time; after 4 h, urmylated substrates are still present, although reduced by roughly 50% (Fig. 5D). This finding suggests that Urm1 is appended to the nucleocytoplasmic shuttling factor CAS, most likely in the cytosol on H₂O₂ treatment, and also indicates that the Urm1-CAS conjugate is rather stable and not prone to accelerated degradation.

Discussion

The carboxyl group of the C-terminal glycine of Urm1 is modified to a thiocarboxylate by the addition of sulfur (9, 10, 12, 13). Thiocarboxylated Urm1 functions as a sulfur donor in tRNA thiolation (9–13). The consequences of thiocarboxylation on the ability of Urm1 to form protein conjugates have not been explored until now. We show here that thiocarboxylated Urm1 also serves as a protein modifier under conditions of oxidative stress in both *S. cerevisiae* and *H. sapiens*. In intact cells as well as in vitro, oxidant treatment results in protein modification by WT Urm1 but not by Urm1 that lacks the glycine residue essential for thiocarboxylate formation. The role of Urm1 in posttranslational modification has been suggested by earlier work in *S. cerevisiae* that described urmylation under normal growth conditions, with the peroxiredoxin Ahp1 the only substrate identified to date (1, 19, 20). It was unclear whether this reaction requires thiocarboxylation of Urm1. We did not detect spontaneous urmylation in mammalian cells except when they were placed in an oxidizing environment. Similarly, exposure of yeast cells to oxidants enhanced Urm1 adduct formation, indicating that oxidant-induced urmylation is a conserved phenomenon in eukaryotes. Thus, the role of thiocarboxylated Urm1 is twofold: It serves as

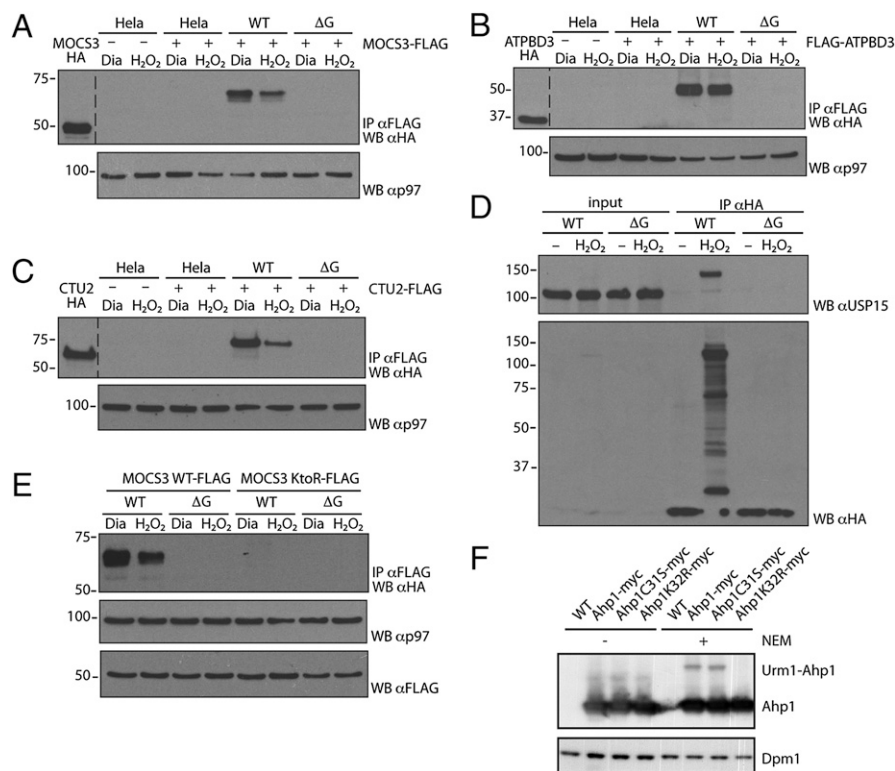


Fig. 4. Urm1 is conjugated to lysine residues in MOCES3, ATPBD3, CTU2, and USP15 in vivo. (A) Nontransduced, HA-Urm1 WT, and HA-Urm1 Δ G HeLa cells were transiently transfected with MOCES3-FLAG and after 24 h treated with either diamide (Dia) or H_2O_2 . MOCES3 was immunoprecipitated through its FLAG tag from SDS lysates, boiled in DTT-containing sample buffer, and resolved by SDS/PAGE. Urmylation was evaluated by anti-HA immunoblotting. The total cell lysate of HeLa cells transfected with MOCES3-HA was included to illustrate the difference in mass between nonurmylated (52 kDa) and urmylated (\sim 65 kDa) MOCES3-FLAG. (B) As described in A, using FLAG-ATPBD3 as the substrate. The total cell lysate of HeLa cells transfected with ATPBD3-HA is shown in the left lane. (C) As described in A, using CTU2-FLAG as the substrate. The total cell lysate from HeLa cells transfected with CTU2-HA is shown in the left lane. (D) HA-Urm1 WT and HA-Urm1 Δ G HeLa cells were treated with H_2O_2 . Samples were processed as in A. Immunoblotting for endogenous USP15 (110 kDa) revealed that USP15 is modified by one or two molecules of WT Urm1 on H_2O_2 addition. (E) HA-Urm1 WT and HA-Urm1 Δ G cells were transiently transfected with WT MOCES3 (MOCES3 WT-FLAG) or mutant MOCES3 in which all lysines have been replaced by arginines (MOCES3 KtoR-FLAG). Cells were processed as in A. (F) Urm1 functions as a lysine-directed modifier and modifies Ahp1 on lysine K32 in vivo. The Δ ahp1 strains were transformed with integrative plasmids expressing different myc-tagged Ahp1 variants under the control of their own *AHP1* promoter. Yeast cells were grown in the absence or presence of NEM, and samples were taken at an OD_{600} of 1.0. Blots were probed with antibodies specific for myc (Upper, Ahp1-myc) or Dpm1 (Lower, loading control).

a sulfur donor in tRNA thiolation and functions as a post-translational modifier under conditions of oxidative stress.

How do these two functions relate to each other? The role of thiomodification of tRNAs remains unclear. Thiolation of tRNAs may stabilize codon-anticodon interactions on the ribosome to improve translational efficiency (17, 32, 33). However, whether the Urm1 pathway contributes to efficient translation of endogenous proteins has not been determined. Translational efficiency and fidelity are reduced during oxidative stress through misacylation of tRNAs as well as via oxidation of a critical cysteine residue in the editing site of certain aminoacyl-tRNA synthetases (34, 35). Although we did not observe an immediate effect of oxidant treatment on tRNA thiolation levels, continuous exposure to oxidants, or high local oxidant concentrations, may alter the balance between thiolation and protein modification by Urm1. During conditions of oxidative stress, the role of Urm1 as a posttranslational modifier may become increasingly important.

The dual role of Urm1 as a sulfur donor and posttranslational modifier emphasizes its place as an evolutionary intermediate between ancient prokaryotic sulfur carriers and the eukaryotic Ub/Ub-like conjugation system (7, 13). Indeed, although Urm1 is thiocarboxylated in a manner similar to the prokaryotic MoeA and ThiS (13), its conjugation to proteins is at least partially analogous to Ub conjugation. Both Ub and Urm1 require ATP-dependent activation of their C-terminal glycine by an E1-like enzymatic activity (5, 15). In addition, we show that urmylation likely requires

a thioester intermediate and that Urm1 is conjugated to lysine residues in substrates through an isopeptide bond, similar to ubiquitylation. Unlike Ub, however, we did not observe poly-urmylation, despite the presence of several surface-exposed lysine residues in Urm1 itself (2, 3). In addition, no E2, E3, or deurmylating enzymes have been identified for Urm1 to date. How does substrate recognition take place in the absence of a ligase? Urm1 is conjugated to a specific site in a limited number of targets. In addition, thiocarboxylate C-terminal modification of EGFP is not sufficient to enable it to engage in conjugate formation. Thus, what determines recognition of substrates by Urm1 remains unclear at present and requires further study. In addition, now that we have defined the conditions that potentiate urmylation, one of the next steps will be to determine whether a deurmylating activity exists.

Two Ub-like small archaeal modifiers (SAMP1 and SAMP2) exist in *Haloflex volcanii* (36). Minimal SAMP conjugation (SAMPylation) occurs when cells are grown under standard conditions, whereas nitrogen limitation causes an increase in the number of SAMP conjugates (36). Thus, both SAMPylation and urmylation occur in response to an environmental cue. We identified three substrates for urmylation that are components of the Urm1 pathway itself. Their archaeal homologs are targets for SAMPylation (36). Furthermore, no apparent E2 or E3 homologs are present in *H. volcanii*, whereas the only E1-like enzyme for SAMP is homologous to Uba4 (36). Although it was not determined whether the C-terminal glycine of SAMP1 or SAMP2 is thiocarboxylated, the analo-

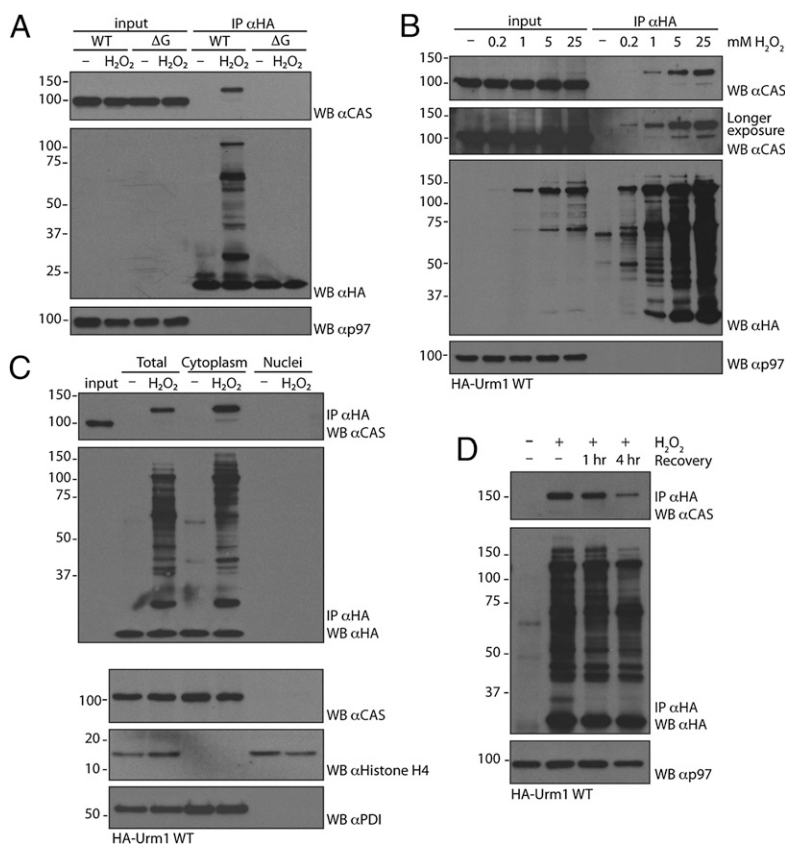


Fig. 5. Urm1 is appended to the nucleocytoplasmic shuttling factor CAS in response to H_2O_2 treatment. (A) HA-Urm1 WT and HA-Urm1 ΔG HeLa cells were treated with H_2O_2 . Urm1 was retrieved from SDS lysates through its HA tag. Immunoprecipitates and whole-cell lysates (input) were boiled in the presence of DTT and separated on a polyacrylamide gel. Anti-CAS immunoblotting showed that CAS (110 kDa) is modified by one, or possibly two, molecules of WT Urm1 (12.5 kDa) in response to H_2O_2 . (B) HA-Urm1 WT cells were treated with increasing concentrations of H_2O_2 for 10 min. Cells were processed as in A. (C) Urmylated CAS resides in the cytosol. HA-Urm1 WT cells were left untreated or treated with 5 mM H_2O_2 . Cells were either lysed directly in an SDS-containing buffer (Total) or lysed in a Nonidet P-40-containing buffer to extract the cytoplasm and membrane fraction (Cytoplasm) followed by disruption of the nuclear pellet in an SDS-based buffer (Nuclei). Urm1 conjugates were immunoprecipitated from these fractions through their HA tag, and urmylation of CAS was demonstrated by anti-CAS immunoblotting. (D) Urmylated CAS persists for several hours. HA-Urm1 WT cells were left untreated or treated with H_2O_2 . After treatment, cells were either immediately harvested or washed and recovered for 1 or 4 h in medium lacking the oxidant. Samples were processed as in A.

gies between SAMPylation and urmylation are striking and either or both may well be archaic ancestors of Urm1. Urm1 homologs have perhaps adapted themselves to respond to a variety of intra- and extracellular cues in the different kingdoms of life.

The generation of oxidants during cellular metabolism may damage DNA, lipids, and proteins (22). Cells have developed several strategies to limit, prevent, or repair such damage (22, 23), one of which is an increase in reversible or irreversible modifications of cysteine residues to protect proteins against oxidizing damage (e.g., glutathionylation, nitrosylation) (37). Oxidative stress also inhibits conjugation of the small Ub-related modifier SUMO to cytosolic targets, although the functional consequences of this decrease are not known (38). What is the function of Urm1 during oxidative stress? Urmylation may modify a subset of proteins to protect them against oxidizing damage and retain a pool of essential functional proteins in a manner similar to glutathionylation. Alternatively, Urm1 might recognize oxidized lysine residues and mark these proteins as damaged, either to target them for proteolysis or to transmit a “danger” signal to the cells. In the latter case, Urm1 may function as “sensor” of redox balance and alert the cell to an intra- or extracellular threat.

Although the function of urmylation of Ahp1 remains unknown, modification of this peroxiredoxin directly links Urm1 to the stress response in yeast, which is induced by alterations in redox balance. It remains equally enigmatic why components of its own pathway, as well as CAS and possibly other members of the nuclear translocation machinery, are modified by Urm1. Nuclear translocation is halted on exposure to oxidants (39). Urmylation of CAS could contribute to an arrest in nuclear transport (e.g., by decreasing the pool of cytosolic importin α). Alternatively, the Urm1-CAS conjugate may not play any particular role during oxidative stress but may merely be a consequence of a block in nuclear translocation. Urmylated CAS may represent a transient conjugate that is stabilized on oxidant exposure and an arrest in nuclear transport.

shRNA-mediated depletion of CAS from HeLa cells results in a G2/M arrest (40), a phenotype similar to that described for Urm1-depleted cells (9). Further studies are required to determine the relationship between CAS and Urm1.

Our data begin to define the requirements for urmylation and the identity of Urm1 substrates in mammalian cells. The conservation of oxidant-induced urmylation from yeast to mammalian cells suggests an important role for Urm1 modification in the response of cells to oxidative damage.

Materials and Methods

Cell Lines and Treatments. HeLa cells were obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% (vol/vol) inactivated FCS and pen/strep at 37 °C, 5% CO_2 . Treatment (Fig. 1C) was as follows: 400 μM diamide for 10 min, serum- and glucose-free DMEM for 5 h, 100 nM rapamycin for 18 h, proteasome inhibition using 25 $\mu g/ml$ MG132 for 6 h, DNA damage by 24.5 Gy of γ -irradiation followed by a 4-h recovery period, heat shock by incubation at 40 °C for 6 h, hypoxia by incubation at 5% O_2 for 2 d, endoplasmic reticulum (ER) stress using 5 $\mu g/ml$ tunicamycin for 18 h, and DMEM lacking any amino acid for 5 h. In all other figures, treatment consisted of cells being washed twice in PBS and treated with 400 μM diamide or 5 mM H_2O_2 in PBS for 10–15 min.

In Vitro Urmylation Assay. For each immunoprecipitation, 200 ng of HA-Urm1-COOH or HA-Urm1-COSH was diluted in Nonidet P-40 lysis buffer [50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40 (pH 7.5)] and immobilized on 16 μL of anti-HA agarose for 2 h at 4 °C. Beads were washed two times in lysis buffer to remove unbound material. HeLa cells were lysed in 1% SDS and diluted to 0.1% SDS in Nonidet P-40 lysis buffer supplemented with Complete protease inhibitors (Roche). Beads were incubated in either HeLa lysate or 0.1% SDS lysis buffer in the presence or absence of 400 μM diamide for 1 h at 4 °C. Beads were washed three times in Nonidet P-40 lysis buffer and eluted by boiling in sample buffer supplemented with DTT.

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