FEBS *Letters*

Urmylation and tRNA thiolation functions of ubiquitin-like Uba4·Urm1 systems are conserved from yeast to man [☆]

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A B S T R A C T

The ubiquitin-like protein Urm1 from budding yeast and its E1-like activator Uba4 have dual roles in protein urmylation and tRNA thiolation pathways. To study whether these are conserved among eukaryotes, we used gene shuffles to replace the yeast proteins by their human counterparts, hURM1 and hUBA4/MOCS3. As judged from biochemical and genetical assays, hURM1 and hUBA4 are functional in yeast, albeit at reduced efficiencies. They mediate urmylation of the peroxiredoxin Ahp1, a known urmylation target in yeast, and support tRNA thiolation. Similar to hUBA4, yeast Uba4 itself is modified by Urm1 and hURM1 suggesting target overlap between eukaryal urmylation pathways. In sum, our study shows that dual-function ubiquitin-like Urm1·Uba4 systems are conserved and exchangeable between human and yeast cells.
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1. Introduction

Ubiquitin related modifier 1 (Urm1) from *Saccharomyces cerevisiae* is a unique member of the ubiquitin-like (Ubl) protein family [1,2]. Thus Urm1 operates in a ubiquitin-like conjugation pathway termed urmylation [1,3–5] and in tRNA thiolation [6–8], combining the sulfur transfer activity typical of prokaryal S-carriers with protein conjugation capacity of eukaryal Ubl's [9]. Similar 'dual-function Ubl's' have also been found recently in prokaryotes [10,11]. As for the tRNA thiomodification role, Urm1 provides sulfur to thiolase (Ncs2-Ncs6) which cooperates with the Elongator pathway in formation of 5-methoxy-carbonyl-methyl-2-thio-uridine (mcm^{5}s^{2}U) at tRNA anticodon wobble positions [12–14]. In this S-relay system, sulfur is mobilized from cysteine by desulfurase Nfs1 and transferred to Uba4, an E1-like Urm1 activator protein [1], or indirectly passed on to Uba4 via sulfurtransferase Tum1

[1,6–8,15]. Either way, S-transfer results in formation of a Uba4 persulfide crucial for Urm1 activation [16] in a process that is ATP dependent and results in S-transfer to the Urm1 C-terminus [5,17]. In this thiocarboxylated form, Urm1 donates the sulfur to the thiolase for thiogroup (s^2) formation in mcm^{5}s^{2}U.

Remarkably, the S-donor role of Urm1 appears to be coupled to its urmylation function, for the latter lysine-directed protein conjugation depends on Urm1 thiocarboxylation [5,16,18]. Thus, the Urm1 thiocarboxylate appears to feed into both the tRNA thiolation and urmylation pathways. Up to date no Urm1-specific E2-conjugating or E3-ligating enzymes typical of ubiquitylation have been identified, meaning that urmylation specificity is an ill-defined issue. In yeast, different Urm1 conjugates are known to form [1,3–5], however, their identities have largely been elusive with the exception of Ahp1, a peroxiredoxin with roles in protection against reactive oxygen species (ROS) [3,5]. In contrast to this, many (>20) human hURM1 targets have been identified [5]. Some of them correspond to homologs of yeast Uba4 (hUBA4/MOCS3), Ncs2 (CTU2), Ncs6 (ATPBD3) and Elongator subunit Elp1 (IKAP) [5]. This indicates that key players for Urm1 activation or thiolation functions and another protein (Elp1/IKAP) involved in wobble uridine modification can be urmylated. Precisely how urmylation influences target protein function is not clear.

However, it is triggered by oxidative stress [5] and urmylation of Ahp1 has been proposed to affect ROS detoxification by the peroxiredoxin [19]. Another option sees Urm1 conjugation as a means to restrict sulfur flow by reducing the pool of free Urm1 available for S-transfer and tRNA thiolation [5,18].

Prompted by recent data showing that tRNA modification functions of Elongator and Urm1 are conserved among yeast and plants [20,21], we studied whether dual roles for Uba4 and Urm1 in protein urmylation and tRNA thiolation can be exchanged between lower (yeast) and higher (human) eukaryotes. Here, we show that when transferred into yeast, human Urm1 (hURM1) and Uba4 (hUBA4) mediate, albeit less efficiently, tRNA anticodon thiolation and support Ahp1 urmylation. In addition to the latter and in analogy to hUBA4 from HeLa cells, we find yeast Uba4 itself can be conjugated to Urm1 and hURM1 suggesting target overlap among eukaryal urmylation pathways. In sum, our data show that dual-function Uba4·Urm1 systems are conserved from yeast to man.

2. Materials and methods

2.1. Yeast strains, general methods and plasmid constructions

Growth of yeast strains (Table S1) was in routine YPD or SC media [22] for 3 days and thermosensitivity was assayed on YPD at 39 °C. Supplemental Table S2 lists primers used for PCR-based protocols [23,24] to generate and diagnose gene deletions or epitope tagged gene fusions. Transformation of yeast with PCR products or plasmids (Table S3) was done as previously described [25]. For construction details of plasmids used to express yeast or human Urm1·Uba4 system components, see Supplementary Data.

2.2. tRNA modification profiling

Total tRNA was isolated from yeast cultures and subjected to LC–MS/MS for modification analysis essentially as previously described [20,26]. Identification of mcm^5 or mcm^5s^2 peaks was according to Kellner et al. [27]. For intersample comparability of the detected modifications, the peak areas of the modified nucleosides, measured in triplicates, were normalized to the UV peak area of uridine.

2.3. Urmylation studies using electrophoretic mobility shift assays (EMSA)

Yeast cells (grown to OD_{600} ∼1) were broken with a bead beater and lysed in a buffer (10 mM K-HEPES pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 2 mM benzamidine) containing complete protease inhibitors (Roche) and 10 mM N-ethylmaleimide (NEM) as previously described [3,4]. Following centrifugation at 16.000g, and prior to SDS–PAGE and Western blot analyses, protein concentration was determined according to Bradford [28]. For EMSAs, PVDF membranes were incubated with primary anti-HA (F-7) and anti-c-Myc (9E10) antibodies (Santa Cruz Biotechnology) and probing involved horseradish peroxidase-conjugated secondary goat anti-mouse or anti-rabbit IgGs (Jackson ImmunoResearch) and the Pierce ECL Western blotting substrate (Thermo Scientific). Loading controls used anti-Cdc19 antibodies (kindly donated by Jeremy Thorner, University of California, USA) to detect pyruvate kinase (Cdc19).

3. Results and discussion

3.1. hURM1, the human homolog of Urm1, urmylates Ahp1 in yeast

Between yeast Urm1 and human URM1 there is ∼42% amino acid identity and 80% similarity (Fig. S1A) [7,9]. To investigate whether this means urmylation is conserved among eukaryotes,

we expressed an HA-tagged version of the human URM1 gene (hURM1) product in the background of a yeast strain lacking URM1 gene function and included, as a control, the HA-tagged yeast URM1 gene. First we studied whether HA-tagged hURM1 was expressed and able to form protein conjugates in yeast. Protein extracts from hURM1 or URM1 expressing cells were subjected to anti-HA Western blots and EMSA. Expression of free forms of HA-tagged hURM1 and Urm1 with molecular weights matching the expected sizes was readily detectable (Fig. 1A). We observed a major anti-HA responsive band at ∼40kDa (Fig. 1A) and additional upshifted signals upon longer exposure times suggesting urmylation of further proteins (Fig. S2). Remarkably, we found the ∼40kDa band that formed in the presence of hURM1, was abolished in uba4Δ cells (Fig. S3) indicating it is an hURM1 conjugate that depends on Uba4, the E1-like Urm1 activator required for urmylation in yeast [1]. Furthermore, the hURM1 conjugate resisted denaturing conditions implying that analogous to Urm1, urmylation by hURM1 involves covalent protein conjugation.

That the major conjugates correspond in size (∼40kDa) suggests Urm1 and hURM1 may urmylate a protein in yeast that is similar, if not identical. Despite clear evidence for multiple urmylation targets (Fig. S2), peroxiredoxin Ahp1 is the only one identified in yeast so far [3–5]. With sizes similar to Ahp1·Urm1 conjugates (36kDa), we next examined whether Ahp1 is indeed conjugated to hURM1 in yeast. To do so, we used ahp1Δ or AHP1-c-myc strain backgrounds that either lack Ahp1 or produce a c-Myc-tagged version of it. As shown by EMSAs, the 40kDa conjugate typical of wild-type (AHP1) was lost in ahp1Δ cells or upshifted, due to the Ahp1 epitope extension, in AHP1-c-myc cells (Fig. 1B). Collectively, this indicates that hURM1 urmylates yeast Ahp1 in a Uba4 dependent fashion. This is identical to Ahp1 conjugation by Urm1 [3,5] and suggests urmylation of Ahp1 is not specific to yeast but can also be mediated by hURM1 even though the human Ubl protein seems not to be involved in peroxiredoxin modification in HeLa cells [5]. Based on weaker signal intensities in relation to Ahp1·Urm1 conjugates from S. cerevisiae (Fig. 1A), however, Ahp1 urmylation by hURM1 appears to be less efficient than with the yeast Ubl protein.

3.2. hURM1 mediates tRNA thiolation in yeast

Given the above data showing hURM1 can urmylate Ahp1 in yeast, we next asked how the human Ubl protein would perform in the tRNA thiolation pathway. In yeast, the Uba4·Urm1 system cooperates with the Elongator complex to attach mcm^5s^2 modifications onto tRNA anticodons [8,12–14]. Hence, loss of Urm1 or Uba4 removes the s^2 thiogroup, while Elongator mutations such as elp3Δ remove the mcm^5 group. Either defect has modest phenotypic consequences but interferes with TOR signaling and protects against cell death by zymocin, a fungal tRNase toxin which cleaves mcm^5-s^2 modified anticodons [1,8,29–31]. While mcm^5s^2 was thought to be essential in yeast [12], we have recently shown that s^2 thiogroup removal in tandem with Elongator inactivation is tolerated but confers thermosensitivity that is a direct consequence of inappropriate tRNA modification [32]. Thus, restoration of the s^2 modification by expression of hURM1 or Urm1 can be monitored by reversion of thermosensitivity shown by an elp3Δurm1Δ double mutant.

We exploited this rationale to study the ability of hURM1 to counter tRNA thiolation defects in yeast. An elp3Δurm1Δ double mutant was allowed to express hURM1 or URM1 and thermosensitivity was compared to an elp3Δ single mutant alone. We found that thermosensitivity at 39 °C of the elp3Δurm1Δ reporter strain was partially rescued by hURM1 and fully complemented by yeast URM1 (Fig. 1C). Such partial suppression in yeast supports a
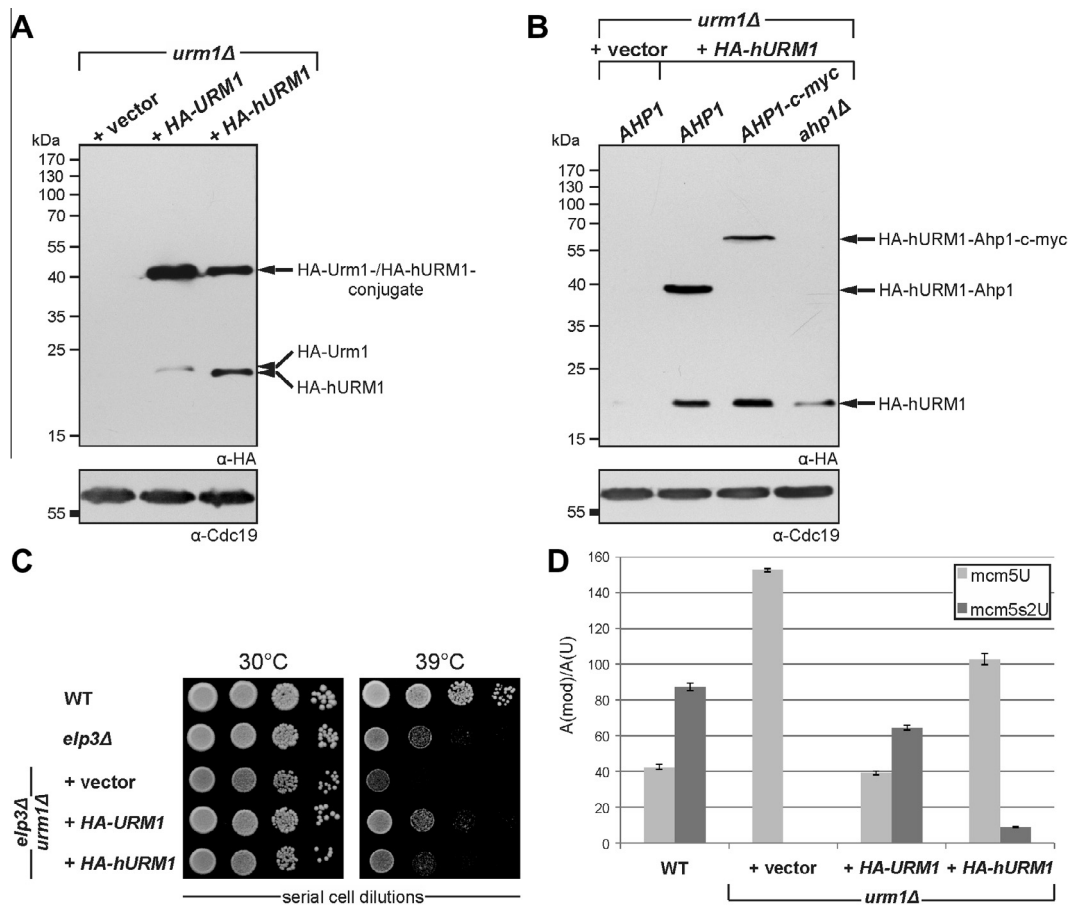
Fig. 1. hURM1 urmylates Ahp1 and supports tRNA thiolation in yeast. (A) Similar to Urm1, hURM1 can form protein conjugates in yeast. Protein extracts obtained from an _urm1Δ_ strain expressing _HA-URM1_, _HA-hURM1_ or no Ubl (empty vector) were separated by SDS–PAGE and immune probed with antibodies specific for the HA tag or Cdc19 (loading control). (B) hURM1 urmylates Ahp1. Protein extracts from an _urm1Δ_ strain expressing _HA-hURM1_ were obtained in _AHP1_ wild-type (WT), _ahp1Δ_ or _AHP1-c-myc_ backgrounds and analyzed as in (A). (C) An _urm1Δ_ linked growth defect is partially rescued by hURM1 in vivo. Growth at 30 °C or 39 °C of an _elp3Δurm1Δ_ double mutant carrying empty vector, _HA-URM1_ or _HA-hURM1_ expression plasmids was compared to WT cells and an _elp3Δ_ single mutant by drop dilution assay on YPD. (D) Albeit at lesser efficiency than Urm1, hURM1 mediates tRNA thiolation ($\sim$14% in relation to Urm1) in yeast. Relative abundance of mcm^{5}s^{2}U and mcm^{5}U in the indicated strain backgrounds was determined by LC–MS/MS.

positive role for hURM1 in the tRNA thiolation pathway. To correlate this directly with tRNA thiolation levels, their relative abundance was compared by mass spectrometry between _urm1Δ_ cells expressing _hURM1_ or _URM1_. As previously shown [7,8,13], _urm1Δ_ cells alone failed to produce s^2 thiogroups (Fig. 1D), a defect associated with increased mcm^5 modification (due to unaltered Elongator activity). Reintroduction of yeast _URM1_ into _urm1Δ_ cells restored mcm^{5}s^2 formation close to wild-type levels while _hURM1_ was less efficient yielding $\sim$14% thiolation in relation to Urm1 from yeast (Fig. 1D). Thus, overall lower restoration of tRNA thiolation by _hURM1_ goes hand-in-hand with partial suppression of _urm1Δ_-linked phenotypes (Fig. 1C). In sum, our data show that although hURM1 performs less efficient than Urm1, the human Ubl protein mediates protein urmylation and supports tRNA thiolation in the context of a yeast cell.

3.3. hUBA4 activates Urm1 for urmylation and tRNA thiolation in yeast

Conservation between hURM1 and Urm1 raised the option that Uba4, the E1-like Urm1 activator [1], might also be replaceable by its human counterpart hUBA4 [16,17] which displays $\sim$38% amino acid identity and $\sim$73% similarity with Uba4 from yeast (Fig. S1B) [7,9]. To test this we co-expressed _hUBA4_ with _HA-URM1_ in an _urm1Δuba4Δ_ reporter strain. As expected, anti HA-Western analysis revealed free forms of Urm1 in all strains except for the empty

vector control, and in the absence of the _UBA4_ gene, formation of Urm1 conjugates was not detectable (Fig. 2A). However, when expression of either _hUBA4_ or yeast _UBA4_ was allowed, extra 40kDa urmylation bands were diagnosed by EMSA. Thus, hUBA4 is capable of activating yeast Urm1 for protein urmylation, even though the signal intensity of the Urm1 conjugate is weak in relation to the one formed when endogenous Uba4 is present. Again, 40kDa band formation essentially required the _AHP1_ gene and an upshifted Ahp1 conjugate was seen with _AHP1-c-myc_ cells (Fig. 2B), indicating Ahp1 urmylation activation is conserved between yeast Uba4 and hUBA4.

To check if hUBA4 also activates the tRNA thiolation role of Urm1, we analyzed whether hUBA4 restored s^2 formation and rescued the thermosensitivity of an _elp3Δuba4Δ_ double mutant [32]. Intriguingly, both human and yeast _UBA4_ suppressed the thermosensitivity at 39 °C of the _elp3Δuba4Δ_ (Fig. 2C). When tRNA modification levels were directly monitored by LC–MS/MS analysis, mcm^{5}s^2 formation was absent from _uba4Δ_ cells, yet redetectable upon reintroduction of _UBA4_ or _hUBA4_ (Fig. 2D). However, and consistent with lower restoration of tRNA thiolation by hURM1 (Fig. 1D), hUBA4 only partially rescued the mcm^{5}s^2 formation defect resulting in $\sim$17% thiolation in relation to yeast Uba4 (Fig. 2D). In sum, and as seen above with hURM1, hUBA4 can replace Uba4 functions, yet its efficiency to do so is reduced in relation to the endogenous E1-like enzyme from _S. cerevisiae_.
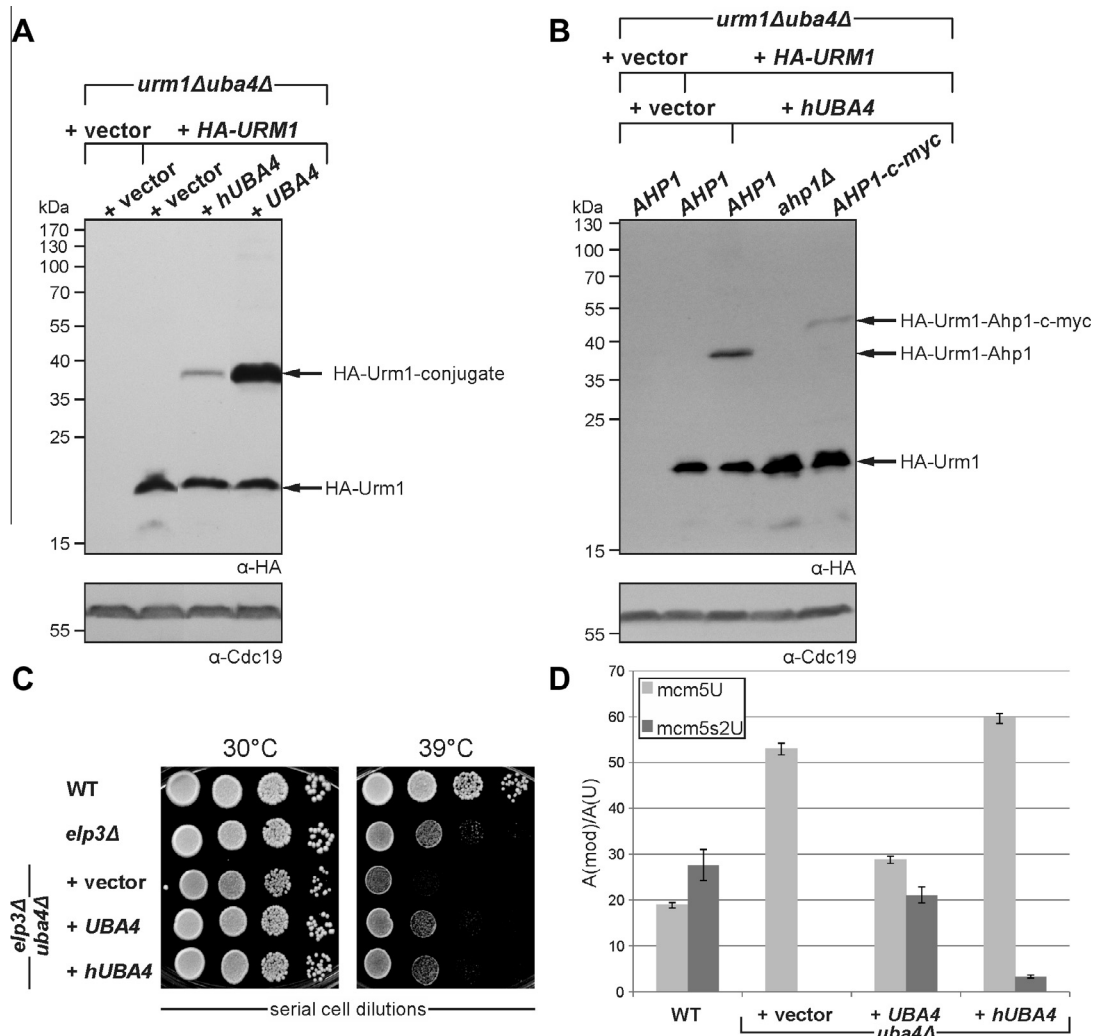
Fig. 2. Ahp1 urmylation and support of tRNA thiolation in yeast by hUBA4. (A) hUBA4 activates protein conjugation by Urm1. Protein extracts from an *urm1Δuba4Δ* double mutant with the indicated vector combinations were analyzed as in Fig. 1 for their capacity to form Urm1 conjugates on the basis of EMSA. (B) hUBA4 mediates urmylation of Ahp1. Protein extracts from an *urm1Δuba4Δ* reporter strain expressing *HA-URM1* or *hUBA4* in the indicated genetic backgrounds (i.e. WT *AHP1*, *ahp1Δ* or *AHP1-c-myc*) were analyzed as in (A). (C) hUBA4 restores temperature sensitivity in an *elp3Δuba4Δ* strain. Growth performance at 30 °C or 39 °C was compared between WT cells, an *elp3Δ* single mutant and an *elp3Δuba4Δ* double mutant carrying the indicated plasmids (i.e. empty vector, *UBA4* or *hUBA4* expression vector). Growth was for 3 days on YPD medium. (D) Though reduced in efficiency, hUBA4 mediates tRNA thiolation (~17% in relation to Uba4) in yeast. Relative abundance of mcm⁵s²U and mcm⁵U in the indicated strain backgrounds was determined by LC–MS/MS.

3.4. Yeast Uba4·Urm1 and human UBA4·URM1 systems are exchangeable

Since hURM1 and hUBA4 alone were found to be conserved but less efficient in urmylation and tRNA thiolation, we asked whether their performance was improvable by co-expression in an *urm1Δuba4Δ* yeast strain. Combined *hUBA4* and *hURM1* expression enabled efficient Ahp1 urmylation (Fig. 3A) and hURM1 conjugate formation was enhanced in relation to expression of either *hURM1* (Fig. 1A) or *hUBA4* (Fig. 2A) alone. In contrast to this, we found that co-expression of hUBA4 and hURM1 did not restore tRNA thiolation to levels seen with the endogenous Uba4·Urm1 system from yeast (Fig. 3B), but was similarly low (~17% in relation to Uba4·Urm1 levels) compared to either hURM1 (Fig. 1D: ~14%) or hUBA4 (Fig. 2D: ~17%) alone.

In conclusion, compared to mixed hybrids (hUBA4·Urm1; Uba4·hURM1) yeast strains expressing the human UBA4·URM1 system display improved protein urmylation but show similar levels of reduced RNA thiolation. Since Western blots indicated

similar protein levels (Fig. S4), such partial reduction in performance is not solely ascribable to expression barriers in the context of a yeast cell. With both the urmylation and tRNA thiolation branches requiring S-transfer onto Uba4 via Nfs1 and Tum1 and Urm1 thiocarboxylation by Uba4 [5,7,15,16], reduced compatibility between human and yeast Uba4·Urm1 systems in tRNA thiolation also appears not to be linked to S-carrier processes upstream of Urm1 activation by Uba4. Rather, we consider it is more likely to do with less congruity between factors downstream of Urm1 activation and required for tRNA thiolation. Perhaps, for full thiogroup (s²) formation, yeast wobble thiolase (Ncs2/Ncs6) prefers S-transfer from Urm1 (and its endogenous Uba4·Urm1 system), while in concert with human UBA4·URM1 or hybrids (hUBA4·Urm1; Uba4·hURM1) the enzyme is catalytically less efficient. In agreement with this, human (CTU2) and yeast (Ncs2) thiolase subunits share only 20% amino acid identity (37% similarity). So the lower conservation of part of the enzyme involved in S-transfer could explain the weaker levels of restored tRNA thiolation that we have measured.
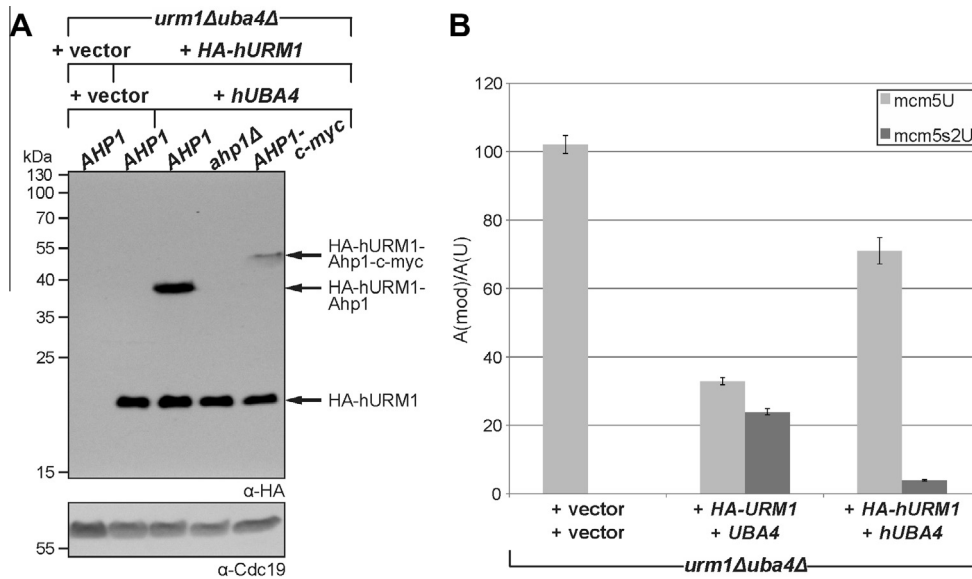
Fig. 3. The human UBA4·URM1 system is functional in yeast. (A) hUBA4 promotes Ahp1 urmylation by hURM1 in yeast. Protein extracts from an *urm1Δuba4Δ* reporter strain expressing *HA-hURM1* and *hUBA4* within the backgrounds of WT *AHP1*, *ahp1Δ* or *AHP1-c-myc* cells were subjected to EMSA using anti-HA and anti-Cdc19 antibodies (loading control). (B) hUBA4·hURM1 partially mediates tRNA thiolation (~17% in relation to yeast Uba4·Urm1). Relative abundance of mcm5s2U and mcm5U in the indicated strain backgrounds was determined by LC–MS/MS.

3.5. Like hUBA4, yeast Uba4 itself is urmylatable

Collectively, our data show Urm1 activation by Uba4 is conserved from yeast to man and peroxiredoxin Ahp1 can be urmylated by either yeast Urm1 or human URM1. This makes it very attractive to speculate that yeast orthologs of hURM1 conjugates identified in HeLa cells [5] may also be urmylated in *S. cerevisiae*. With hUBA4 among these lysine-directed urmylation targets [5], yeast Uba4 itself may be modified by Urm1. Indeed, we found by EMSA that Urm1 or hURM1 can be conjugated to yeast Uba4 (Fig. 4), an observation further supported by co-purification of Uba4 with TAP-tagged Urm1. That hUBA4 and Uba4 are modified by the very protein that both activate in HeLa and yeast cells, respectively, is further reinforced by reports showing that prokaryal Uba4 orthologs (TtuC, UbaA) are also modified by Urm1-like modifiers (TtuB, SAMP) [10,33] that they apparently activate. Whether such 'auto-urmylation' of Uba4·Urm1 systems reflects a feedback loop regulating the flow of sulfur for their respective S-transfer (thiolation) and protein modification activities [11,18] is unclear but an attractive option that remains to be elucidated in the future. Intriguingly, with hUBA4 and Uba4 as well as subunits of human wobble thiolase (CTU2/Ncs2; ATPBD3/Ncs6) all shown to be urmylatable [5], it appears that urmylation involves proteins that are themselves required for hURM1/Urm1 activation and downstream tRNA thiolation functions. Although the significance of this phenomenon is unclear, the observations that TtuA and NcsA, bacterial and archaeal tRNA thiolase orthologs, are modified by TtuB and SAMP (see above), respectively [10,11,33–35], strongly suggest this conserved aspect is biologically relevant for dual-function ubiquitin-like family proteins from all three domains of life.

Interestingly, although Ahp1 is a major urmylation target in *S. cerevisiae*, regardless of whether yeast or human Uba4·Urm1 systems are involved in its conjugation, no such urmylation of human peroxiredoxin has been detected [5]. It therefore appears that while the human UBA4·URM1 system is functional in yeast, proteins that are urmylated in yeast and human cells may not necessarily be conserved. That urmylation of Ahp1 proposedly affects the role of the peroxiredoxin in ROS protection [19] may correlate with previous data and our own evidence showing that ROS
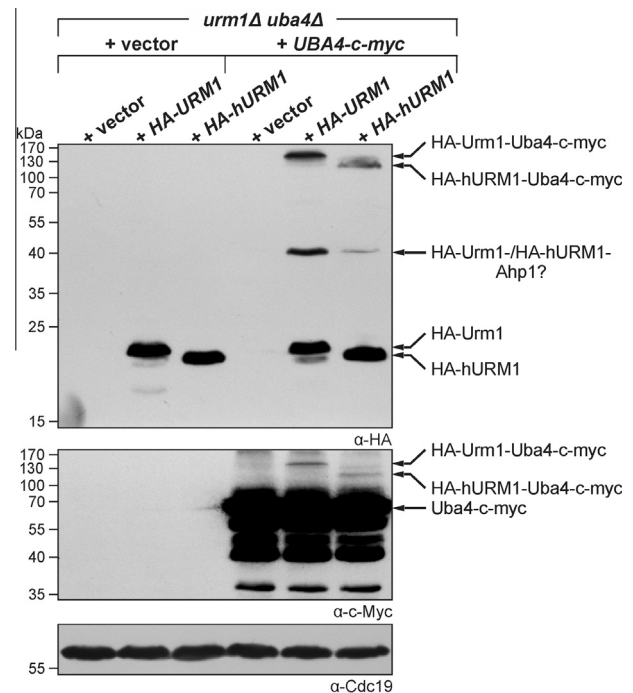

Fig. 4. Yeast Uba4 is modified by Urm1 and hURM1. Protein extracts from an *urm1Δuba4Δ* double mutant expressing either *HA-URM1* or *HA-hURM1* in combination with *UBA4-c-myc* and respective empty vector controls were subjected to EMSA using antibodies specific for HA, c-Myc or Cdc19 (loading control). Following parallel anti-HA and anti-c-Myc immune blots, formation of conjugates between Uba4 and Urm1 or Uba4 and hURM1 is indicated. In addition, anti-HA responsive bands at ~40kDa which are likely urmylated forms of Ahp1 (see Figs. 2B and 3A) are indicated by a question mark.

induces hURM1 conjugation in HeLa cells [5] and that various ROS sources trigger differential urmylation in yeast (Fig. S5). Clearly, further investigations are needed to identify these conjugates, to reveal the complement of a cell that is urmylated and to provide insights into the functional consequences of urmylation. Finally, in addition to hURM1 activation, hUBA4/MOCS3 also

activates the S-carrier MOCS2 for molybdenum cofactor synthesis [15,16,36]. With inappropriate tRNA modification and molybdopterin levels being linked to health conditions and neurodegeneration [37–39], a reassessment of such biomedical scenarios is justified, particularly in the light of a link between activation by hUBA4 of S-carriers involved in thiolation processes and the conservation of dual-function Uba4·Urm1 systems reported here.

Conflict of interest

None of the authors has any potential conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.02.024.

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