Vms1 and ANKZF1 peptidyl-tRNA hydrolases release nascent chains from stalled ribosomes

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Ribosomal surveillance pathways scan for ribosomes that are transiently paused or terminally stalled owing to structural elements in mRNAs or nascent chain sequences10–2. Some stalls in budding yeast are sensed by the GTase Hbs1, which loads Dom34, a catalytically inactive member of the archaeo-eukaryotic release factor 1 superfamily. Hbs1–Dom34 and the ATPase Rli1 dissociate stalled ribosomes into 40S and 60S subunits. However, the 60S subunits retain the peptidyl-tRNA nascent chains, which recruit the ribosome quality control complex that consists of Rqc1–Rqc2–Ltn1–Cdc48–Ufd1–Npl4. Nascent chains ubiquitylated by the E3 ubiquitin ligase Ltn1 are extracted from the 60S subunit by the ATPase Cdc48–Ufd1–Npl4 and presented to the 26S proteasome for degradation3–9. Failure to degrade the nascent chains leads to protein aggregation and proteotoxic stress in yeast and neurodegeneration in mice10–14. Despite intensive investigations on the ribosomal quality control pathway, it is not known how the tRNA is hydrolysed from the ubiquitylated nascent chain before its degradation. Here we show that the Cdc48 adaptor Vms1 is a peptidyl-tRNA hydrolase. Similar to classical eukaryotic release factor 1, Vms1 activity is dependent on a conserved catalytic glutamine. Evolutionary analysis indicates that yeast Vms1 is the founding member of a clade of eukaryotic release factor 1 homologues that we designate the Vms1-like release factor 1 clade.

Yeast Cdc48, an AAA + (ATPases associated with diverse cellular functions) ATPase that is conserved across eukaryotes and archaea, is a protein unfoldase15,16 that engages in myriad cellular functions through the binding of adaptors such as Ufd1–Npl4, which bind both Cdc48 and ubiquitylated substrate proteins. A protein A-based ‘non-stop’ substrate reporter (PrANS) encoded by an mRNA lacking a stop codon (Fig. 1b) accumulates on the ribosomal 60S subunit as a ubiquitylated species linked to tRNA when Cdc48 or Ufd1–Npl4 function is compromised17, suggesting that Cdc48–Ufd1–Npl4 somehow extracts the ubiquitylated nascent chain from the 60S tunnel6–8.

Two pieces of evidence implicated Vms1, which contains a Cdc48-binding VIM motif17,18, in the ribosome quality control (RQC) pathway. First, vms1Δ and RQC-deficient mutations cause a synthetic growth defect when combined with mutations that impair degradation of non-stop mRNA8,19. Second, vms1Δ mutants, like rqc2Δ20, are sensitive to cycloheximide treatment. We extended this by showing that vms1Δ, like ltn1Δ (Ltn1 is also known as RRK1) and cdc48Δ, displayed sensitivity to hygromycin, which binds the decoding centre (Extended Data Fig. 1a).

To investigate further the role of Vms1 in the degradation of non-stop proteins, we examined PrANS levels. PrANS conjugated to tRNA (PrANS–tRNA) accumulated in vms1Δ cells, but not in mutants lacking other Cdc48 adaptors (Fig. 1c). Additionally, vms1Δ cells accumulated high molecular mass forms of PrANS. Treatment with RNase or deubiquitylase enzymes (Extended Data Fig. 1c) and pull-downs with ubiquitin-binding TUBE resin (Extended Data Fig. 1d) confirmed that high molecular mass PrANS species were tRNA-linked ubiquitin conjugates (Ub–PrANS–tRNA).

Splitting of stalled 80S ribosomes is a prerequisite for the stable association of Ltn1 with 60S subunits. Ltn1 then ubiquitylates the nascent chains before their degradation by 26S proteasomes21. To determine whether Ub–PrANS–tRNA accumulated on 60S subunits in vms1Δ cells, we fractionated cell lysates on sucrose gradients. Immunoblotting for PrANS and the 60S protein Rpl3 revealed that Ub–PrANS–tRNA accumulated maximally in the first few Rpl3-containing fractions, which marks the position of 60S subunits21 (Fig. 1d). By contrast, non-ubiquitylated PrANS–tRNA that accumulated in both vms1Δ and vms1Δltn1Δ cells was enriched on 80S subunits and polysomes. These data confirm that Ub–PrANS–tRNA accumulated on 60S subunits in vms1Δ in a manner that was dependent on Ltn1.

If Vms1 is directly involved in releasing stalled PrANS, it should bind ribosomes. To investigate this prediction, we immunoblotted sucrose gradient fractions from cells expressing Vms1 tagged with a triple haemagglutinin epitope (Vms1–HA3) and Rli1 was restricted to 60S fractions4, whereas Vms1–HA3 was detected across the entire gradient (Fig. 1e). This behaviour suggests a broader function for Vms1 beyond its role in the RQC pathway. Notably, the 60S peak of Vms1–HA3 was diminished and shifted towards lighter fractions in ltn1Δ cells (Fig. 1e), suggesting that in addition to Cdc48–Ufd1–Npl44, Ltn1 also promotes the recruitment of Vms1 to 60S subunits. However, unlike Cdc48–Ufd1–Npl4, Vms1 was not identified as a component of the RQC complex4. Consistent with this, Vms1 did not associate directly with Ufd1–Npl4 (Extended Data Fig. 1e–h).

Given the exceptionally strong accumulation of PrANS–tRNA in vms1Δ mutants, we wondered whether Vms1 might have any relationship to known peptidyl-tRNA hydrolases. We carried out sensitive iterative sequence profile searches with PSI-BLAST and hidden Markov models seeded with the core RNase H fold domain of the archaeal and eukaryotic release factor 1 (RF1) proteins and the catalytically inactive Dom34 proteins, which are both members of the archaeo-eukaryotic RF1 (aeRF1) superfamily (see ‘Computational analyses’ in Methods). Notably, these searches recovered the central globular region of Vms1 orthologues. Reciprocal PSI-BLAST searches with this region recovered several members of the aeRF1 superfamily. This relationship was confirmed with a profile–profile comparison using the HHpred program with an alignment of the central conserved region of Vms1 run against the Protein Data Bank (PDB) database, which significantly recovered human eRF1 (PDB accession number 1DT9; P = 4 × 10–5). This showed that the central domain of Vms1 had a putative catalytic loop bearing a conserved glutamine comparable to the classic active site of aeRF1 proteins (Fig. 2a, b). An aeRF1-like topology was observed in the recently reported crystal structure of the middle domain of Vms1, but the putative active-site loop was not resolved22. The conserved glutamine in the eRF1 protein coordinates a water molecule in the A site at the peptidyl transferase centre (PTC). Nucleophilic attack of the peptidyl-tRNA in the P site by this water molecule releases the completed polypeptide chain23,24. In addition to the glutamine in the putative active site, Vms1 also contains conserved vicinal arginines...
in the α-helix immediately following the active-site loop (Fig. 2a, b). Equivalent arginines in eRF1 have been implicated in the stabilization of the active-site loop23, and stimulation of GTase eRF323. Based on these similarities, we designated Vms1 as the founding member of the Vms1-like release factor 1 (VLRF1) clade within the aeRF1 superfamily. In addition to the eukaryotic orthologues of Vms1, the VLRF1 clade also contains members from certain bacteria, predominantly bacteroidetes and more divergent versions in certain Archaea (Extended Data Fig. 3d). Notably, C-terminal region is characterized by a run of three ankyrin repeats, and certain SBDS paralogues, which function late in 60S subunit maturation17. This suggests that Vms1 might contact the ribosome with its Rei1-like zinc-finger domain (Fig. 2c). Meanwhile, the Vms1 C-terminal region is characterized by a run of three ankyrin repeats, a treble-clef fold zinc-binding domain (VTC; lost in fungi) and a VIM motif that binds Cdc4817, 18 (Fig. 2c). To probe the role of these other sequence features, we generated mutants including DNKR (lacking four Rei1-like zinc-finger residues predicted to interact with the 60S subunit; see legend to Extended Data Fig. 3c for details), RR (lacking the conserved vicinal arginines in the catalytic domain) and ΔVIM, and expressed the proteins in a vms1Δ strain. All mutant proteins were expressed comparably to wild type (Extended Data Fig. 3a). To assess the effects of the mutations, we performed sucrose gradient fractionation of ribosomes. Compared to vms1Δ rescued by wild-type VMS1HA3, all mutants accumulated Ub–PrANS–tRNA in 60S fractions (Extended Data Fig. 3c). The RR mutant exhibited the strongest accumulation, as well as conspicuous spreading of Ub–PrANS–tRNA into the 80S peak, which was also observed with the ΔVIM mutant. Notably, the DNKR mutant protein was shifted to lighter fractions, with depletion from monosome and polyosome fractions, consistent with a role for the zinc-finger in binding ribosomes (Extended Data Fig. 3d). Notably, the cycloheximide sensitivity of the various mutants mimics the data obtained with the reporter, with the ΔAS and RR mutants being most sensitive (Extended Data Fig. 3b).

To address conclusively the hypothesis that Vms1 is a novel peptidyl-tRNA hydrolase, we sought to reconstitute peptidyl-tRNA hydrolytic activity in vitro. Polyhistidine (His6) tags were added to the N-terminus of wild-type Vms1 and the Vms1–Q295L mutant. The proteins were then expressed in bacteria and purified (Extended Data Fig. 2a, b). Native lysates (10 μg protein/μl) were separated on sucrose gradients and fractions were resolved on Tris–glycine gels and immunoblotted with PAP and an anti-Rpl3 antibody. Molecular masses (MM) 250, 150, 50 and 23 kDa are indicated by marks overlaid on the gel. Model substrate PrANS–tRNA (Fig. 3a, left), which sedimented with ribosomes (Fig. 3a, right), was used to study the non-stop pathway.

To verify that Vms1 is a novel peptidyl-tRNA hydrolase we generated two separate point mutations (Q295L and Q295P) of the predicted catalytic glutamine, which abrogate eRF1 function in budding yeast23. We also generated a putative catalytic region of Vms1 and other VLRF1 proteins is separated on sucrose gradients and fractions were resolved on Tris–glycine gels and immunoblotted with PAP and an anti-Rpl3 antibody. Molecular masses (MM) 250, 150, 50 and 23 kDa are indicated by marks overlaid on the gel. Model substrate PrANS–tRNA (Fig. 3a, left), which sedimented with ribosomes (Fig. 3a, right), was used to study the non-stop pathway.

### Table: Ribosome splitting

| Yeast | Hbs1 | Dom34 | Rei1 | Rqc1 | Rqc2 | Ltn1 | Cdc48 | Ufd1 | Npl4 | Vms1 |
|-------|------|-------|------|------|------|------|-------|------|------|------|
| Mammals | HBS1L | PELO | ABCE1 | TCF25 | NEMF | LT1N | VCP | UFD1 | NLOC4 | ANZK1 |

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C2H2-type zinc-finger of Vms1 is specifically related to those of Rei1 flanked by additional N- and C-terminal domains. The N-terminal mutants were extremely sensitive to hygromycin and cycloheximide. Vms1–Q295L was depleted from low molecular mass fractions (Fig. 3c). Consistent with the biochemical defect, the vms1–Q295L, vms1–Q295P and vms1ΔAS mutants were extremely sensitive to hygromycin and cycloheximide (Fig. 3d). The point mutants thus phenocopy vms1Δ, both genetically and biochemically.
Fig. 2 | Vms1 is a member of the aeRF1 superfamily and the founding member of the VLRF1 clade. a. Multiple sequence alignment of the aeRF1 superfamily; protein names, organism abbreviations and accession numbers are indicated on the left, and families are indicated in boxes on the bottom right. Atul, Archaeoglobus fulgidus; Aper, Aeropyrum pernix; Atha, Arabidopsis thaliana; Aful, Archaeoglobus fulgidus; Aper, Aeropyrum pernix; Atul, Archaeoglobus fulgidus; Dom43, Cenarchaeum symbiosum; CHei, Candidatus Heimdallarchaeota archaeon; Shum, Homo sapiens; Sinomonas humi; Nbac, Nitrospirae bacterium; Ocya, Oscillatoriales sp. A56; Mvac, Methanosarcina barkeri sp. A56; Mvac, Methanosarcina barkeri sp. A56; Vacuolata, Cyclobacterium marinum; b. Vms1 is a member of the aeRF1 superfamily and the founding member of the VLRF1 clade; baERF1, bacteroidetes VLRF1 family; Cmar, Cyclobacterium marinum; CHei, Candidatus Heimdallarchaeota archaeon; Hsap, Homo sapiens; Halostagnocila sp. A56; Mvac, Methanosarcina barkeri sp. A56; Nbac, Nitrospirae bacterium; Ocya, Oscillatoriales sp. A56; aVLRF1, archaeal VLRF1 family; bVLRF1, bacteroidetes VLRF1 family; Cmar, Cyclobacterium marinum; CHei, Candidatus Heimdallarchaeota archaeon; Hsap, Homo sapiens; Halostagnocila sp. A56; Mvac, Methanosarcina barkeri sp. A56; Nbac, Nitrospirae bacterium; Ocya, Oscillatoriales sp. A56; vms1, Vms1 domain architectures.

For the substrate, we used 60S fractions from vms1Δ cells expressing PrANS. Recombinant Vms1 and the substrate were mixed, and after incubation, peptideyl-tRNA was precipitated using hexadecyl-trimethylammonium bromide (CTAB). His6–Vms1 promoted the release of both Ub–PrANS–tRNA and PrANS–tRNA species from 60S subunits (Fig. 3a). By contrast, His6–Vms1-Q295L was unable to release modified substrate. This assay was reproducible but required extensive manipulations that resulted in spontaneous hydrolysis of the substrate during its preparation. In addition, it was difficult to reliably quantify heterogeneous ubiquitin conjugates by immunoblot. Therefore, we turned to in vitro translation in rabbit reticulocyte lysate, which has been used to reconstitute ubiquitylation of RQC substrates. These earlier studies demonstrated that ubiquitylation of the nascent chain is delayed by approximately 15 min after translation. Because the Vms1-dependent reaction (Fig. 4a) did not display strong dependence on substrate ubiquitylation, we ran brief translation reactions with a non-stop template encoding Flag-tagged CRP with a 30-nucleotide poly(A) tail (Flag–CRPNSKn) to generate stalled nascent chains that had not yet been ubiquitylated, which simplified quantification. The major translation product (Fig. 4b) was confirmed to be tRNA-conjugated Flag–CRPNSKn using CTAB precipitation and RNase treatment (Extended Data Fig. 4b). Consistent with a previous report, the cyanobacterium; Scer, Saccharomyces cerevisiae; Shum, Sinonomas humi. Residues are coloured per consensus conservation, residue characteristics are indicated on the bottom consensus line (h, hydrophobic; l, aliphatic; p, polar; +, positively charged; b, big; u, tiny; s, small), key residues and features are marked at the top (* indicates the catalytic glutamine, † indicates arginine residues conserved across multiple families and ‡ indicates the arginine that is conserved in the VLRF1 family). b. Topology diagram of the aeRF1 RNase H fold domain with key features labelled. c. Domain architectures of proteins containing the Rei1 C2H2-type zinc-finger. Ank, ankyrin repeat; ZnF, zinc-finger domain.

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The major translation product (Fig. 4b) was confirmed to be tRNA-conjugated Flag–CRPNSKn using CTAB precipitation and RNase treatment (Extended Data Fig. 4b). Consistent with a previous report, the stalled Flag–CRPNSKn–tRNA was associated maximally with 80% fractions (Extended Data Fig. 4c).

To assay for eRF1-like peptideyl-tRNA hydrolytic activity, wild-type or mutant His6–Vms1 was incubated with 35S-labelled substrate for increasing periods of time (Fig. 4b). De-acylation of Flag–CRPNSKn–tRNA with concomitant formation of unmodified substrate was observed within three minutes (Fig. 4b, c) and at concentrations as low as 25 nM His6–Vms1 (Fig. 4d, quantified in Extended Data Fig. 4d), whereas essentially no hydrolysis above background was observed with His6–Vms1-Q295L. Because recombiant yeast Vms1 was functional in this heterologous system, we next tested ANKZF1, the human Vms1 orthologue. Purified ANKZF1 (Extended Data Fig. 4a) catalysed deacylation, whereas the active site mutant ANKZF1-Q246L was inactive (Fig. 4e and Extended Data Fig. 4e).

Like vms1Δ mutants, cdc48 mutant cells accumulate Ub–PrANS–tRNA on 60S subunits. This raises the question as to what the role of Cdc48 is in the hydrolysis of peptideyl-tRNA. We were unable to establish ATP-dependence for the in vitro deacylation reaction (data not shown), which perhaps is not surprising because the ATPase Rli1 accelerates nascent chain release by eRF1 in the presence of non-hydrolysable ATP. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest that Cdc48 facilitates presentation of hydrolysable ATP 5. We suggest...
Fig. 3 | Point mutations in the conserved, putative peptidyl-tRNA hydrolase active site of Vms1 phenocopy vms1Δ. a, Schematic of experimental design. Brown circles, ubiquitin; green circles, tRNA. Left blot, lysates from vms1Δ cells expressing either wild-type Vms1–HA3 or the active site mutants were fractionated on a NuPAGE gel, and immunoblotted to detect protein A and Rpl3. Middle blot, ribosomes were isolated on sucrose cushions, and aliquots were served as a loading control.

b, Native lysates (10 μg ml−1) from vms1Δ expressing either wild-type Vms1–HA3 Δ or the Q295L mutant (right) were fractionated on sucrose gradients and immunoblotted to detect protein A and Vms1–HA3. Hexokinase (HK) was immunoblotted to detect mutant Vms1 and Rpl3. They were run at the same time as wild-type Vms1–HA3 in Fig. 1e and the exposures are identical. All western blots are representative of two biological replicates.

c, Sucrose gradient fractions from vms1Δ expressing Vms1–Q295L–HA3 were immunoblotted to detect mutant Vms1 and Rpl3. They were run at 30 °C for 3 days. Images shown are representative of three biological replicates.

d, Schematic of PrANS–tRNA. a, PrANS–tRNA is released after deacylation. The emerging nascent chain is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our experiments indicate that the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our data indicate that in vivo, the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our data indicate that in vivo, the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our data indicate that in vivo, the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our data indicate that in vivo, the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our data indicate that in vivo, the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1. Our data indicate that in vivo, the leading stalled ribosome on non-stop poly(A) mRNA that has its A site occupied could be the monosome substrate for Vms1 (see Extended Data Fig. 5 for model). Cleavage by an unidentified endonuclease generates ribosomes with empty A sites on truncated mRNA that are known to be rescued by Dom34–Hbs1–Rli1Δ. Jammed nascent chain–tRNA on these split 60S subunits is ubiquitylated by Ltn1 and the tRNA linkage is hydrolysed by Vms1.
Fig. 4 | Peptidyl-tRNA hydrolase activity of Vms1 and ANKZF1 depend on the catalytic glutamine residue. a, Left, experimental design. Brown circles, ubiquitin; green, tRNA. Lysate from vms1Δ cells expressing PrANS was fractionated on a sucrose gradient, and 60S fractions were pooled and pelleted. Top right, His6–Vms1 or His6–Vms1–Q295L was added and incubated for 10 min at 30 °C. Ub–PrANS–tRNA was precipitated with CTAB, and immunoblotted with PAP to detect protein A. Bottom right, shorter exposure from which the discrete peptidyl-tRNA band was quantified (lane five contained additional 1 mM GTP). Western blot is representative of two biological replicates. b, WT, His6–Vms1; Q295L, His6–Vms1–Q295L. c, Titration of Vms1. Reactions and processing as in b. d, Wild-type ANKZF1 and ANKZF1–Q246L mutant were analysed as in b, except that the hydrolase reaction was performed at 37 °C. The image shown is representative of two biological replicates. Gel source images and quantification data are provided in Supplementary Fig. 1.

Discovery of Vms1 as the founding member of the VLRF1 clade reveals that multiple different functional peptidyl-tRNA hydrolase release factors belonging to the aeRF1 superfamily coexist in eukaryotic cells. We posit that VLRF1 and Dom34 evolved to protect the translation apparatus from errors in transcription and post-transcriptional processing that generate defective mRNAs.

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Author contributions R.V., K.M.R. and R.J.D. designed experiments, J.M.R. performed mass spectrometry analyses, L.A. and A.M.B. did the computational analyses, K.M.R. performed the mutagenesis analyses, R.S.O. generated all the yeast strains and R.V. performed all the biochemical experiments. R.V. and R.J.D. supervised research, R.V., R.J.D. and L.A. wrote the paper and all authors participated in editing the manuscript. All figure schematics were generated by K.M.R.

Competing interests R.V., K.M.R., A.M.B., R.S.O., J.M.R. and L.A. declare no competing interests. R.J.D. is currently Senior Vice President of discovery research at Amgen and a Visiting Associate at the California Institute of Technology (Caltech).

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**METHODS**

**Yeast strains and culture conditions.** All strains used in the current study are listed in Extended Data Table 1. They were derived from W303 or S288C backgrounds and grown as described.

**Western blot analyses.** The following antibodies were used: PAP (Sigma, P1291), anti-Flag M2 (Sigma, F-1804), anti-TAP (Thermo Fisher, CAB1001) and anti-HA 3F10 (RocheSigma, 12013819001). Anti-Rp32 and -Rpl3 antibodies were a gift from J. Warner.

**Ribosome isolation and sub-fractionation.** The sucrose cushion method for the isolation of ribosomes; the binding of ribosomal preparations to TUBE-UBA resin; sucrose density gradient centrifugation; and CTAB precipitation of peptide-tRNA from sucrose gradient fractions were performed as previously described. TUBE resin was purchased from Boston Biochem (AM-130). CTAB was purchased from Sigma (52365).

**Site-directed mutagenesis.** All expression constructs for mutational analysis were generated using the Qu5-Site-Directed Mutagenesis Kit (NEB E0655S4) following the manufacturer’s instructions. Primers were designed using the online tool NEBaseChanger (http://nebasechanger.neb.com). Mutations were confirmed by Sanger sequencing. All plasmids are listed in Extended Data Table 1.

**Purification of His6–Vms1 and His6–Vms1-Q295L from bacteria.** Wild-type and mutant Vms1 (Extended Data Table 1) were cloned into the bacterial PET-28a vector in frame with the N-terminal His6 tag. Expression was induced in Rosetta cells using 1 mM IPTG at 16 °C for 16h. Cell pellets were brought up in lysis buffer containing 50 mM Hepes, pH 8.0, 0.5 M NaCl, 20 mM imidazole and 0.5% Triton, sonicated, and centrifuged at 34,000g for 20 min in a Sorvall SS34 rotor. Supernatants were loaded onto a Ni-NTA affinity resin (Qiagen) for 1 h at 4 °C. Bound proteins were washed four times in lysis buffer and eluted in 25 mM Hepes, pH 7.5, 150 mM NaCl, 5% glycerol and 250 mM imidazole. Imidazole was removed by dialysis and proteins were concentrated using Amicon Ultra centrifugal filters.

**Purification of ANKZF1–Flag–Myc and ANKZF1(Q246L)–Flag–Myc.** Plasmids were constructed that contained the cytomegalovirus (CMV) promoter followed by sequences encoding wild-type (Origene) or Q246L ANKZF1 (current study; confirmed by sequencing) tagged with a Flag–Myc epitope sequence at the 3′ end of the open reading frame. These constructs were transiently transfected into human embryonic kidney HEK293 cells (ATCC, tested negative for mycoplasma contamination). After 72h, proteins were purified using a previously published protocol for Flag-tagged mammalian proteins.

**In vitro transcription and translation.** PCR products were generated using 3× Flag–CRP (Extended Data Table 1) as a template with the reverse primer either containing a stop codon (3× Flag–CRPStop), or lacking a stop codon but with a 30-nucleotide poly(T) extension (3× Flag–CRPNSK). PCR products were transcribed and translated in vitro using TNT T7 Quick rabbit reticulocyte lysate (Promega, L5340) in the presence of radioactive methionine (Perkin Elmer, NEN-4709) on ice. Reaction mixtures were typically for 30 min at 30 °C, at which time recombinant proteins were added for another 10 min. Aliquots were resolved by SDS-PAGE, and dried gels were exposed either to film or to a PhosphorImager screen.

**Ribosomal isolation and sub-fractionation.** The sucrose cushion method for the isolation of ribosomes; the binding of ribosomal preparations to TUBE-UBA resin; sucrose density gradient centrifugation; and CTAB precipitation of peptide-tRNA from sucrose gradient fractions were performed as previously described. TUBE resin was purchased from Boston Biochem (AM-130). CTAB was purchased from Sigma (52365).

**Data availability.** Gel source images for Figs. 1, 3, 4 and Extended Data Figs. 1, 3 and 4 are available in Supplementary Fig. 1. Source Data for quantifications in Figs. 4 and 8 are provided in the online version of the paper. All other data supporting the findings of this study are available from the corresponding authors upon reasonable request.

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Extended Data Fig. 1 | Mass spectrometry analysis of Vms1 required for release of Ub–PrANS–tRNA from ribosomes. a, Serial tenfold dilutions of exponential cultures were spotted on YPD plates with or without 100 μg ml⁻¹ hygromycin B and allowed to grow at 30 °C for 3 days. Data shown are representative of three biological replicates. b, Ponceau S stained nitrocellulose filter used as loading control for Fig. 1c. c, Lysate from vms1Δ cells expressing PrANS (input lysate, lane five) was fractionated on a sucrose gradient and 60S fractions were pooled and mock-treated (lanes one and three) or pretreated with the deubiquitylating enzyme Usp2c (1 μM; lane two) or 100 μg ml⁻¹ RNase A (lane four) at 30 °C for 20 min before CTAB precipitation. The pellets were immunoblotted with PAP. d, Ribosomes from the indicated strains were isolated using sucrose cushions and aliquots were immunoblotted to detect PrANS and Rpl3. The remainder was bound to TUBE resin. The adsorbed fractions were immunoblotted with PAP. All lanes in the left and right panels are from the same blots. The dashed lines indicate cropping of lanes not pertinent to the current study. Uncropped gel source images are provided in Supplementary Fig. 1. Data in c and d are representative of two biological replicates. e–h, Mass spectrometry analysis of TAP-tagged Cdc48, Vms1 and Ufd1. e, Relative abundance of each Cdc48 adaptor co-immunoprecipitated with Cdc48 relative to all adaptor proteins identified. f, Schematic illustrating the estimated stoichiometry of Cdc48–adaptor complexes. g, Relative stoichiometry of associated proteins that co-immunoprecipitated with Cdc48, Vms1 and Ufd1. Samples were normalized to the iBAQ value of the bait protein and are presented as percentage of the bait protein. Protein iBAQ values from the untagged control were subtracted from the tagged immunoprecipitation samples. h, Coomassie blue-stained gel of samples used for mass spectrometry analysis. All mass spectrometry data (e–h) are representative of two biological replicates.
Extended Data Fig. 2 | Vms1 is the founding member of the VLRF1 clade. a, Extended sequence alignment of aeRF1 superfamily with representatives from all families and clades (compared to the limited subset shown in Fig. 2a). b, Phylogenetic tree depicting relationships within the aeRF1 superfamily; colouring matches clade labels in Fig. 2a.

In the classical aeRF1 clade, two branches contain eukaryotic orthologues (eRF1) and archaeal orthologues (aRF1), respectively. Of the bacterial (baeRF1) versions, certain members are misannotated as ‘Host_attach’ in the Pfam database while most cannot be detected by existing profiles.

The total number of prokaryotic representatives of the VLRF1 clade in the non-redundant database (NCBI, as of 1 December 2017) is 1,044. Of these, the archaeal VLRF1 family (aVLRF1) has 279 members, actinochloroflexi VLRF1 family (acVLRF1) has 669 and the bacteroidetes VLRF1 family (bVLRF1) has 96. Notable domain architectures and conserved gene neighbourhoods are shown to the right of tree. A gene encoding a ribosome hibernation factor (HPF-like or YfiA-like) that facilitates inactive ribosome aggregation frequently co-occurs and is predicted to function with the baeRF1 domains. The labels below the architecture diagrams list the well-characterized clade representatives, PDB structure accessions and phyletic distributions. The dashed outline indicates domains that are not universally present.
Extended Data Fig. 3 | Mutagenesis of the non-catalytic domains of Vms1. a, SDS lysates of cells were immunoblotted with anti-haemagglutinin antibodies. Ponceau S staining of the blot shows the equivalency of the extracts. The RR mutant is R313A, R314A. The DNKR mutant contains the following four mutations in the zinc-finger domain: D94A, N99A, K101A, and R102A. The ∆VIM mutant is deleted for amino acids 622–625. b, Serial tenfold dilutions of wild-type and vms1Δ cells transformed with the indicated vms1 alleles were spotted on YPD plates containing 10 or 25 ng ml⁻¹ cycloheximide and incubated at 30 °C for 3 days. Data are representative of three biological replicates. c, Native lysates (10 A260 units each) were subjected to sucrose gradient analysis. In each case fractions 10–23 were resolved and immunoblotted to detect protein A and Rpl3. Identical exposures are shown for all panels. d, Native lysates of vms1Δ cells expressing wild-type Vms1–HA3 or Vms1–HA3–DNKR were subjected to sucrose gradient analysis. Fractions were immunoblotted for Vms1 and Rpl3. MW, molecular weight marker. All western blot data are representative of two biological replicates. Gel source images are available in Supplementary Fig. 1.
Extended Data Fig. 4 | In vitro reconstitution of Vms1 peptidyl-tRNA hydrolase activity. **a**, Coomassie blue-stained gels of the indicated purified proteins used for in vitro reconstitution. **b**, Analysis of ribosome–nascent chain complexes generated by translation in reticulocyte lysate. PCR products encoding Flag–CRPNS followed by a 30-nucleotide poly(A) sequence and Flag–CRPStop (with a stop codon) were transcribed and translated in reticulocyte lysate in the presence of 35S-labelled methionine. Completed translation reactions were treated (or not treated), fractionated using SDS–PAGE, and visualized by autoradiography. Lane one, no treatment; lane two, RNase A treatment; lane three, CTAB treatment; lane four, immunoprecipitated with anti-Flag resin. For lanes three and four, the pellet fraction was analysed. **c**, Sucrose gradient analysis of reticulocyte translation reactions. Flag–CRPNSKn was transcribed and translated in 200 μl reticulocyte lysate for 30 min. Lysates were layered onto 2-ml 10–50% sucrose gradients and centrifuged using a Beckman SW55 rotor for 80 min. Eleven fractions (200 μl each) were collected from the top by hand. Aliquots were analysed for fractionation of 35S-labelled substrate and Rpl8 by autoradiography and immunoblotting, respectively. **d**, Quantification of two independent biological replicates of the yeast His6–Vms1 and His6–Vms1–Q295L titration reactions shown in Fig. 4d. **e**, Quantification of the human ANKZF1 and ANKZF1–Q246L titration reactions shown in Fig. 4e. Data are representative of two biological replicates. Gel source images are provided in Supplementary Fig. 1 and Source Data are available with the online version of this paper.
Extended Data Fig. 5 | Working model of Vms1 function at stalled ribosomes. In non-stop decay of mRNAs lacking a stop codon, ribosomes translate the poly(A) tail and stall after translating several lysines. The A site as a consequence is occupied by an AAA codon. Our data suggest that Vms1 can potentially hydrolyse peptidyl-tRNA chains on this leading stalled ribosome without prior splitting by Dom34–Hbs1–Rli1. One of the known responses to stalling is endonucleolytic cleavage of the mRNA by an as-yet unidentified endonuclease. The cleavage reaction generates a truncated transcript. Lagging ribosomes that translate up to the cleavage site stall with an empty A site. Such stalls are recognized by Dom34–Hbs1 that together with Rli1 dissociate the 80S ribosome into the 40S subunit and the 60S subunit, which contains the nascent peptidyl-tRNA. Dissociation allows for stable association of the RQC complex members Rqc1–Rqc2 and the E3 ubiquitin ligase Ltn1. Rqc2 adds non-templated alanine and threonine residues to the C-terminal end of the nascent chain to extrude sequences in the exit tunnel past the active site of Ltn1, which ubiquitylates lysine residues in the emerging nascent chain and with the aid of Cdc48, optimizes the conformation of Vms1 at the PTC such that it can hydrolyse the tRNA. The ubiquitylated nascent chain is engaged by Ufd1–Npl4 bound to Cdc48 that together unfold and extract the nascent chain. Dissociation of Rqc2 allows Vms1 to access the 60S subunit, resulting in hydrolysis of the peptidyl-tRNA. Recruitment of Cdc48–Ufd1–Npl4 to the ubiquitylated nascent chain ensures its efficient extraction from the 60S subunit. It is unclear whether the action of Vms1 on 80S ribosomes is coupled in some manner to ribosome splitting.
Extended Data Table 1 | *Saccharomyces cerevisiae* cell lines and plasmids used

| PDL Number | Alias | Source | Genotype |
|------------|-------|--------|----------|
| 0569       | S288C | Open Biosystems | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, a |
| 5070       | S288C pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, [pGAL-ProteinA-nonstop-PGK3'UTR-URA3], a |
| 6646       | ubx3Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx3:KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 6647       | ubx4Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx4:KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 6648       | ubx5Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ubx5:KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 6654       | ufd2Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ufd2::KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 6651       | ufd3Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ufd3::KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 6640       | vms1Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 5510       | ltn1Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ltn1::KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], a |
| 6670       | ufd1-1 pProtein A Nonstop | This Study | his4-519, ura3-52, ade1-100, leu2-3, 112, ufd1-1, TRP+, pGAL-ProteinA-nonstop-PGK3'UTR-URA3 |
| 6722       | ltn1Δ vms1Δ pProtein A Nonstop | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, ltn1::KANMX, [pGAL-ProteinA-nonstop-PGK3'UTR-URA3], a |
| 6771       | vms1Δltn1Δ pVMS1-HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, ltn1::KANMX, [YCplac111-VMS1-3HA], a |
| 5445       | vms1 Δ | Open Biosystems | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, a |
| 5400       | ltn1 Δ | Open Biosystems | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, ltn1::KANMX, a |
| 6698       | vms1Δ pVMS1 HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-VMS1-3HA], a |
| 6732       | vms1Δ pvm11 C295L HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-C295L-3HA], a |
| 6701       | vms1Δ pProtein A Nonstop vms1-C295L-HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-C295L-3HA], a |
| 6705       | vms1Δ pProtein A Nonstop vms1-C295L-3HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-C295L-3HA], a |
| 6733       | vms1Δ pvm11 C295P | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-C295P-3HA], a |
| 6731       | vms1Δ pVMS1 ASΔ3HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-ActiveSitΔ3-3HA], a |
| 6740       | vms1Δ pvm11 Δb2Δ-625 (vmΔ) 3HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-Δb2Δ-625-3HA], a |
| 6739       | vms1 Δ pvm11 Δnir3 Δ3HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-Δnir3-3HA], a |
| 6738       | vms1 Δ pvm11 RPS313/314AA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [YCplac111-vms1-RPS313/314-3HA], a |
| 6707       | vms1Δ pProtein A Nonstop pvm1 ΔasΔ3HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], [YCplac111-vms1-ASΔ3-3HA], a |
| 6706       | vms1Δ pProtein A Nonstop pvm1- ΔasΔ265-3HA | This Study | his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, vms1::KANMX, [pGAL-ProteinA-Nonstop-PGK3'UTR-URA3], [YCplac111-vms1-C295P-3HA], a |

**Plasmid** | **Relevant Vector** | **Source**
--- | --- | ---
RD82764 | pGAL-ProteinA-nonstop-PGK3'UTR-URA3 | Ambro VanHoof
RD82266 | YCplac111-VMS1-3HA | Alexander Buchberger
RD82267 | YCplac111-vms1 ΔAA74-102-HA | This Study
RD82298 | YCplac111-vms1 Δnir3-HA | This Study
RD82299 | YCplac111-vms1 ASΔ3-HA | This Study
RD82302 | YCplac111-vms1 RR313/314AA | This Study
RD82652 | pET28a-VMS1 | Hai Rao
RD83336 | pET28a-vms1 C295L | This Study
RD83339 | pCMV5-ANKZF1-mycDDK | OriGene
RD83357 | pCMV5-ankZF1C295L-mycDDK | This Study
RD85299 | T7-SaFLAG-CRP | This Study

Top, list of yeast strains used in this study; bottom, list of plasmids used in this study.
Experimental design

1. Sample size
   Describe how sample size was determined.

   No sample size calculation was performed because the size of the effect was unknown. Experiments were repeated (biological replicates) to confirm results, or done in duplicate as in Figs. 4b, 4d.

2. Data exclusions
   Describe any data exclusions.

   No data was excluded from the analyses.

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   All findings have been replicated twice (western blots and PhosphorImager analyses), or thrice (spot viability assays).

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   Not relevant to the current study. There were no experimental groups.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   The experimenter was aware of the identity of the samples being analyzed. Blinding was not relevant in the current study since the Figures show the raw data from the experiments and thus the reader can directly assess the accuracy of the interpretations by the experimenter.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a

   Confirmed

   ☒ The exact sample size \( (n) \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)

   ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

   ☒ A statement indicating how many times each experiment was replicated

   ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)

   ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons

   ☒ The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted

   ☒ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)

   ☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

- Commercial Prism software. Otherwise, all publicly available software: BLAST, PSIBLAST, RPSBLAST, HHpred, HMMER3, KALGN, FastTree, MaxQuant, Dalilte, PyMol 1.8.2.0 (Open Source). The central conclusion of the MS is supported by all publicly available software.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

- Peroxidase Anti-Peroxidase Soluble Complex (PAP; used to detect Protein A, Sigma, # P1291), Anti-Flag M2 (Sigma # F-1804), anti-TAP (Thermo Fisher # CAB1001, and anti-HA 3F10 (Roche/Sigma # 12013819001). Antibodies were validated by using untagged or untransformed controls. Anti-Rpl32 and Rpl3 were a gift from Jonathan Warner. These have been validated by immunoblotting sucrose gradient ribosomal fractions as well as comparing mobility on gels with tagged controls.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

- All yeast cell line sources are listed in Table 1. 293T cells used for the expression and purification of hAnkzf1 were from ATCC (DTC#264).

b. Describe the method of cell line authentication used.

- All yeast cell lines were confirmed by selection on drop out media or genomic PCR, or temperature-sensitivity, if the strain was temperature sensitive. ATCC certification for 293T cells.

c. Report whether the cell lines were tested for mycoplasma contamination.

- 293T cells used for expression and purification of hAnkzf1 tested negative for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human research participants.