SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIAL AND METHODS

Yeast culture and media
YPD medium was composed of 10 g/l Bacto yeast extract (Becton Dickinson, USA), 20 g/l Bacto peptone (Becton Dickinson, USA) and 2% (w/v) D-glucose. Synthetic complete (SC+D) media was composed of 1.7 g/l Difco yeast nitrogen base without amino acids and (NH₄)₂SO₄ (Becton Dickinson, USA), 5.0 g/l (NH₄)₂SO₄ (Sigma), 0.03 g/l isoleucine, 0.15 g/l valine, 0.04 g/l adenine, 0.02 g/l histidine, 0.1 g/l leucine, 0.02 g/l methionine, 0.05 g/l phenylalanine, 0.2 g/l threonine, 0.04 g/l tryptophan, 0.03 g/l tyrosine, 0.02 g/l uracil, 0.1 g/l glutamic acid, 0.1 g/l aspartic acid, 0.02 mg/ml arginine, 0.03 mg/ml lysine (all amino acids were bought from Sigma), 2% (w/v) glucose). All yeast cultures were grown to mid-log phase and for at least four doublings. Similarly, in the case of heat-stress treatment, all cultures were exposed to 37 °C for at least four doublings unless stated otherwise.

SILAC labeling
SILAC strains were exponentially grown in SC-K-R+D supplemented with 0.2 g/l proline and 0.02 mg/ml arginine, 0.03 mg/ml lysine or 0.02 mg/ml ¹³C₆,¹⁵N₂-arginine (Sigma), 0.03 mg/ml ¹³C₆,¹⁵N₂-lysine (Sigma). The precultures were also grown in the labelled medium and cells were grown for at least 4 doublings in the main culture to ensure complete labelling. After harvesting, equal OD₆₀₀ of light and heavy cultures were mixed and subsequently processed together.

Protein extraction and digestion for MS
After harvesting yeast cells were treated with 1.85 M NaOH, 7.6% (v/v) β-mercaptoethanol for 10 min on ice then with an equal volume of 50% (w/v) trichloroacetic acid (CCl₃COOH) for 20 min on ice. The precipitated proteins were pelleted and washed with acetone at least three times. Tryptic digestion was carried out using the FASP method (1) with the sequencing grade modified trypsin (V5113, Promega). Digested peptides were purified on C18 MacroSpin columns (The Nest Group).

Peptide fractionation by isoelectric focusing (OffGel)
C18 purified peptides were fractionated in 12 fractions by isoelectric focusing on an OffGel Fractionator (G3100AA, Agilent) as per manufacturer instructions, except that the strips used were pH 3-11 NL, 13 cm (17-6003-75, GE Healthcare) and the ampholytes were 2% IPG buffer pH 3-11 NL (17-6004-40, GE Healthcare).

LC-MS-MS/MS
C18 cleaned peptides were resuspended in 0.1% CF₃COOH for the LC-MS-MS/MS analysis. An Ultimate3000 uHPLC system (Dionex, Thermo Scientific) was used for the online reverse phase liquid chromatography. Coated-tip fused silica columns of 50 cm length (PF-360-75-10-CE-5, New Objective) were in-house packed with C18 silica beads (Magic C18, 200 Å, 3 µm, Michrom Bioresources). A 250 nl/min gradient of buffer B (0.1% (v/v) HCOOH, 90% (v/v) CH₃CN, 3% (v/v) DMSO) in buffer A (0.1% (v/v) HCOOH, 2% (v/v) CH₃CN, 3% (v/v) DMSO) ranging from 2% to 35% over 240 min for the OffGel fractionated samples or for 360 min for unfraccionated samples was used to resolve peptides. The column oven compartment was maintained at 45 °C. The chromatography setup was directly coupled to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Finnigan) configured for top-15 data dependent acquisition (DDA) by collision-induced fragmentation (CID). MS1 scans were done in profile mode at the FT-MS resolution of 60,000 and MS/MS scans were done in centroid mode with the IonTrap Rapid Scan Rate. The precursor ion intensity threshold for
triggering fragmentation was set at 500 arbitrary units. Dynamic exclusion was enabled with a repeat count set at 1, repeat duration at 30 s, exclusion list size of 500 and exclusion duration of 90 s. Preview mode for FT-MS master scans, monoisotopic precursor selection and charge state screening with +1 rejection were enabled.

**Protein identification and quantification**

MS data were processed using the Trans-proteomic pipeline (2). Raw files were converted to the mzXML file format (3) and searched using Comet (4) against the Saccharomyces Genome Database protein database. For the searches, carboxyamidomethylation (57.022 Da) of Cys was set as a static modification and $^{13}\text{C}_6,^{15}\text{N}_2$-Lys (8.01419892 Da), $^{13}\text{C}_6,^{15}\text{N}_4$-Arg (10.008252778 Da) and oxidation of Met (15.99491463 Da) were set as variable modifications. Semi-tryptic digestion was set with a maximum of two missed cleavages. The mass error tolerances used were 25 ppm and 0.4 Da for MS and MS/MS, respectively. PeptideProphet and ProteinProphet were used for the evaluation of peptide and protein probabilities, respectively (5, 6). Protein abundance ratios were estimated by XPRESS (7). Results were stored in an in-house developed database.

**Data normalisation and statistical analysis of differential abundance**

Protein and peptide abundance ratios were imported into R. Peptides not passing the 1% FDR threshold were removed and the remaining peptide ratios were median normalised. Next, the peptides that were consistently quantified across label switch conditions were used to calculate protein abundance ratios. Subsequently, proteins meeting 1% FDR and having at least two quantified peptides were retained and protein abundance ratios were median normalised. Finally, statistical significance of changes in protein abundance was calculated using an empirically moderated Bayes t-test and p-values were adjusted for multiple testing by Benjamini and Hochberg procedure. The FDR threshold used for statistical significance of protein abundance ratios was 1% (adjusted p-value = 0.01).

**Random Forest analysis for codon importance**

Random Forest analyses of the codon content for the mRNAs corresponding to significantly changing proteins were done in R using the random forest implementation of the party package (v0.9-99992). The abundance of all codons except the stop ones were used as variables in the analysis. The number of trees in the forest was 1,000 and the number of predictor variables was set to square root of the total number of variables.

**Quantitative PCR**

Ribo-pure yeast kit (AM1926, Life Technologies) was used to extract the total RNA from exponentially growing yeast cells. The cDNA library was prepared using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (4374966, Life Technologies) according to the manufacturer's protocols. Estimation of the mRNA levels was done using the quantitative real time PCR with the predesigned TaqMan gene expression assays on an Applied Biosystems 7900HT Fast Real-Time PCR System.

**Western blotting**

Exponentially growing yeast cells were harvested by centrifugation and lysed by treatment with 1.85 M NaOH, 7.6% β-mercaptoethanol for 10 min on ice followed by protein precipitation with 50% (w/v) TCA for 15 min on ice. Precipitated protein pellets were washed with acetone, air-dried and resolubilised in 10% (w/v) SDS, 1.0 M unbuffered Tris and reduced with an equal volume of 0.2 M DTT, 30% (v/v) glycerol, 0.002% (w/v) bromophenol blue for 30 min at 65 °C. The denatured protein samples were separated by poly-acrylamide gel electrophoresis, transferred onto a nitrocellulose membrane and probed with specific antibodies (mouse-anti-HA antibody (HA.11 Clone 16B12; MMS-101R, Covance), mouse-
anti-Actin antibody (ab8224, Abcam) and goat-anti-mouse IgG, IgA, IgM (H+L) horseradish peroxidase conjugate (A-10668, Invitrogen) using standard procedures.

**Extraction of total RNA and enrichment for bulk tRNA**
For small scale extraction, 25 ml of an exponentially growing yeast culture was harvested by centrifugation and resuspended in 0.5 ml of 150 mM NaCl solution. The cells were lysed by bead beating (G8772, Sigma Aldrich) in 750 µl saturated phenol for 7 min. Subsequently, 75 µl of chloroform were added and the mixture was vortexed for 1 min. After centrifugation the aqueous phase was transferred to a new tube and re-purified with 500 µl phenol and 50 µl chloroform. The tubes with organic phases were re-extracted with 2 ml 150 mM NaCl. The aqueous phases were pooled and loaded into a Nucleobond AX20 anion exchange columns (Macherey-Nagel, 740511). Prior to sample loading, the columns were equilibrated with 3 ml of column buffer (10 mM Tris pH 6.8, 15% (v/v) Ethanol, 200 mM KCl). The bound RNA was washed with 6.0 ml of column buffer. tRNA was eluted with 1.5 ml elution buffer (10 mM Tris pH 6.5, 15% Ethanol, 650 mM KCl) and concentrated by precipitation with 2.5x volumes of Ethanol at −20 °C overnight. The precipitated tRNA was washed extensively in 80% (v/v) ice-cold ethanol and air-dried. The tRNA pellet was resuspended in nuclease free water (Applied Biosystems, AM9930). For large scale applications, 1 litre of yeast culture was grown and the volume of the various reagents used was scaled proportionally. Nucleobond AX500 columns were used for the enrichment of bulk tRNA.

**Purification of specific tRNAs**
3'-biotinylated DNA oligonucleotide probes, specific for the amino-acyl arm of the tRNAs to be purified, were purchased from Invitrogen. Streptavidin-sepharose resin (17-5113-01, GE Healthcare) was used at an approximate ratio of 1 nmole of probe to 10 µl of the resin slurry. Prior to coupling, the streptavidin beads were extensively washed at room temperature; first in coupling buffer (5 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1 M NaCl) for 10 min, then twice in 0.1 M NaOH, 0.05 M NaCl for 2 min each, then once in 0.1 M NaCl solution for 2 min and finally, twice in coupling buffer for 5 min each. Following the washes, the probe was added to the resin in coupling buffer and incubated for 45 min at 37 °C on a thermostirrer. The coupled resin was washed once with coupling buffer and 4 times with tRNA binding buffer (30 mM HEPES-KOH (pH 7.5), 15 mM EDTA (pH 8.0), 1.2 M NaCl). About 400 µg of bulk tRNA dissolved in tRNA binding buffer was added and the mixture was incubated at 65 °C for 15-20 min to partially denature the tRNAs. Then, the mixture was moved to a rotating-wheel for 1 h at room temperature to allow annealing of the tRNAs with immobilised probes. Subsequently, the resin was washed 5 times with tRNA wash buffer (100 mM NaCl, 2.5 mM HEPES-KOH (pH 7.5), 1.25 mM EDTA (pH 8.0)) at 37 °C for 5 min each. Finally, the tRNAs were eluted twice in elution buffer (20 mM NaCl, 0.5 mM HEPES-KOH (pH 7.5), 0.25 mM EDTA (pH 8.0)) at 70 °C for 15 min each. The eluted tRNAs were precipitated overnight at −20 °C with 0.1 volumes of 20% (w/v) NaOAc and 2.5x volumes of ethanol.

The probes used were; 5'-CTCCGATAAGGGAGTTGAGCCCGGTCTC-3' for Sc-tElUUC, 5'-AGGTCTATCCCGGATTCCGAAACCGGGTTGT-3' for Sc-tQlUG and 5'-CCTCACGATGGGGGTTGCAACCCATAATCT-3' for Sc-tRlUCU.

**RNA mass spectrometry analysis**
10 µg of a specific tRNA were digested and dephosphorylated in a 50 µl reaction volume with 2U of Nuclease P1 (N7000-USB, Stratech), 300U of bacterial alkaline phosphatase (18011-015, Life Technologies) in the presence of 0.9 mM ZnSO4 at 37 °C for 90-120 min. 15 µl of 0.5 M NH4HCO3 was added and incubated at 37 °C for 90-120 min. The reaction mixture was acidified using HCOOH, the nucleosides were purified over graphite TopTips (Glygen Corp.) and dried in a vacuum dryer.
For the mass spectrometry analysis, the nucleosides were resuspended in water and separated by liquid chromatography over an Hypercarb graphite column (35005-100065, Thermo Scientific) connected to an UltiMate 3000 uHPLC system (Dionex) running a 1.0 μl/min gradient of buffer B (0.1% (v/v) HCOOH, 90% (v/v) CH₃CN) in buffer A (0.1% (v/v) HCOOH, 2% (v/v) CH₃CN) ranging from 1% to 37% over 45 min. The column-oven temperature was maintained at 65 °C throughout the run. A fused silica emitter (PicoFrit columns, PF360-75-10-N-5, New Objective) was used to spray eluting nucleosides into an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Finnigan) operating at a source voltage of 1.8 kV. MS1 scans were acquired in profile mode at an FT-MS resolution of 100,000. The MS was running in data-independent acquisition mode (DIA) using collision-induced fragmentation (CID) with an isolation width set at 2.0 m/z and normalised collision energy of 35.0. MS/MS spectra were acquired in centroid mode with the IonTrap Normal scan rate. Raw MS files were converted to the mzXML format (3) and analyzed by an in-house developed software to identify and quantify the nucleosides. The identifications were validated by manually comparing the fragmentation spectra with previously reported ones (9, 10).

APM supplemented denaturing PAGE
0.5 to 1.0 μg of bulk tRNA was mixed with an equal volume of loading buffer (AM8547, LifeTechnologies) and electrophoresed through a 10% acrylamide gel containing 7 M urea, 0.5X TBE and 50 μg/ml [(N-acryloylamino)phenyl] mercuric chloride (APM) (synthesised in house according to the procedure described in (11)) at 200 V for 90 min. The gels were pre-run for 15 minutes at 200 V and each well was washed by pipetting electrophoresis buffer multiple times. After electrophoresis, the gels were stained with SYBR Gold (Invitrogen) diluted 1:10,000 in 0.5X TBE for 5 min before imaging.

tRNA northern blot analysis
Electrophoretically separated tRNAs were transferred to a positively charged nylon membrane (Hybond-N+, GE Healthcare) using a semi-dry blotter (Fastblot, biometra) at a constant current of 400 mA for 40 min in 0.5X TBE as the transfer buffer. Transferred tRNAs were cross linked using a UV crosslinker (C-1000 UVP) at an energy setting of 1200 J for 30 s, while the membrane was still moist. Subsequently, the membrane was prehybridised using 5ml of PerfectHyb Plus (Sigma) buffer with 1x ProtectRNA RNAse inhibitor and 0.1 μg/μl ssDNA for 2 h in a 50 ml centrifuge tube. 10 pmol of oligonucleotide probe was labeled with 10U of T4 Polynucleotide Kinase (New England Biolabs) and 5 µl of 3000 Ci/mmol 10 uCi/µl ϒ³²P ATP (PerkinElmer) at 37 °C for 60 min. Labelling was quenched by 1 µl of 0.5 M EDTA at 65 °C for 20 min. The labeled probes were cleaned using the illustra MicroSpin G-25 Columns (GE Healthcare). After pre-hybridisation the labeled probe was added directly to the tubes and incubated overnight at 55 °C in a hybridisation oven. The membrane was washed twice for 10 min each and again twice for 30 min each at 55 °C using 10 ml wash buffer (3x SSC, 25 mM NaH₂PO₄ pH 7.5, 5% SDS, 10x Denhardt’s reagent) pre-warmed to 37 °C. Finally, the membrane was washed for 8 min in high stringency buffer (1x SSC, 10% SDS) pre-warmed to 37 °C and exposed to the X-Ray Film.

The probes used were; 5'-CTCCTCATAGGGGCTCGAACC-3' for Sc-tK₃U₃,U, 5'-AGGTCTTACCCGGAATTGAAACC-3' for Sc-tQ₃U₃,G, 5'-CGCCCAAACAGGGACTTGAACCC-3' for Hs-tK₃U₃,U, 5'-GGTCCACCCAGATTGAACTCGG-3' for Hs-tQ₃U₃,G, 5'-CGACTCTGGTGGATCGAACC-3' for Hs-tR₃U₃,U and 5'-TGCGTGTCGGCAGGAATCGAACC-3' for Hs-tG₃U₃,C.
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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1: Filtering and quality control of the 30 °C vs. 37 °C comparison. The SILAC labelling design for replicate number 2, was switched (denoted by label design -1). The means of log2 of ratios of each peptide from the two designs were compared and the peptides that were not
consistently quantified across the label switch conditions were removed. (A) and (B) represent the distributions of peptide ratios before and after the label switch filter in the form of a scatter plot of binned hexagonal tiles. The tiles colour indicates the number of peptides in each bin. (C) MA plots for the three biological replicates. M: log₂(peptide ratio); A: log₂(light peptide area) + log₂(heavy peptide area) / 2. (D) and (E) Box-and-Whisker plots of the protein ratios from the three biological replicates before and after median normalisation respectively. The box represents the inter quartile range (50% of the data) with the solid-line in the centre of the box representing the median of the distribution of protein ratios. (F) Dynamic range of protein expression after stringent filtering. The histogram illustrates the number of quantified proteins passing the filtering criteria in relation to their copy per cell number (14). The red and blue dashed lines represent the median and mean in the linear scale, respectively.

Supplementary Figure S2: 30 °C vs 37 °C proteomics data quality check and normalisation. (A) Scatter plot of the statistically significant protein ratios in common in the present study and the heat shock response study by Nagaraj et al. (15). (B) Heat-map comparison of statistically significant changes in protein abundances from this study and in mRNA abundances estimated from Gasch et al. (16). Micro-array analyses ct1 29 °C/37 °C along with two repeats of ct2 37 °C vs. 29 °C were treated as replicates and a Bayes moderated t-test was used to determine significant changes. Only significant changes in common are plotted. (C - D) Word clouds showing the most significantly over-represented (p-value < 0.001) GO terms for Biological Processes from (C) up- or (D) down-regulated proteins; analysed by FunSpec (17). GO terms in common with those significantly over-represented in the wild-type vs. urm1Δ analysis (presented in (8)) are shown in red.

Supplementary Figure S3: Manual validation of Urm1p identification and quantification. (A) and (B) are the annotated MS/MS spectra for the heavy and light versions of the only Urm1p peptide identified and quantified in the wt 30 °C/37 °C proteomics analysis. The annotated spectra were obtained from the Lorikeet Spectrum Viewer for Comet (available as part of the TPP). (C) and (D) are the extracted ion chromatograms obtained from XPRESS for the light and heavy Urm1p peptide. The abundance of Urm1p at 30 °C and 37 °C is not significantly different as judged by one-sample t-test (p-value = 0.39).

Supplementary Figure S4: Manual validation of Ncs2p identification and quantification. Shown are the annotated MS/MS spectra, obtained from the Lorikeet Spectrum Viewer for Comet, and the extracted ion chromatograms (XICs), obtained from XPRESS, for the indicated peptides of Ncs2p that were identified and quantified in the WT 30 °C/37 °C proteomics analysis. Refer to Table 2 and Supplementary Table S2 for the mean Ncs2p protein abundance ratio and the associated statistical significance.

Supplementary Figure S5: Manual validation of Ncs6p identification and quantification. Shown are the annotated MS/MS spectra, obtained from the Lorikeet Spectrum Viewer for Comet, and the extracted ion chromatograms (XICs), obtained from XPRESS, for the indicated peptides of Ncs6p that were identified and quantified in the WT 30 °C/37 °C proteomics analysis. Refer to Table 2 and Supplementary Table S2 for the mean Ncs6p protein abundance ratio and the associated statistical significance.

Supplementary Figure S6: tRNA hypotiolation and crosstalk between the URM1 and ELP pathways. (A) WT cells, growing exponentially at 30 °C, were diluted into prewarmed YPD medium and incubated at 37 °C. Samples were taken at the indicated time points, processed to extract bulk tRNA and analysed by [p-(N-acrylamino)-phenyl]mercuric chloride (APM) supplemented denaturing PAGE. ON: over-night. (B) Electropherogram of bulk tRNA isolated from wild-type, urm1Δ or elp1Δ cells and analysed by APM-dPAGE. APM interacts and retards the thiolated tRNAs that appear as a slow moving band at the top of the gel. (C) TEUJC runs as a single band in a PAGE after purification.

Supplementary Figure S7: MS and MS/MS spectra for mcm⁵U and mcm⁵s⁵U. (A) MS spectra of mcm⁵U and mcm⁵s⁵U nucleoside and nucleobase are shown. (B) MS/MS spectra of mcm⁵s⁵U and mcm⁵U nucleobases obtained after CID based fragmentation. Spectra were annotated by matching against the published fragmentation products of the modified uridines (9,10).
Supplementary Figures S8, S9 and S10: Extracted ion chromatograms for mcm$^5$U and mcm$^5$s$^2$U. tRNA tQ$^{UUU}$ (S8), tE$^{UUC}$ (S9) and tR$^{UCU}$ (S10) were purified from wild-type yeast cells grown in either rich medium at 30 °C, or rich medium at 37 °C, or in absence of sulfur amino acids at 30 °C, and were digested and dephosphorylated to nucleosides and analysed by LC-MS-MS/MS. Shown here are the extracted ion chromatograms (XICs) for the m/z values corresponding to protonated nucleoside (MH$^+$) and nucleobase (BH$^+$) of tQ$^{UUU}$, tE$^{UUC}$ or tR$^{UCU}$. The area under the XIC was used to estimate the abundance of the corresponding nucleoside. Note that the overlapping nucleoside-nucleobase elution profiles are generated by post-source fragmentation of the N-glycosidic bond. This overlap is used as a criterion for the identification of a nucleoside in addition to its MS/MS spectra (9).

Supplementary Figure S11: Volcano plots for codon biases. Each volcano plot in the figure shows the abundance ratio and statistical significance of the proteins whose corresponding genes are among the top 2% of yeast genes with the highest frequency of the indicated codon. The dotted red line (y=2) indicates 1% FDR. The black dotted line (x=0) indicates a protein ratio of 1.

Supplementary Figure S12: The tRNA recognising AGA is thiolated in higher eukaryotes. APM-dPAGE and northern blot analyses of the bulk tRNAs isolated from HEK-293 cells.

Supplementary Figure S13: An NCS2 mutant deregulates tRNA thiolation. Electropherogram from APM-dPAGE analysis of the bulk tRNA isolated from wild-type (WT) or ncs2Δ cells transformed with either an empty plasmid (pEV), a plasmid with wild-type NCS2 (pNCS2), or one with ncs2_A212T (pncs2_A212T) and grown under the indicated conditions.

Supplementary Figure S14: Over-expression of tRNAs does not affect their thiolation. Electropherogram from APM-dPAGE analysis of bulk tRNA isolated from wild-type (WT) cells transformed with either a high-copy empty plasmid (pEV) or the same plasmid with tRNA genes for tK$^{UUU}$, tQ$^{UUU}$ and tE$^{UUC}$ (ptKQE) and grown under the indicated conditions.

Supplementary Figure S15: Filtering and quality control of the 30 °C vs 37 °C + ptKQE analysis. SILAC labelling design for replicate number 2, was switched (denoted by label design -1). The means of log$_2$ of ratios of each peptide from the two designs were compared and the peptides that were not consistently quantified across the label switch conditions were removed. (A) and (B) represent the distributions of peptide ratios before and after the label switch filter in the form of a scatter plot of binned hexagonal tiles. The tiles colour indicates the number of peptides in each bin. (C) Peptide level MA plots. M is equal to the log$_2$ of peptide abundance ratios (30 °C/37 °C + ptKQE) and A is equal to 1/2 * log$_2$ of the product of peptide abundances from the two conditions. Systematic inter-sample variances were checked and removed using median normalisation. (D) and (E) Box-and-Whisker plots of the protein ratios from the three biological replicates before and after median normalisation respectively. The box represents the inter quartile range (50% of the data) with the solid-line in the centre of the box representing the median of the distribution of protein ratios.
Supplementary Figure S1

A  Peptide ratios before filtering

B  Peptide ratios after filtering

C  MA plots of peptide ratios

D  Protein ratios before normalization

E  Protein ratios after normalization

F  Distribution of log_{10}(copies per cell)
Supplementary figure S4

**MS/MS and XICs for NCS2 peptide MESLINEK**

sequence identified: R.MESLINEK\textsubscript{136.11}, M
m/z = 486.2515, z = 2
ratio(30 °C/37 °C) = Heavy/Light = 1.57

**MS/MS and XICs for NCS2 peptide NLAQFLINVEETNVKPNCLIAR**

sequence identified: R.NLAQFLINVEETNVK\textsubscript{136.11} PNC\textsubscript{160.03} LIAR\textsubscript{166.11}, K
m/z = 858.7991, z = 3
ratio(30 °C/37 °C) = Heavy/Light = 1.97
Supplementary figure S5

MS/MS and XICs for NCS6 peptide CGYLSSNNICK
sequence identified: R.C₁₆₀.₀₃ GYLSSNIC₁₆₀.₀₃ K₁₃₆.₁₁ A
m/z = 662.2992, z = 2
ratio(30 °C/37 °C) = Heavy/Light = 4.31

MS/MS and XICs for NCS6 peptide VSQLCELCHSR
sequence identified: K.VSQLC₁₆₀.₀₃ ELC₁₆₀.₀₃ HSR₁₆₆.₁₁ K
m/z = 463.5518, z = 3
ratio(30 °C/37 °C) = Heavy/Light = 4.44
Supplementary Figure S6

A

| Temp. (°C) | 30 | 37 | 37 | 37 | 37 | 30 |
|------------|----|----|----|----|----|----|
| Time (h)   | 1.5| 3.0| 4.5| 6.0| ON| ON|

mcm<sup>5</sup>s<sup>2</sup>U

APM + dPAGE

B

WT | um1Δ | elp1Δ

mcm<sup>5</sup>s<sup>2</sup>U | s<sup>2</sup>U

APM + dPAGE

C

IE<sub>loc</sub> | ladder | bulk IFNA

[300] [150] [80] [50]
Supplementary figure S7

A

mcm\textsuperscript{5}s\textsuperscript{2}U: nucleoside
theoretical mass: 333.0751
retention time: 56.95 min

mcm\textsuperscript{5}s\textsuperscript{2}U: nucleobase
theoretical mass: 201.0328
retention time: 56.95 min

mcm\textsuperscript{5}s\textsuperscript{2}U: nucleoside
theoretical mass: 317.0979
retention time: 40.73 min

mcm\textsuperscript{5}s\textsuperscript{2}U: nucleobase
theoretical mass: 185.0557
retention time: 40.8 min

B

\text{m/z}

mcm\textsuperscript{5}s\textsuperscript{2}U

(BH\textsuperscript{+} - CH\textsubscript{3}OH)

168.96930

(BH\textsuperscript{+} - CH\textsubscript{2}OCHO)

141.05627

mcm\textsuperscript{5}U

(BH\textsuperscript{+} - CH\textsubscript{3}OH)

153.03264

(BH\textsuperscript{+} - CH\textsubscript{2}OCHO)

125.04382
Supplementary figure S8

XICs for nucleoside and nucleobases from tQ^{UUG}

30 °C, 37 °C, 30 °C, no S-AA

Intensity (arbitrary units)

Retention time (s)
Supplementary figure S9

XICs for nucleoside and nucleobases from tE^UUC

30 °C

| Retention time (s) | Intensity (arbitrary units) |
|-------------------|-----------------------------|
| 0                 | 1x10^4                      |
| 1                 | 5x10^7                      |
| 2                 | 1x10^10                     |
| 3                 | 2.4x10^20                   |
| 4                 | 8.7x10^20                   |
| 5                 | 5x10^7                      |
| 6                 | 1x10^10                     |

37 °C

| Retention time (s) | Intensity (arbitrary units) |
|-------------------|-----------------------------|
| 0                 | 1x10^4                      |
| 1                 | 5x10^7                      |
| 2                 | 1x10^10                     |
| 3                 | 1.9x10^20                   |
| 4                 | 6.8x10^20                   |
| 5                 | 5x10^7                      |
| 6                 | 1x10^10                     |

30 °C, no S-AA

| Retention time (s) | Intensity (arbitrary units) |
|-------------------|-----------------------------|
| 0                 | 1x10^4                      |
| 1                 | 5x10^7                      |
| 2                 | 1x10^10                     |
| 3                 | 2.1x10^20                   |
| 4                 | 7.4x10^20                   |
| 5                 | 5x10^7                      |
| 6                 | 1x10^10                     |

MH^+ 268.10, BH^+ 136.06, mcm^3s^U MH^+ 333.07, BH^+ 201.03, mcm^5U MH^+ 317.10, BH^+ 185.05
Supplementary figure S10

XICs for nucleoside and nucleobases from tRUUC

Intensity (arbitrary units)

Retention time (s)
Supplementary Figure S12

APM-dPAGE

mcm^5s^2U

APM-dPAGE

NB

α-tK_{UUU}  α-tQ_{UUG}  α-tR_{UCU}  α-tG_{UCC}
### Supplementary Figure S13

|          | ncs2Δ | WT | ncs2Δ |
|----------|-------|----|-------|
| pncs2A212T | +     | +  | +     |
| pNCS2    |       | +  |       |
| pEV      | +     | +  | +     |
| S-AA (1xMet) | +    | +  | +     |
| temp (°C) | 30    | 30 | 30    |
Supplementary figure S14

| Temp. (°C) | 30 | 37 | 30 | 37 |
|-----------|----|----|----|----|
| pEV       | +  | +  | +  | +  |
| ptKQE     | +  | +  | +  | +  |
| mcm^5s^2U |    |    |    |    |
Supplementary Figure S15

**A** Peptide ratios before filtering

**B** Peptide ratios after filtering

**C** MA plots of peptide ratios

**D** Protein ratios before normalization

**E** Protein ratios after normalization