Supplementary material for:

tRNA 3' shortening by LCCR4 as a response to stress in *Trypanosoma brucei*

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Supplementary methods

Deacylation of tRNAs

Chemical deaminoacylation of tRNAs was done by incubating total RNA samples at 37°C for 30min in the presence of 100mM Tris-HCl buffer pH 9. Afterwards tRNAs were EtOH precipitated and resuspended in water.

RACE

3' RACE was done using the TrueSeq small RNA library preparation kit according to the manufacturer's instructions (Illumina). Briefly, 3 μg of deaminoacylated total RNA extracted from cells growing either exponentially or in PBS for 2 h (nutritional stress) was used as input, an adaptor was ligated to the 3' end using the provided T4 RNA ligase and reverse transcribed. Amplification was performed using one of the provided indexed primers and oligonucleotide 27 for $tRNA^{Val}$. The PCR product was run on a gel, extracted, ligated to pGEMT easy vector (Promega) and positive colonies sequenced.

CCA stem loop ligation

Analysis of the integrity of the CCA-tail was done as described in (16). Briefly, total RNA was deacylated by incubation in 100 mM Tris pH 9.5 for 30 min at 37°C and then precipitated. The RNA was resuspended in water and 1-2 μg ligated to a fluorescently labeled oligonucleotide (5'-pCgc act gct txt tgc agt gcg tgg n, where C is RNA and x is T bearing Cy3) by using T4 DNA ligase. After 1 h incubation at 16°C the reaction was separated on an 8% polyacrylamide/urea gel and the fluorescence detected using a G-box system with green LED lightning and the appropriate filter. Finally, the gels were stained with ethidium bromide and imaged under UV light.

CAE tagging and localization

The *T. brucei* CCA-adding enzyme was amplified from genomic DNA using oligos 31 and 59 and cloned in frame in a vector containing the GFP sequence (for C-terminal tagging) and allowing inducible expression. Cells expressing the GFP-tagged CAE were grown exponentially or exposed to nutritional stress. Approximately 10^6 cells were harvested, washed and resuspended in PBS and let adhere to a glass slide for 10 min. After fixation for 10 min in PBS containing 4% paraformaldehyde cells were washed 3x 5 min with PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min.

Cells were washed 3x 5 min with PBS and blocked with PBS containing 2% BSA for 30 min. Anti GFP antibodies (1/100 in PBS/BSA, Roche) were incubated for 45 min followed by 3x 5 min washes with PBS and 45 min incubation with the secondary antibody (1/100 in PBS/BSA, anti-mouse LT680 Licor). After washing (3x 5 min with PBS) samples were air dried and mounted using Vectashield containing DAPI. Images were obtained using a DFC360 FX monochrome camera (Leica Microsystems) and a DMI600B microscope (Leica Microsystems). Image analysis was done using LAS X software (Leica Microsystems) and ImageJ.

Chromatography and analysis of fractions

Cells were grown exponentially and then stressed on PBS for 2h. Cell lysates were prepared as described before (15) using 1 ml of ribosome buffer A (20 mM Tris pH 7.6, 120 mM KCl, 2 mM $MgCl_2$) for 3-4x10^9 cells. Lysates (0.5 ml) were run in a 25 ml Superose6I column using ribosome buffer A at 0.5 ml/min flow. For analysis of the fractions 25 μl of 4 fractions were pooled and *in vitro* tRNA cleavage activity tested as described in Material and Methods. Positive fractions were pooled, concentrated and loaded in a Superdex200 column. Elution was performed using the same buffer and fractions tested for activity as before. A positive fraction and 2 negative fractions (eluting immediately before and after the positive ones) were analyzed by mass spectrometry and deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021533. Proteins exclusively detected in the positive fractions were considered.

SILAC proteomics and immunoprecipitation

To identify proteins interacting with LCCR4 a stable isotope labeling by amino acids in cell culture (SILAC)-based approach was followed (23). Briefly, cells were grown in a defined media containing 5.55 mM glucose as well as arginine and lysine either in their light (unlabeled) or heavy ($^{13}C_6{}^{15}N_4$-l-arginine; $^{13}C_6{}^{15}N_2$-lysine) versions (*Cambridge Isotope Laboratories*). All cultures were grown in the presence of 10–15% (vol/vol) dialyzed FCS (*BioConcept*) for 4 days to ensure complete labeling of proteins. Then cells were stressed for 2h in PBS and samples collected from uninduced cultures and from cultures that had been induced with tetracycline for expression of LCCR4-HA for 4 days. Cell extracts were prepared and LCCR4-HA immunoprecipitated (IP) as described before. The same procedure was applied to cell extracts obtained from uninduced cells. Extra washes with 1M NaCl were included at the end of the IP to decrease non-specific binding (LCCR4 remained active after these washes). After IP proteins were eluted by boiling in 1x Laemmli buffer, the eluates from both purifications were mixed in a 1:1 ratio, separated in a 10% SDS-PAGE and proteins identified by MS. The procedure was done in duplicate.

Blue Native gels

For blue native PAGE analysis cell lysates were prepared as described for affinity purification. Soluble protein corresponding to 10^7 cells was separated on an 8-16% gradient gel as described (23). After electrophoresis the gel was incubated briefly in SDS-PAGE running buffer, proteins blotted onto a PVDF membrane and LCCR4-HA detected by western blot as described in the Materials and Methods

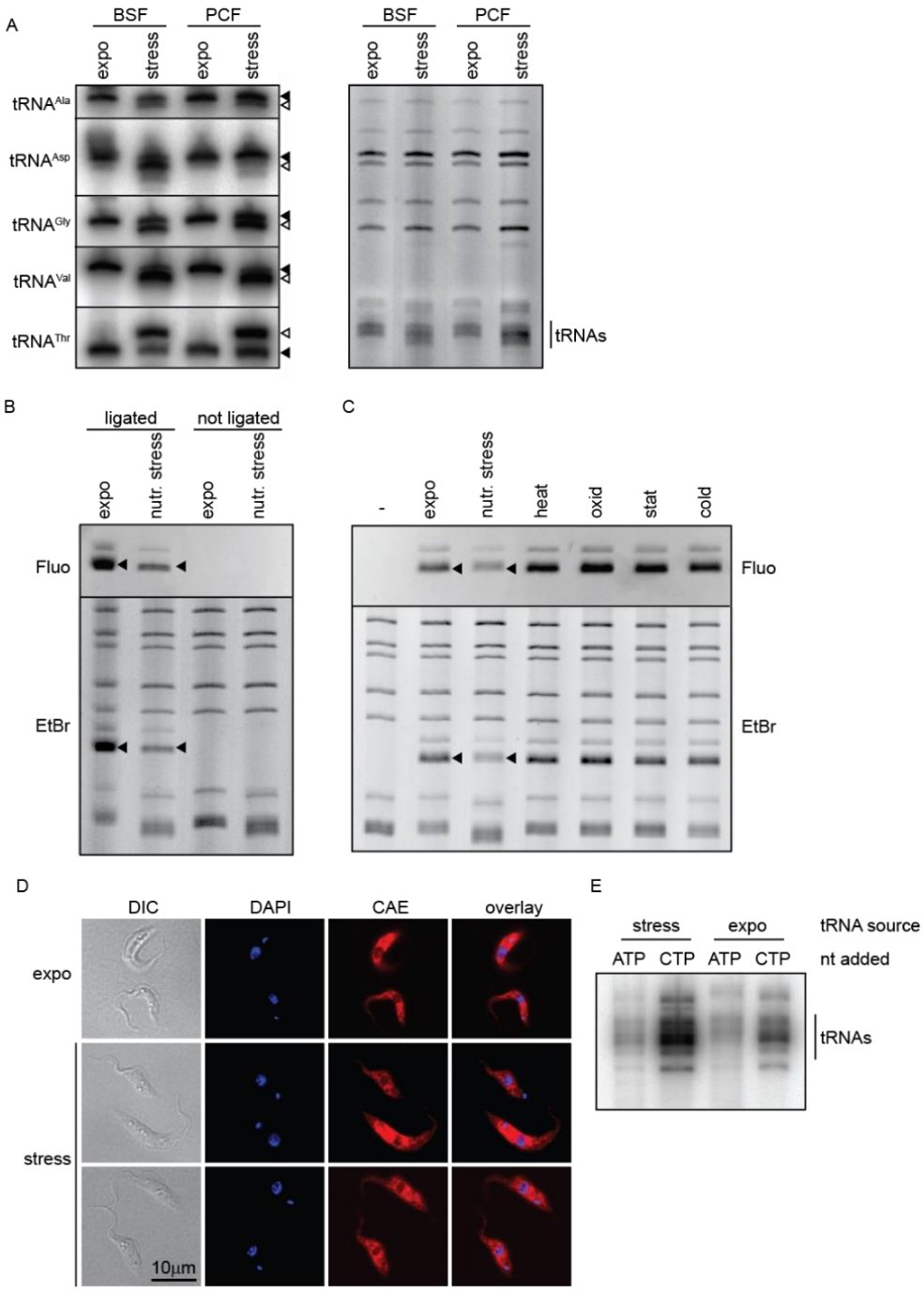
section. When indicated RNase treatment was performed by adding 1 μg of RNase to the sample and incubated for 10min on ice.

Analysis of tRNA shortening in yeast

Wild type *S. cerevisiae* (strain BY4742: MATalpha, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0) was grown in YPD media to OD_{600} 0.8, harvested and RNA extracted using Trizol according to the manufacturer's instructions. Nutritional stress was applied by harvesting wild type cells, washing then with 1 x PBS and incubating then in 1 x PBS for 15 min at 30°C.

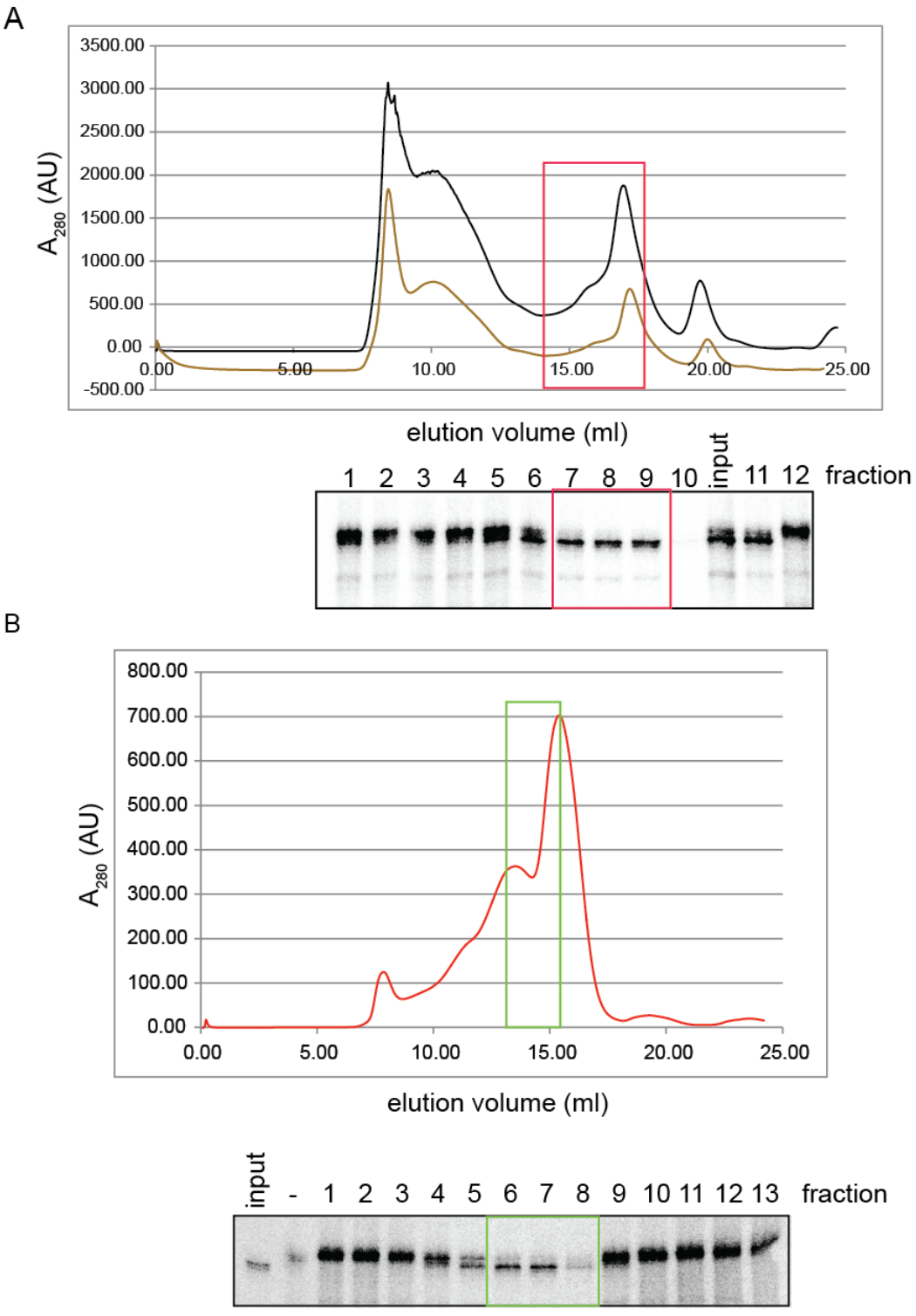
For *S. cerevisiae* Ccr4 overexpression the open reading frame was amplified using oligonucleotides 183 and 184 (oligonucleotide 183 encodes the triple HA-tag) and cloned into the pESC-HIS vector (Agilent Technologies). Growth, selection and induction was performed following the vector instructions. Cells were harvested by centrifugation, washed with water and resuspended in 1 volume of buffer E (20 mM HEPES pH8, 350 mM NaCl, 10% Glycerol, 0.1% Tween 20). Cells were ground using a Spex 6770 freezer/mill. After thawing, cell lysates were diluted 1:1 (vol:vol) with buffer containing 20 mM pH 7.6 HEPES and 2 mM $Mg(AcO)_2$ and cell debris removed by centrifugation at 16000 *xg* for 10 min. For *in vitro* cleavage assays 50 μl of cell lysates were incubated for the indicated time points at 30°C, total RNA was extracted using phenol/chloroform and run in 12% polyacrylamide/urea gels. tRNA shortening was investigated by staining with ethidium bromide and northern blot using oligo Sc $tRNA^{Glu}$ or Sc $tRNA^{Phe}$.

Supplementary Figure S1



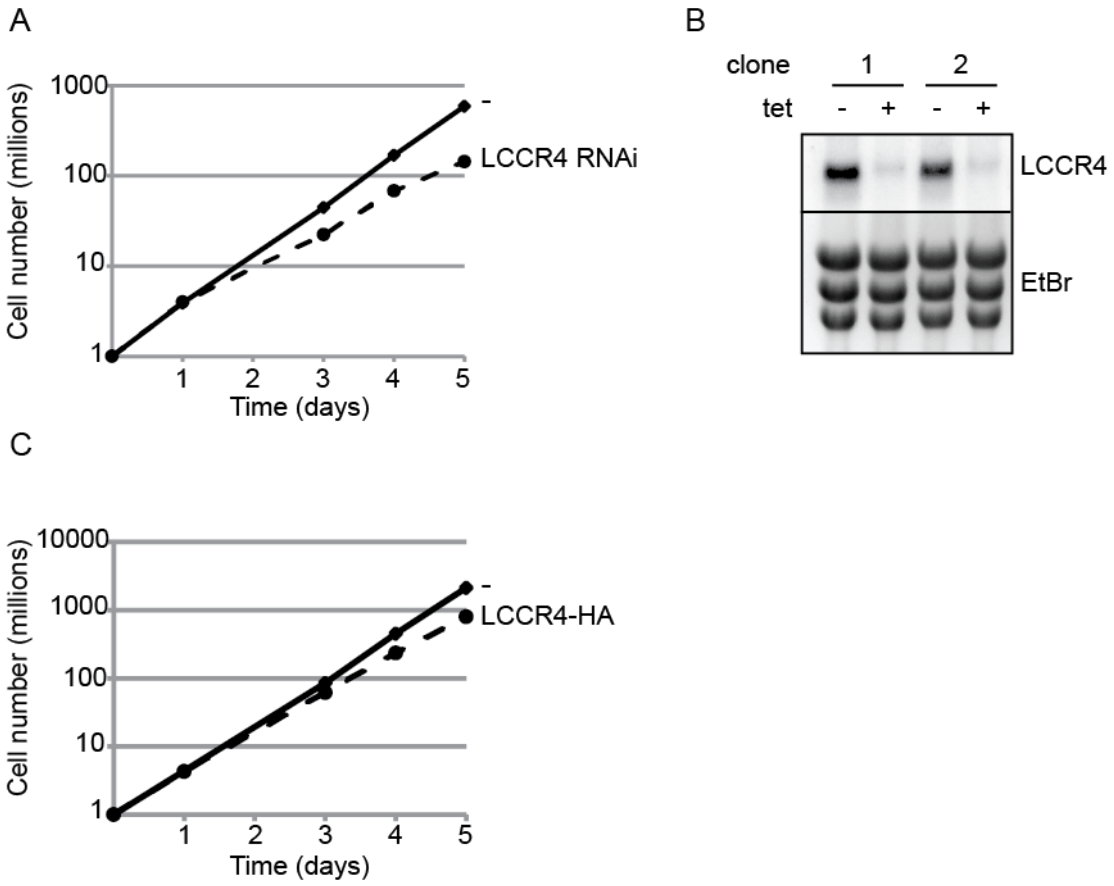
Supplementary Figure S1: Shortening of tRNAs happens in the main life stages of *T. brucei.* (A) Northern blot analyses of tRNAs on total RNA extracted either from bloodstream (BSF) or procyclic (PCF) forms growing exponentially (expo) or subjected to nutritional stress (stress). Full length tRNAs and 3' trimmed tRNAs are indicated with filled and open arrowheads, respectively. The right panel depicts the ethidium bromide stain of a representative gel where the bulk of the cellular tRNAs is indicated. (B) CCA-stem loop ligation using RNA samples extracted from cells growing exponentially (expo) or after nutritional stress (nutr. stress). The upper panel shows the fluorescence while the lower panel corresponds to the ethidium bromide stain of the same gel. Ligated tRNAs are marked with filled arrowheads. (C) As in (B) but using RNA extracted from cells growing exponentially (expo) or after nutritional stress (nutr. stress), heat shock (heat), oxidative stress (oxid), stationary phase (stat) or cold shock (cold). (D) Immunofluorescence analysis of the cellular distribution of the CAE (red) upon stress. Cell nuclei and kinetoplasts are stained with DAPI (blue). (E) The activity of the tagged CCA-adding enzyme was tested on tRNAs extracted from cells exposed to nutritional stress (stress) or growing exponentially (expo) using either γ^{32}P-ATP or γ^{32}P-CTP.

Supplementary Figure S2



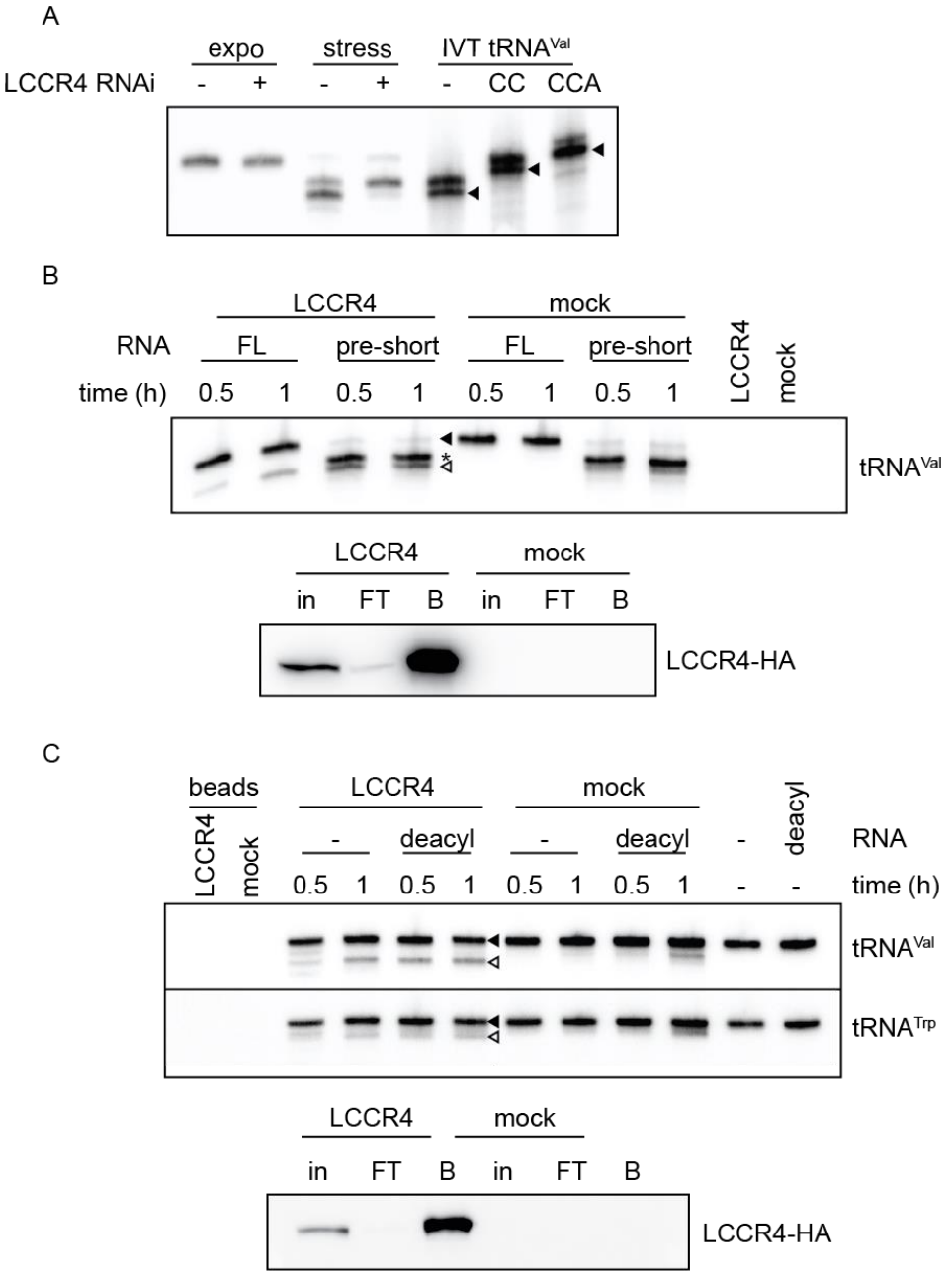
Supplementary Figure S2. Fractionation and *in vitro* tRNA cleavage activity of total cell lysates. Total cell lysates were fractionated in consecutive Superose 6I (A) and Superdex 200 (B) columns and the tRNA cleavage activity was tested *in vitro* on a 5' labeled $tRNA^{Val}$ (lower panels). (A) Elution profiles showing absorbance at 280 nm in arbitrary units (AU) vs. elution volume for two independent runs (black and brown profiles). Fractions showing tRNA cleavage activity are marked within a red frame. (B) Positive fractions from (A) were concentrated, run on a Superdex 200 column and the tRNA trimming activity was tested. Fractions having tRNA cleavage activity are framed in green.

Supplementary Figure S3



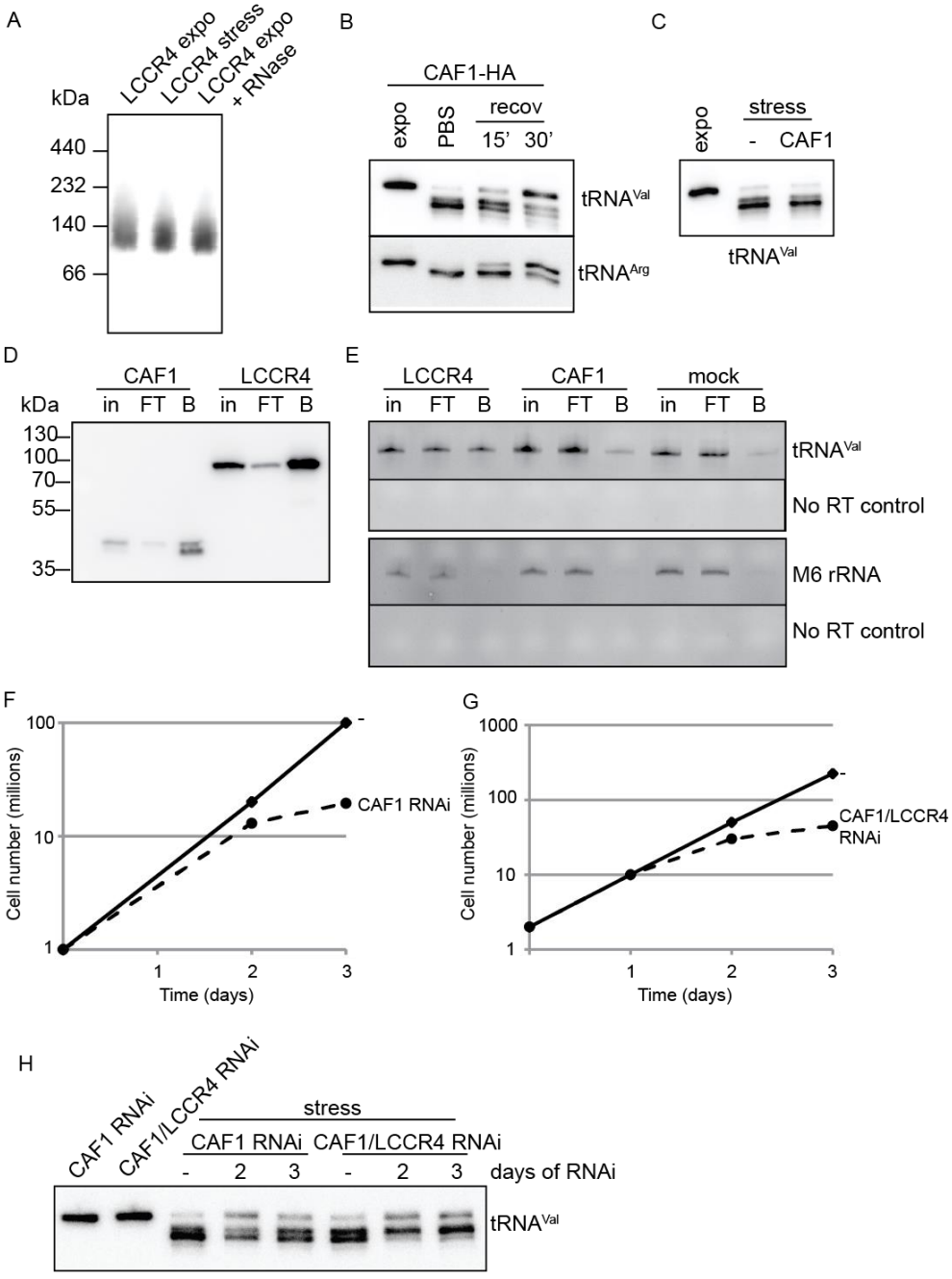
Supplementary Figure S3. (A) Growth curve of cells uninduced (black line) or induced (dashed line) for LCCR4 RNAi. (B) Northern blot showing the depletion of LCCR4 mRNA upon 2 days of RNAi induction. The bottom panel showing the rRNA stained with ethidium bromide serves as loading control. (C) Growth curve of untreated cells (black line) or cells induced for overexpression of LCCR4-HA (dashed line).

Supplementary Figure S4



Supplementary Figure S4. (A) Northern blot analysis comparing the sizes of $tRNA^{Val}$ extracted from uninduced or induced LCCR4 RNAi cells growing exponentially (expo) or under nutritional stress (stress). *In vitro* transcribed $tRNA^{Val}$ without a CCA-tail (-) or with CC or CCA tails serve as size markers. The presence of double bands is probably due to the untemplated addition of nucleotides by the T7 RNA polymerase at the 3' termini and the predicted correct band is marked with an arrowhead. (B) LCCR4-HA was purified from stressed cells and its activity tested using total RNA extracted from cells growing exponentially (FL: full length) or from LCCR4-depleted cells and stressed (pre-short: pre-shortened). $tRNA^{Val}$ was detected by northern blot. The bottom panel shows a western blot analysis of LCCR4-HA in the samples used above. (C) As in (B) but using as substrate total RNA either left untreated (-) or subjected to alkaline deacylation (deacyl). $tRNA^{Val}$ and $tRNA^{Trp}$ were detected by northern blot. The bottom panel shows a western blot analysis of LCCR4-HA in samples used above. In all panels full length tRNAs and 3' trimmed tRNAs are indicated with filled and open arrowheads, respectively, while intermediates of trimming are marked with an asterix. in: input, FT: flow-through; B: bead-bound.

Supplementary Figure S5



Supplementary Figure S5. (A) Total cell lysates of cells expressing LCCR4-HA growing exponentially, under stress and treated or not with RNase A were run on a coomassie blue native gel (8-16%). Proteins were blotted and LCCR4-HA detected by western blot. (B) Total RNA extracted from cells overexpressing CAF1-HA growing exponentially (expo), subjected to nutritional stress (stress), or allowed to recover in normal media for 15 or 30 min after stress was analyzed by northern blot for $tRNA^{Val}$ and $tRNA^{Arg}$. (C) Total RNA extracted from cells overexpressing untagged CAF1 or not growing exponentially (expo) or subjected to nutritional stress (stress), was analyzed by northern blot for $tRNA^{Val}$. (D) CA1-HA was immunoprecipitated and samples obtained from each purification step analyzed by western blot using anti HA antibodies. LCCR4-HA serves as positive control. in: input, FT: flow through, B: beads. (E) CAF1-HA was immunoprecipitated and its interaction with $tRNA^{Val}$ investigated by RT-PCR. Immunoprecipitation of LCCR4-HA serves as positive control for the experiment. RT-PCR of M6 rRNA serves as specificity control. (F-G) Growth curves of cells allowing inducible depletion of CAF1 (F) or CAF1 and LCCR4 simultaneously. (H) RNAi of CAF1 or CAF1 and LCCR4 simultaneously was induced for 2 and 3 days and total RNA was extracted from cells growing exponentially or under nutritional stress (stress). Shortening of $tRNA^{Val}$ was investigated by northern blot.

Supplementary Figure S6

A

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LCCR4  MARHPNTAIVLQPTNSAIITLDLCLQLGGPSAPLWRKSVARDENEAADRAIERLRILIAQSAGDKKSKKT 70
CNOT6  ---------------------------------------------------------------------- 0

LCCR4 SREEILKNCQPIEVLGVSSEGEQILNEENG---LTKLDTSVSNKEFWRNVRQMLIGSTVVPVKYNVPTIT 137
CNOT6 -----------MRLIGMPKEKYDPPDPRRIYTIMSAEEVANGKKSHWAELEISGRVRSLSTSLWSLTHLT  59
                :.::*: .*  :  : ..    ::  :.: .:*..* :: .      ::    :.: :*
LCCR4 AVVPPSALYVGAPAVCAGITALFTTEVDVQYEWCARAAPKSPAGTGASSDATVRVISTKPVFTPTTRELG 207
CNOT6 ALHLNDNYLSRIPPDIAKLHNLVYL------DLSSNKLRSLPAELGNMVSLR-ELLLNNNLLRVLPYELG 122
      *:   .      *   * :  *.        : .:.   . **  *   .   .:: .: ::     **
LCCR4 ESLFLRVLPEPGSDLC-----------------TQVELPQVRAEVPPMDRWTQT---KQSVEAPSFRMV 256
CNOT6 RLFQLQTLGLKGNPLSQDILNLYQDPDGTRKLLNFMLDNLAVHPEQLPPRPWITLKERDQILPSASFTVM 192
      . : *:.*   *. *.                      ::   *: *  *   *      .* : : ** ::
LCCR4 TYNVLHEEFCSTSSAKKTIYPFATDDILSLEYRQSRIVQELLAYNGDIICLQECGKKVYQQFFSRVMTQY 326
CNOT6 CYNVLCDKY-----ATRQLYGYCPSWALNWEYRKKGIMEEIVNCDADIISLQEVETEQYFTLFLPALKER 257
       **** :::     *.: :* :. .  *. ***:. *::*::  :.***.***  .: *  :*  .:.:
LCCR4 GYEGCYTNKNG---------GVREGCACFWRRSRFFLQEKDEFPLNWSTMEKEHPALAAEVTRHPELKEA 387
CNOT6 GYDGFFSPKSRAKIMSEQERKHVDGCAIFFKTEKFTLVQKHTVEFNQVAMANSDGSEAM-------L-NR 319
      **:* :: *.             :*** *:: .:* * :*. . :*  :* :.. : *         * :
LCCR4 LENVTSIGALVL-------------LKDNATNEELVVGNTHLFYHANACHIRLLQVYMLLHKLKSRSD-- 442
CNOT6 VMTKDNIGVAVVLEVHKELFGAGMKPIHAADKQLLIVANAHMHWDPEYSDVKLIQTMMFVSEVKNILEKA 389
          : .  .**. *:           . * :: *:*.*:*:.:. : ..::*:*. *:: ::*.  :
LCCR4 -----------SRRGVVLCGDFNFTHTTGGYKLVTTGRTEASHHSWAKGEVFHWGCDRMLGINAAEGAGG 501
CNOT6 SSRPGSPTADPNSIPLVLCADLNSLPDSGVVEYLSNGGVADNHKDFKELR-------------------- 439
                .   :***.*:*    :*  : ::.* .  .*:.: : .
LCCR4 SAEVETGLDVRRGKGESATCASKNNKNDTNNNNESNGNSVDASTSCAATSADTGVASSVKADYRPPFDVF 571
CNOT6 ----------------YNECL--------------------MNFSCNGKN------GSSEG--------- 458
                       *                       . ** ...      .* :.
LCCR4 RADMEAPMQFTDAYGVTDPEMPWTNYTMTFREVIDYVFFNPDRLLVVQTI-PIPPESELSE-NVALPNRK 639
CNOT6 --RITHGFQL--KSAYENNLMPYTNYTFDFKGVIDYIFYSKTHMNVLGVLGPLDPQWLVENNITGCPHPH 524
        :   :*:    .  :  **:****: *: ****:*:.  :: *: .: *: *:  :.:  .. *: :
LCCR4 YPSDHVALIADLSYK---------------- 654
CNOT6 IPSDHFSLLTQLELHPPLLPLVNGVHLPNRR 555
       ****.:*:::*. :
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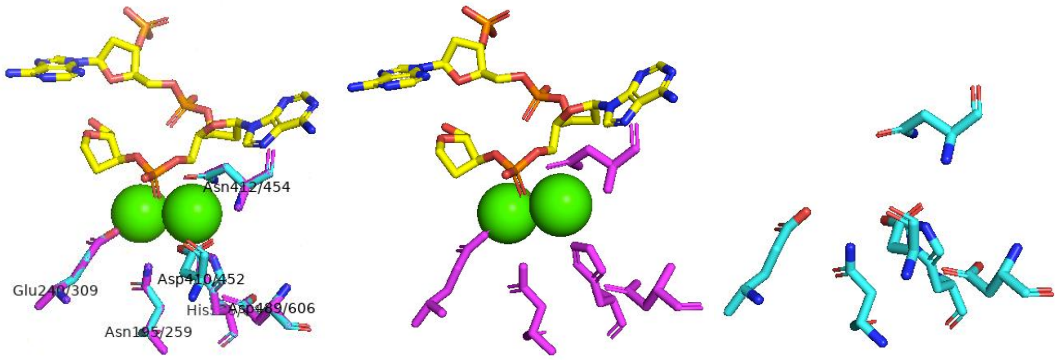
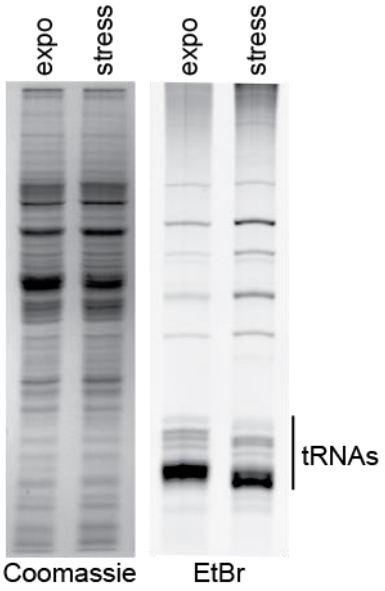
B



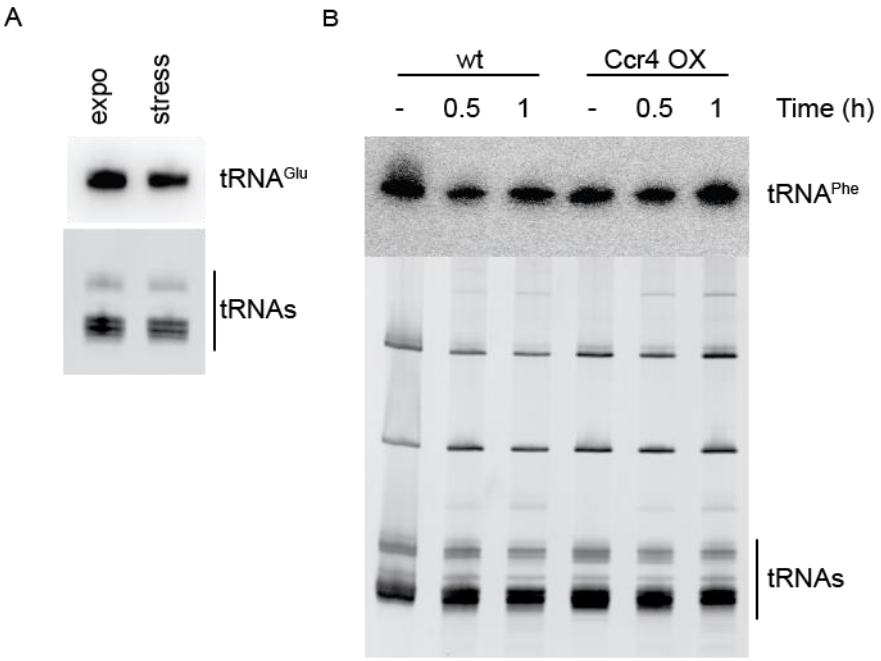
Supplementary Figure S6. (A) Alignment of human CNOT6L and *T. brucei* LCCR4 done with CLUSTAL O (1.2.4) multiple sequence alignment tool. Conserved residues important for function are highlighted in green. (B) The structure of *T. brucei* LCCR4 was predicted using the available structure of CNOTL6 bound to Mg^{2+} and DNA poly(A) as template (pdb 3ngo). The residues present in the active site of CNOTL6 (magenta) and LCCR4 (light blue) are shown, with the residues number corresponding to each protein separated by a bar. Green spheres: Mg^{2+} atoms. poly(A) is colored by atom (yellow: C, red: O, blue: N). Structure overlay was done using pymol.

Supplementary Figure S7



Supplementary Figure S7. Comparison of S100 fractions used for *in vitro* aminoacylation analysis. Equal volumes of S100 fractions prepared from cells growing exponentially (expo) or under nutritional stress (stress) were run on SDS-PAGE gels and the amount of proteins assessed by Coomassie staining (left panel). Total RNA was prepared from equal amounts of S100 fractions, run in polyacrylamide/urea gels and the amount and integrity of the RNAs assessed by ethidium bromide staining (right panel). The region of the tRNAs is marked on the right.

Supplementary Figure S8



Supplementary Figure S8. No tRNA trimming is detected in yeast. (A) *S. cerevisiae* were stressed by incubation in PBS for 15 min. tRNA shortening was assessed by running total RNA in 12% polyacrylamide/urea gels, stained with ethidium bromide followed by northern blot against $tRNA^{Glu}$. (B) tRNA shortening in yeast lysates prepared from wt cells or cells overexpressing Ccr4 was assessed by incubating total cell lysates at 30°C for the indicated times. Total RNA was extracted, run in 12% polyacrylamide/urea gels and stained with ethidium bromide followed by northern blot against $tRNA^{Phe}$. Upper panels: northern blot; bottom panels: ethidium bromide staining.

Supplementary Table S1: Sequences of oligonucleotides used in this study.

Oligo name	**Sequence (5'-to-3')**
Ala (AGC)	GGCGCTCTACCATCTGAGCTACA
Arg (ACG)	GATGCTCTTCCATTGAGCCAC
Asn (GUU)	GAATTGAACCCGGGTCACCCGCG
Asp (GUC)	CGCTGCCGACTGCGCCAGGGA
Cys (GCA)	CACCCGGGTTTGAACCGGGGAC
Gln (CUG)	GGACTCGAACCAGGGTTATCGGAT
Glu (UUC)	AGCCAGATGTTCTAACCGTT
Gly (TCC)	CTTACCGTTGGACCACGATTGC
His (GUG)	AACGTGAGGGAAGACCGGGAATCGA
Ile (AAU)	TGCTCCCAACAGGGGTCGAACCTGTGA
Leu (CAA/UAG/CAG/AAG)	GTGGGGTTTGAACCCACGCC
Lys (CUU/UUU)	GGGATCGAACCCACGACCACACG
Met (CAU)	GCGCTGCCGACTGCGCCACGCTCGC
Met in (CAU)	GCGCTTCCCCTGCGCCACGGTGC
Phe (GAA)	CTCTCCCAACTGAGCTATCGCGGC
Pro (CGG/AGG)	GAATCATGCCACTAGACCA
Sel (UCA)	ACCCAGCACCAGCTGAGCTCATCG
Ser (AGA/CGA/UGA/GCU)	TTCGAACCTGCGCGCGAGATC
Thr (AGU)	ATCGAACCCCCGACCTCCGTCTT
Trp (CCA)	GGGATTGAACCTGCGACCCCTGG
Tyr (GUA)	CCAGCGACCCTGTGATACCCGC
Val (CAC)	CCTAACCACTAGACGACCATCGC
39	CAGAGCCCACCAGATAAGAGAAGT
40	AATGCTCGAGATGTTGCTACCAAAGAAGG
41	ATGGTACCCAGAACTCTATTGGGGTATC
43	GGATCCTAATACGACTCACTATAGCGATGGTCGTCTAGTGG
51	TACGACGGGCGGGGATCG
99	ACGAAGCTTGGATCCCAAAGCAATCTGTGGAAGC
100	CTTCTAGACTCGAGCCAACCACCAACTCTTCG
133	GCTCTAGAATGGCACGGCACCCCAAC
139	GCCTCGAGTTTGTACGACAAATCGGCAATC
143	GCTCTAGAATGATGCAGTATGGTGGC
144	GCCTCGAGGCTATGACCCTTTACCGC
151	ACGAAGCTTGGATCCTCTGCCTCACAGAGGATG
152	CTTCTAGACTCGAGGAGGAATCTTCACAGAGCC
153	TTATACCTCCGTCTCTTCC
165	GTCTTATGTGGGGACTTCGCCTTTACCCACACAACGGG
166	CCCGTTGTGTGGGTAAAGGCGAAGTCCCCACATAAGAC
Sc $tRNA^{Phe}$	GATCTTCAGTCTGGCG
Sc $tRNA^{Glu}$	CTCCACGGTGAAAGC
CCA stem loop ligation	pCGCACTGCTTXTTGCAGTGCGTGGN

Supplementary Table S2: Proteins found exclusively in the active fraction that are either predicted to have an RNA-related activity or contain a domain associated with nucleic acid metabolism (ProteomeXchange Consortium identifier PXD021533)

Gene ID	Description	Tested
Tb927.10.1630	atp-binding cassette sub-family e member 1	-
Tb927.10.6800	developmentally regulated GTP-binding protein, putative	-
Tb927.11.7890	zinc finger CCCH domain containing protein 44	-
Tb927.7.970	NMD3 family, putative	-
Tb927.10.6680	member of the NOL1/NOP2/sun family of proteins	-
Tb927.6.1900	essential nuclear protein 1, putative	-
Tb927.11.11770	Eukaryotic translation initiation factor 4E-3	-
Tb927.9.9020	ribosome-interacting GTPase 2, putative	-
Tb927.4.2430	Endonuclease/Exonuclease/phosphatase family, putative (LCCR4)	+
Tb927.9.8780	tRNA nucleotidyltransferase, putative	+
Tb927.9.10570	predicted zinc finger protein	-
Tb927.10.810	pentatricopeptide repeat domain containing protein, putative	-
Tb927.11.360	ATP-binding protein, putative	-
Tb927.6.3250	C2H2 type zinc-finger (2 copies), putative	-
Tb927.1.1470	conserved protein, unknown function	-
Tb927.8.5720	Met-10+ like-protein, putative	-
Tb927.11.4860	Ring finger domain containing protein, putative	-
Tb927.11.10750	pre-mRNA-splicing factor CWC22, putative	-
Tb927.8.1500	hypothetical protein, conserved	-
Tb927.11.14580	Bifunctional Monophosphate RNA Kinase/RNA guanylyltransferase	-
Tb927.11.2370	mRNA export factor MEX67	+
Tb927.3.5320	UBX domain-containing protein	-
Tb927.3.3230	NOL1/NOP2/sun family, putative	-
Tb927.9.9220	RNase P: PRORP1	-
Tb927.3.1100	exonuclease, putative	+
Tb927.11.8290	DIS3-like exonuclease, putative	+
Tb927.8.1410	pre-rRNA-processing protein tsr1 homolog	-
Tb927.6.3250	C2H2 type zinc-finger (2 copies), putative	-
Tb927.11.10860	Nin one binding (NOB1) Zn-ribbon like, putative	-

Supplementary Table S3: LCCR4-HA interacting partners (ProteomeXchange Consortium identifier PXD021540)

Gene ID	Putative function
Tb927.11.13070	O-phosphoseryl-tRNA(Sec) selenium transferase, putative (SepSecS)
Tb927.2.2520	Mitochondrial outer membrane protein porin 2/ VDAC2
Tb927.4.840	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase, putative
Tb927.6.1870	Eukaryotic translation initiation factor 4E-4