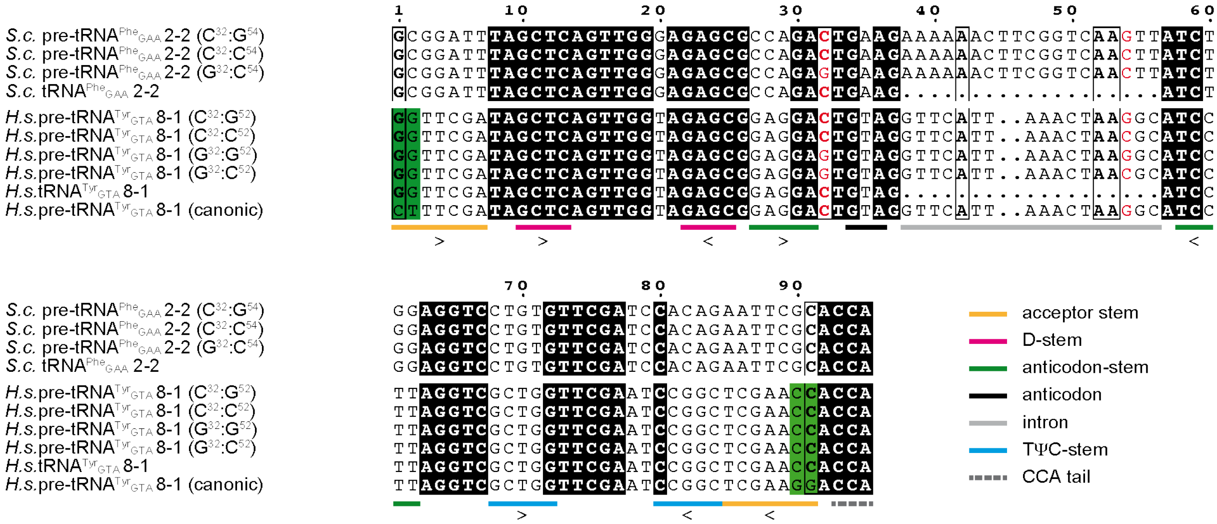
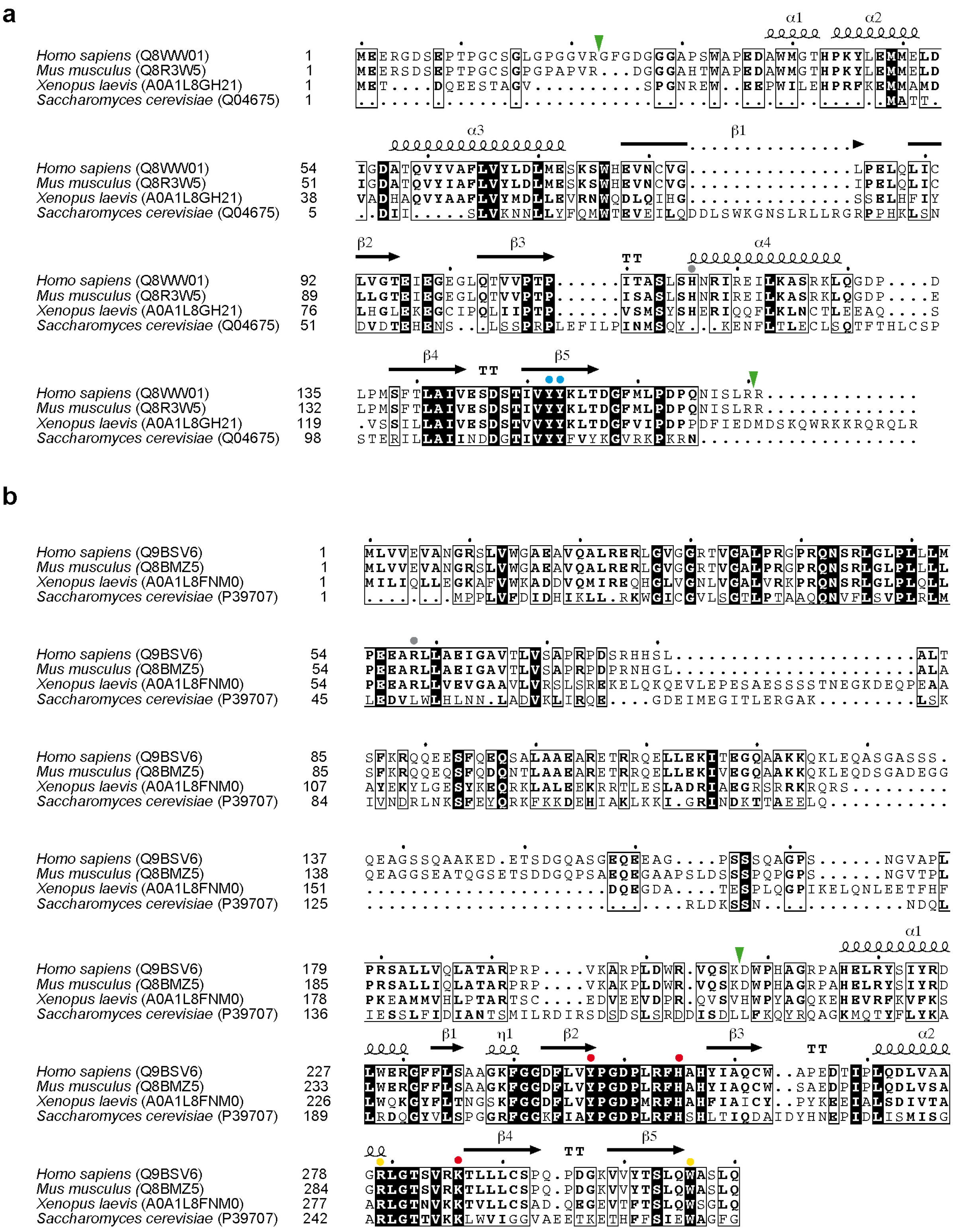
Supplementary Figures, Tables, and Source Data Files

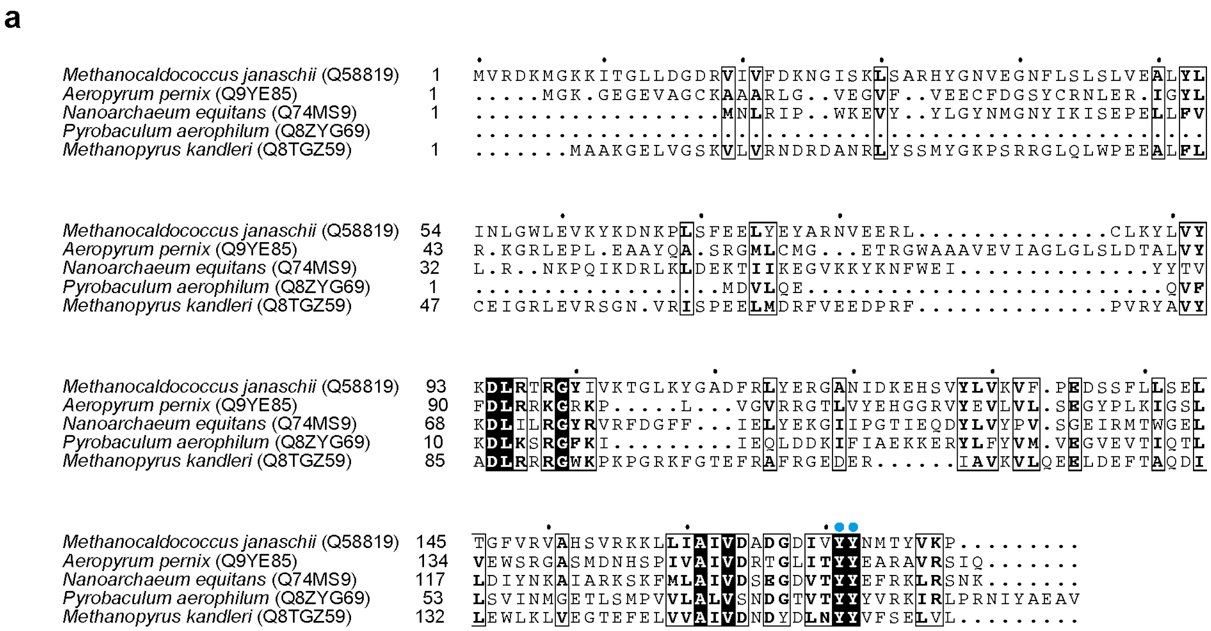


Supplementary Fig. 1 | Sequence comparison of pre-tRNA and tRNA molecules. Sequence alignments were performed using Clustal Omega, edited in Jalview and colored by conservation using ESPript 3.0. A–I base pair residues are colored in red. Predicted stem structures, anticodon, intron and CCA tail are indicated by colored bars. Ribonucleotides modified for efficient *in vitro* transcription are boxed in green and compared to the canonical sequence.



Supplementary Fig. 2 | Sequence conservation of TSEN15 and TSEN34. Sequence alignments were performed using Clustal Omega and colored by conservation using ESPript 3.0. **a**, The TSEN15 sequence alignment includes orthologues from *Homo sapiens* (UniProtKB Q8WW01), *Mus musculus* (UniProtKB Q8R3W5), *Xenopus laevis* (UniProtKB A0A1L8GH21), and *Saccharomyces cerevisiae* (UniProtKB Q04675). **b**, The TSEN34 sequence alignment includes orthologues from *Homo sapiens*

(UniProtKB Q9BSV6), *Mus musculus* (UniProtKB Q8BMZ5), *Xenopus laevis* (UniProtKB A0A1L8FNM0), and *Saccharomyces cerevisiae* (UniProtKB P39707). Tryptic sites identified from limited proteolysis experiments are shown by green arrow heads. The YY-motif is indicated by blue circles, residues of the catalytic triad are highlighted by red circles, and residues possibly involved in the cation-π-interaction are shown as yellow circles. Residues mutated in PCH (TSEN15$^{\mathrm{H116Y}}$, TSEN34$^{\mathrm{R58W}}$) are indicated by grey circles. Helices and strands are numbered sequentially according to the TSEN15–34 X-ray crystal structure and are indicated above the alignments. TT – β-turn.



Supplementary Fig. 3 | Sequence conservation of Archaeal α_4 and $(\alpha\beta)_2$ endonucleases highlighting the YY-motif. Sequence alignments were performed using Clustal Omega and colored by conservation using ESPript 3.0. The sequence alignment includes orthologues from *Methanocaldococcus jannaschii* (UniProtKB Q58819), *Aeropyrum pernix* (UniProtKB Q9YE85), *Nanoarchaeum equitans* (UniProtKB Q74MS9), *Pyrobaculum aerophilum* (UniProtKB Q8ZYG6), and *Methanopyrus kandleri* (UniProtKB Q8TGZ5). The YY-motif is indicated by blue dots.

Supplementary Tables

Supplementary Table 1 | Masses of protein subunits and complexes observed in native MS spectra. The experimentally determined and theoretically calculated masses as well as the mass differences are given. A larger mass difference (*) originates from incomplete desolvation and can be in part attributed to the high phosphorylation state of the TSEN54 subunit (Extended Data Fig. 1c).

Composition	**Experimental mass** (Da)	**Theoretical mass** (Da)	**∆ mass** (Da)
TSEN			
TSEN2–15–34–54	165573 ±130	164416	1157 (*)
unassigned	104865 ±48		
HSP70	71461 ±6	71432	29
TSEN15–34	52389 ±15	52350	39
TSEN15	18693 ±4	18698	-5
TSEN/CLP1			
unassigned	466075 ±127		
unassigned	417988 ±36		
TSEN2-15-34-54-2xCLP1	261096 ±182	259822	1274 (*)
TSEN2-15-34-54-1xCLP1	212967 ±98	212119	848 (*)
unassigned	123948 ±28		
unassigned	104818 ±6		
HSP70	71521 ±3	71432	89
TSEN15–34	52412 ±7	52350	62
CLP1	47776 ±5	47703	73
TSEN15	18704 ±0	18698	6

Subunits	**Uniprot KB**	**Theoretical mass** (Da)
TSEN15	Q8WW01	18698
TSEN34	Q9BSV6	33652
TSEN2	Q8NCE0	53247
TSEN54	Q7Z6J9	58819
CLP1	Q92989	47703
HSP70	Q9U639	71432

Supplementary Table 2 | Protein identification by LC-MS/MS. The protein masses, the number of identified peptide sequences, the number of observed spectra, and the sequence coverage are given for TSEN subunits and CLP1 of purified TSEN, TSEN/CLP1 and proteolyzed TSEN15–34 complexes.

			TSEN			**TSEN/CLP1**		
Protein	**UniProtKB**	**Mass** (Da)	**Peptide sequences** (#)	**Spectra** (#)	**Sequence coverage** (%)	**Peptide sequences** (#)	**Spectra** (#)	**Sequence coverage** (%)
TSEN15	Q8WW01	18629	9	140	47.4	11	180	73
TSEN34	Q9BSV6	33631	53	907	100.0	58	1583	100
TSEN2	Q8NCE0	53213	99	1228	98.5	86	1946	99
TSEN54	Q7Z6J9	58783	67	771	84.8	68	1132	95
CLP1	Q92989	47615				75	1314	100

			Proteolyzed TSEN15–34		
Protein	**UniProtKB**	**Mass** (Da)	**Peptide sequences** (#)	**Spectra** (#)	**Sequence coverage** (%)
TSEN15	Q8WW01	18629	9	92	87
TSEN34	Q9BSV6	33631	14	275	34

Supplementary Table 3 | Masses of proteolytic fragments of TSEN 15 andTSEN34 obtained from denaturing. The experimentally determined and theoretically calculated masses as well as the mass difference are given.

Protein fragment	**Experimental mass** (Da)	**Theoretical mass** (Da)	**Δ mass** (Da)
TSEN15 (residues 23 to 170)	16313.9±1.0	16314.7	-0.8
TSEN15 (residues 23 to 171)	16469.8±0.9	16470.9	-1.1
TSEN34 (residues 208 to 310)	11614.8±0.7	11615.2	-0.4

Supplementary Table 4 | Identification of proteolytic fragments of TSEN15 and TSEN34 by LC-MS/MS. The position in the protein sequence, the sequence of observed tryptic peptides and the number of spectra for each peptide are given. The preceding and following amino acids are given for each peptide sequence.

TSEN15

Residues	Peptide sequence	# spectra
23-45	R.GFGDGGGAPSWAPEDAWMGTHPK.Y	22
46-74	K.YLEMMELDIGDATQVYVAFLVYLDLMESK.S + Oxidation (M)	4
75-118	K.SWHEVNCVGLPELQLICLVGTEIEGEGLQTVVPTPITASLSHNR.I	6
119-127	R.IREILKASR.K	7
121-127	R.EILKASR.K	7
128-154	R.KLQGDPDLPMSFTLAIVESDSTIVYYK.L	13
155-170	K.LTDGFMLPDPQNISLR.R	17
155-171	K.LTDGFMLPDPQNISLRR.-	16

TSEN34

Residues	Peptide sequence	# spectra
204-220	R.VQSKDWPHAGRPAHELR.Y	1
208-220	K.DWPHAGRPAHELR.Y	37
221-230	R.YSIYRDLWER.G	61
231-253	R.GFFLSAAGKFGGDFLVYPGDPLR.F	1
240-253	K.FGGDFLVYPGDPLR.F	6
254-279	R.FHAHYIAQCWAPEDTIPLQDLVAAGR.L	7
280-286	R.LGTSVRK.T	4
280-298	R.LGTSVRKTLLLCSPQPDGK.V	8
286-298	R.KTLLLCSPQPDGK.V	75
286-310	R.KTLLLCSPQPDGKVVYTSLQWASLQ.-	1
287-298	K.TLLLCSPQPDGK.V	68
287-310	K.TLLLCSPQPDGKVVYTSLQWASLQ.-	3
299-310	K.VVYTSLQWASLQ.-	3

Supplementary Table 5 | X-ray data collection, refinement, and validation statistics. The structure of TSEN15–34 was determined from one protein crystal. Values in parentheses are given for highest-resolution shell.

	TSEN15–34
Data collection	
Space group	P 1 21 1
Cell dimensions	
a, b, c (Å)	34.85, 69.28, 94.79
α, β, γ (°)	90, 98.31, 90
Resolution (Å)	28.3 - 2.1(2.175 - 2.1)
R_{merge}	0.07428 (0.8863)
$I / \sigma I$	13.37 (1.44)
Completeness (%)	0.99 (0.99)
Redundancy	5.9 (5.9)
Refinement	
Resolution (Å)	28.3 - 2.1
No. reflections	25898 (2576)
R_{work} / R_{free}	19.18 (30.49) / 25.28 (36.27)
No. atoms	3636
Protein	3516
Ligand/ion	12
Water	120
B-factor (average, Å^2)	60.17
Protein	60.10
Ligand/ion	78.56
Water	60.32
R.m.s. deviations	
Bond lengths (Å)	0.009
Bond angles (°)	0.99
Validation	
Ramachandran plot	
Favored (%)	97
Allowed (%)	2.5
Outliers (%)	0.2
Rotamer outliers (%)	1
Clash score	9.26

Supplementary Table 6 | DSF data analyzed by ProteoPlex. T_d – denaturing temperature.

TSEN complex	**T_d - Boltzman (°C)**	**T_d - ProteoPlex (°C)**	**R^2 (fit to data)**	**R^2 (fit to 2-state unfolding)**
TSEN (wt)	51.0	52.1	0.99973	0.99899
TSEN (T2^{Y309C})	44.8	46.3	0.99979	0.99955
TSEN (T34^{R58W})	44.1	46.5	0.99955	0.99902
TSEN (T54^{S93P})	46.5	48.7	0.99935	0.99818
TSEN (T54^{A307S})	49.6	50.7	0.99978	0.99951

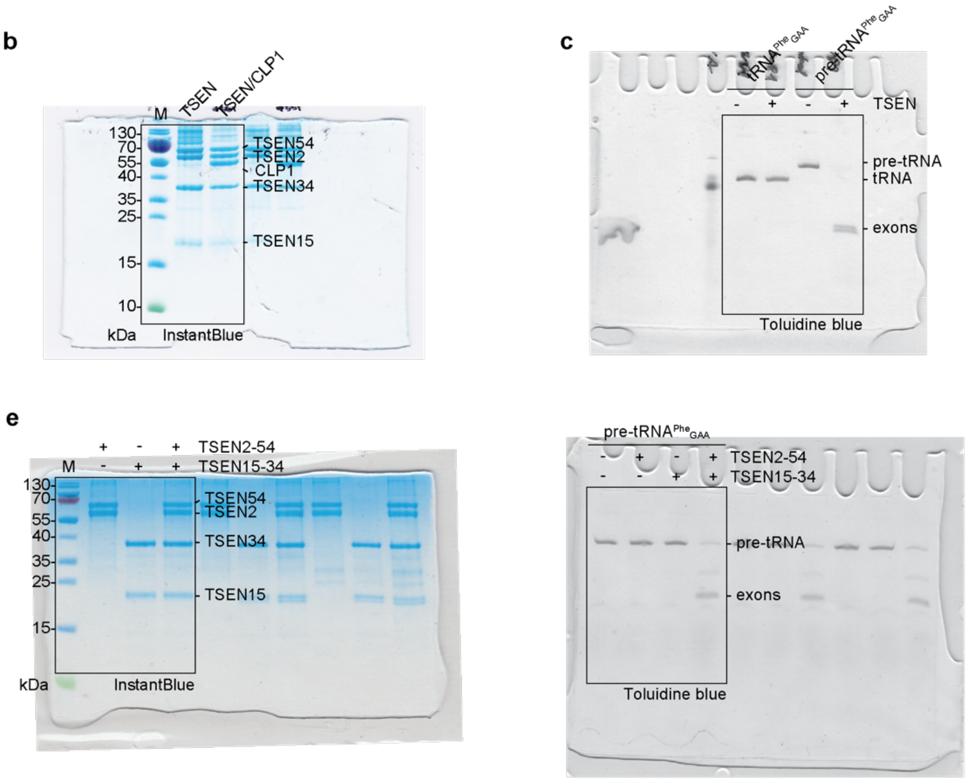
Supplementary Table 7 | List of patient-derived primary fibroblast cells used in this study.

Cell line	Mutations	Description	Zygosity
Ba1	*TSEN54 c.919G / 919G*	control	homozygous
Ba2	*TSEN54 c.919G / 919G*	control	homozygous
Ba3	*TSEN54 c.919G / 919G*	control	homozygous
Ba5	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba8	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba9	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba10	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba12	*TSEN54 c.919G / 919G>T*	parent of Ba19	heterozygous
Ba13	*TSEN54 c.919G / 919G*	control	homozygous
Ba14	*TSEN54 c.919G / 919G*	control	homozygous
Ba15	*TSEN54 c.919G / 919G*	control	homozygous
Ba17	*TSEN54 c.919G / 919G>T*	parent of Ba19	heterozygous
Ba18	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba19	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba20	*TSEN54 c.919G>T / 923delC* p.(Pro318Gln fsX23)	PCH4 patient	compound heterozygous
Ba245	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba1230	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
Ba1613	*TSEN54 c.919G / 919G>T*	parent of Ba1597	heterozygous
Ba1614	*TSEN54 c.919G / 919G>T*	parent of Ba1597	heterozygous
Ba1597	*TSEN54 c.919G>T / 919G>T*	PCH2 patient	homozygous
T1 (BAB3846)	*CLP1 c.419G / 419G>A*	parent of BAB3402	heterozygous
T3 (BAB3402)	*CLP1 c.419G>A / 419G>A*	patient	homozygous

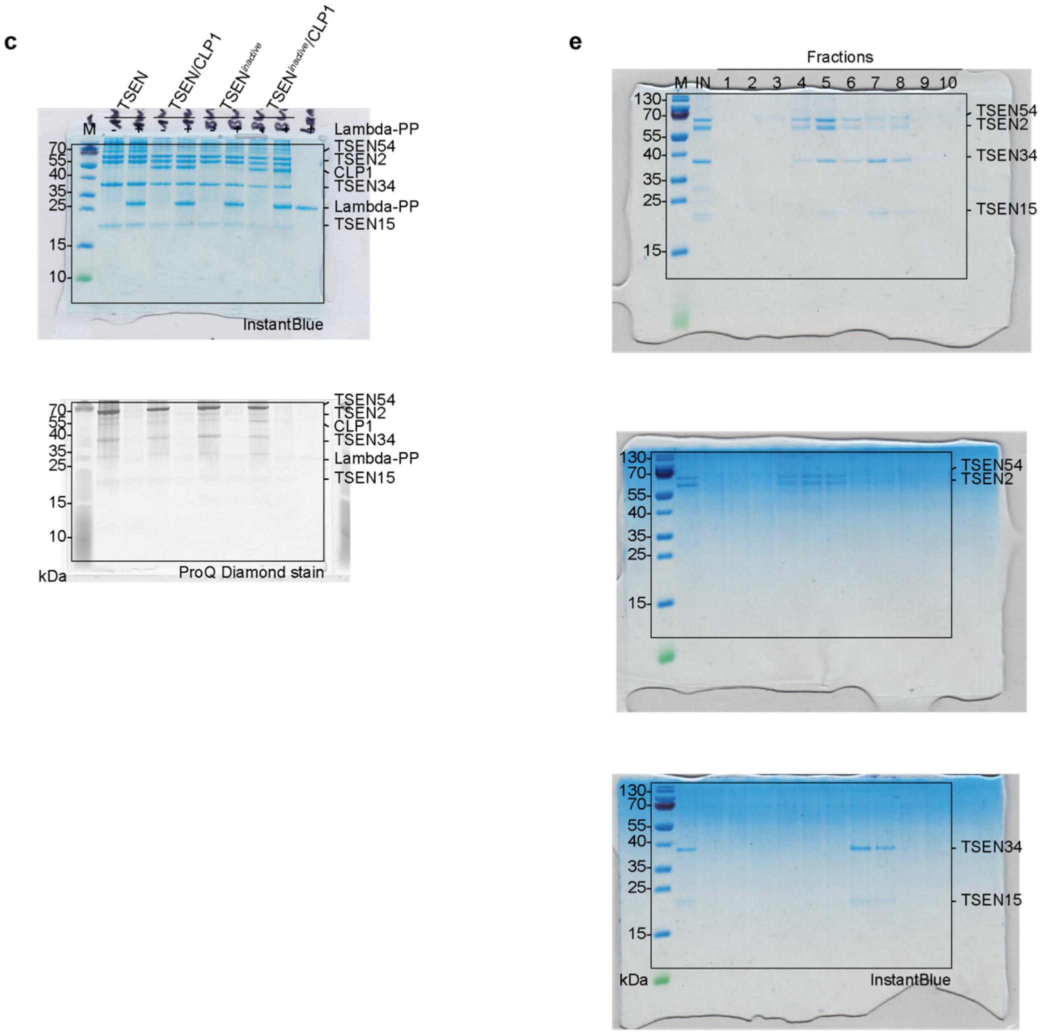
Supplementary Table 8. Hydro-tRNAseq data (separate file).

Source Data

Source Data 1 | Uncropped images as shown in Fig. 1.



Source Data 2 | Uncropped images as shown in Extended Data Fig. 1.



Source Data 3 | Uncropped images as shown in Fig. 2.

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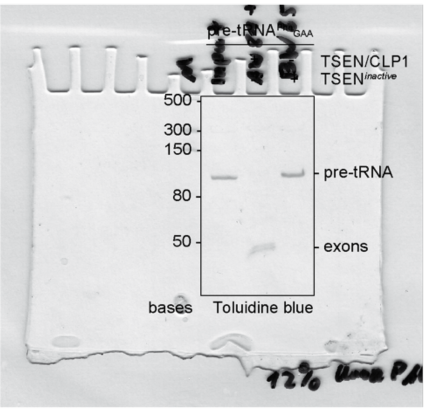

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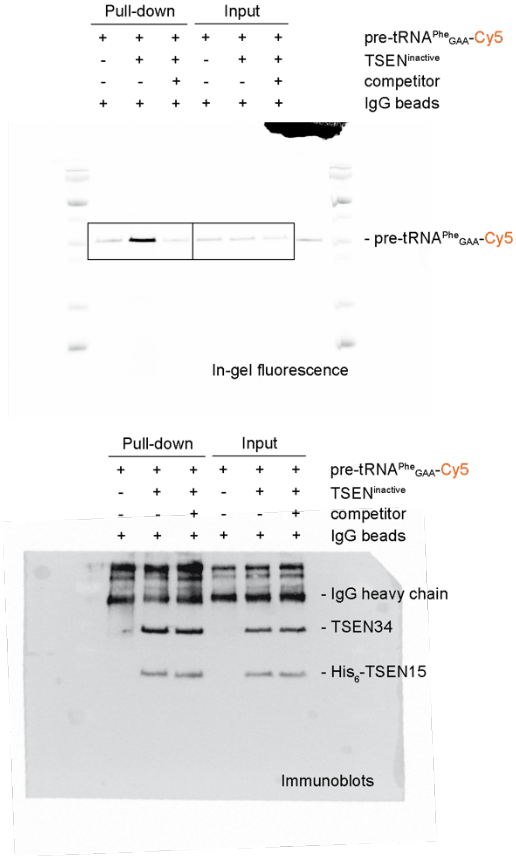

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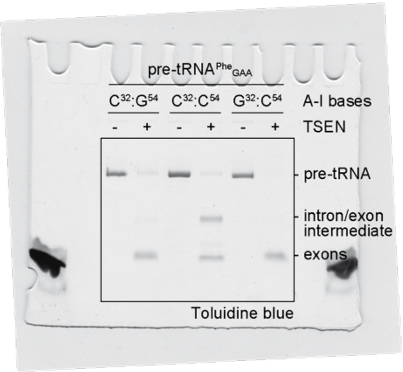

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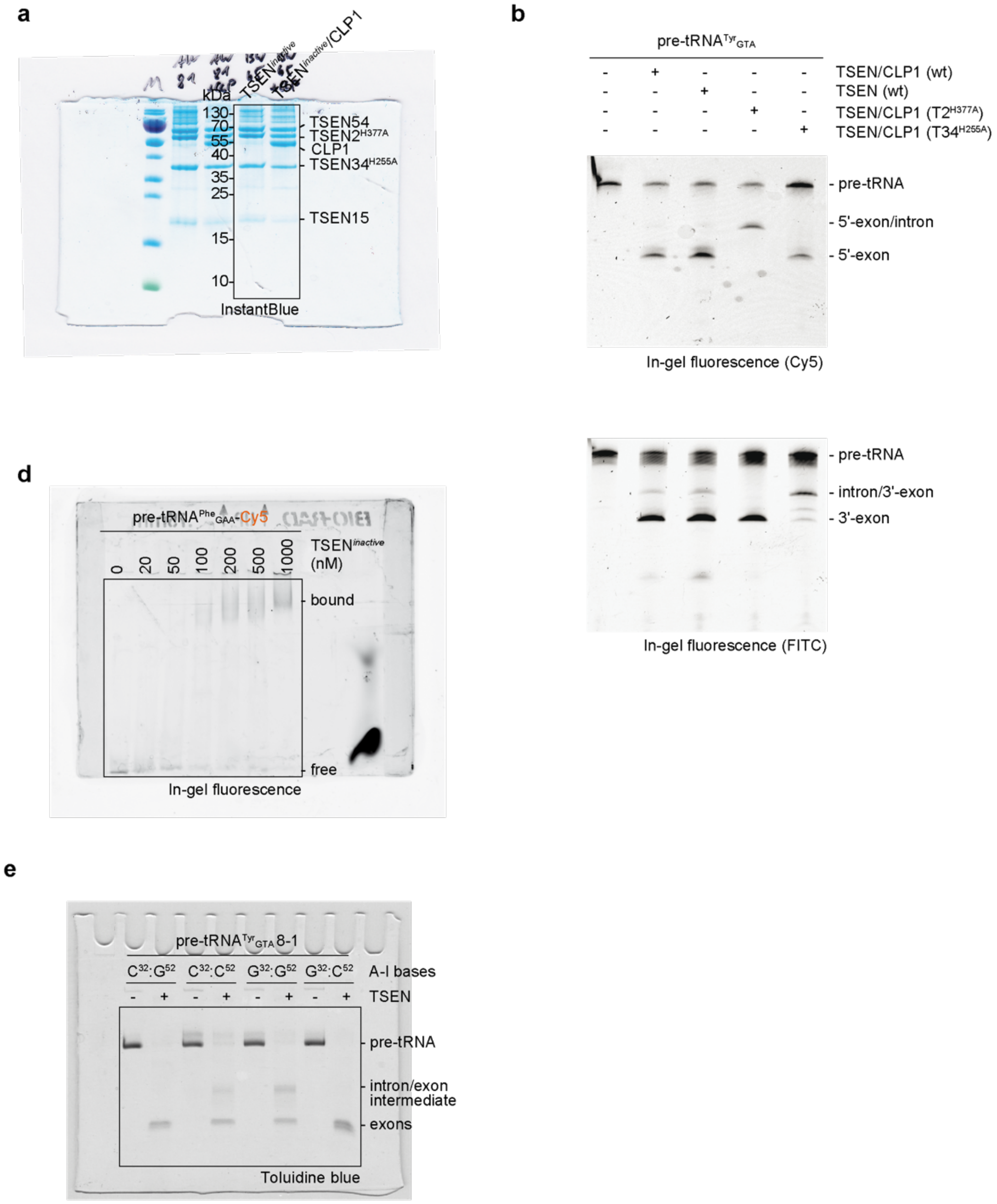

Source Data 5 | Uncropped images as shown in Fig. 3.

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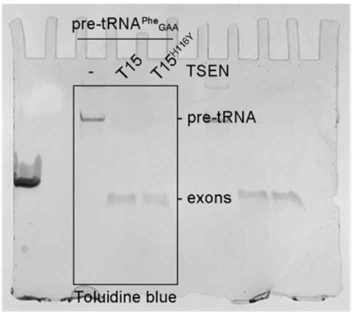
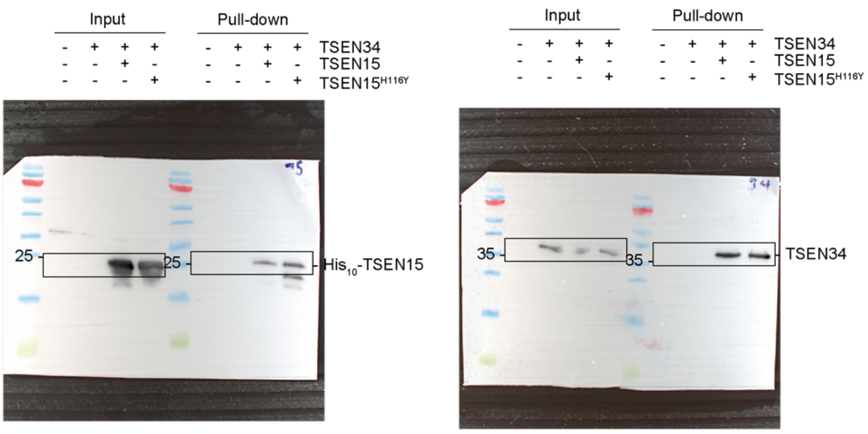


e



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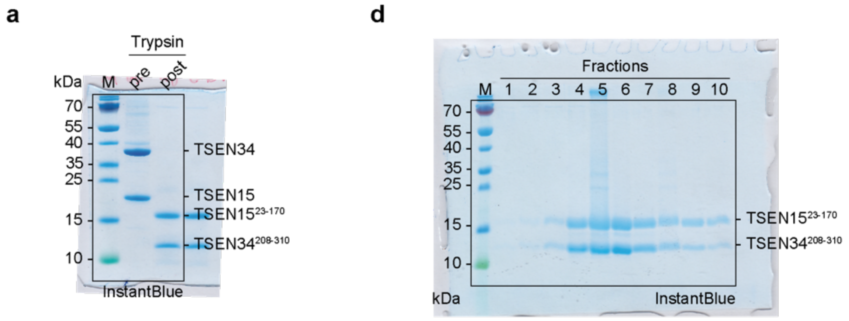

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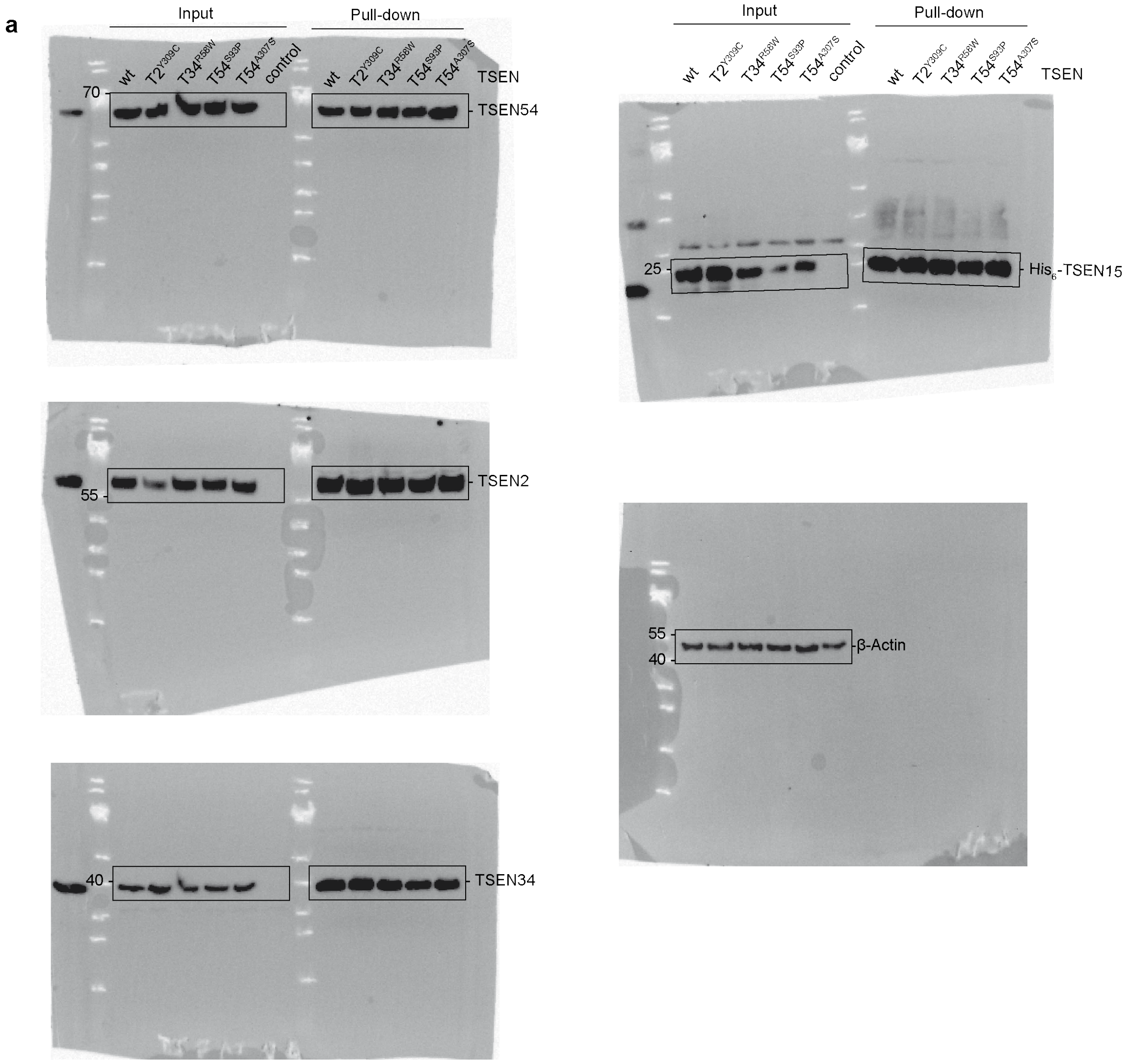
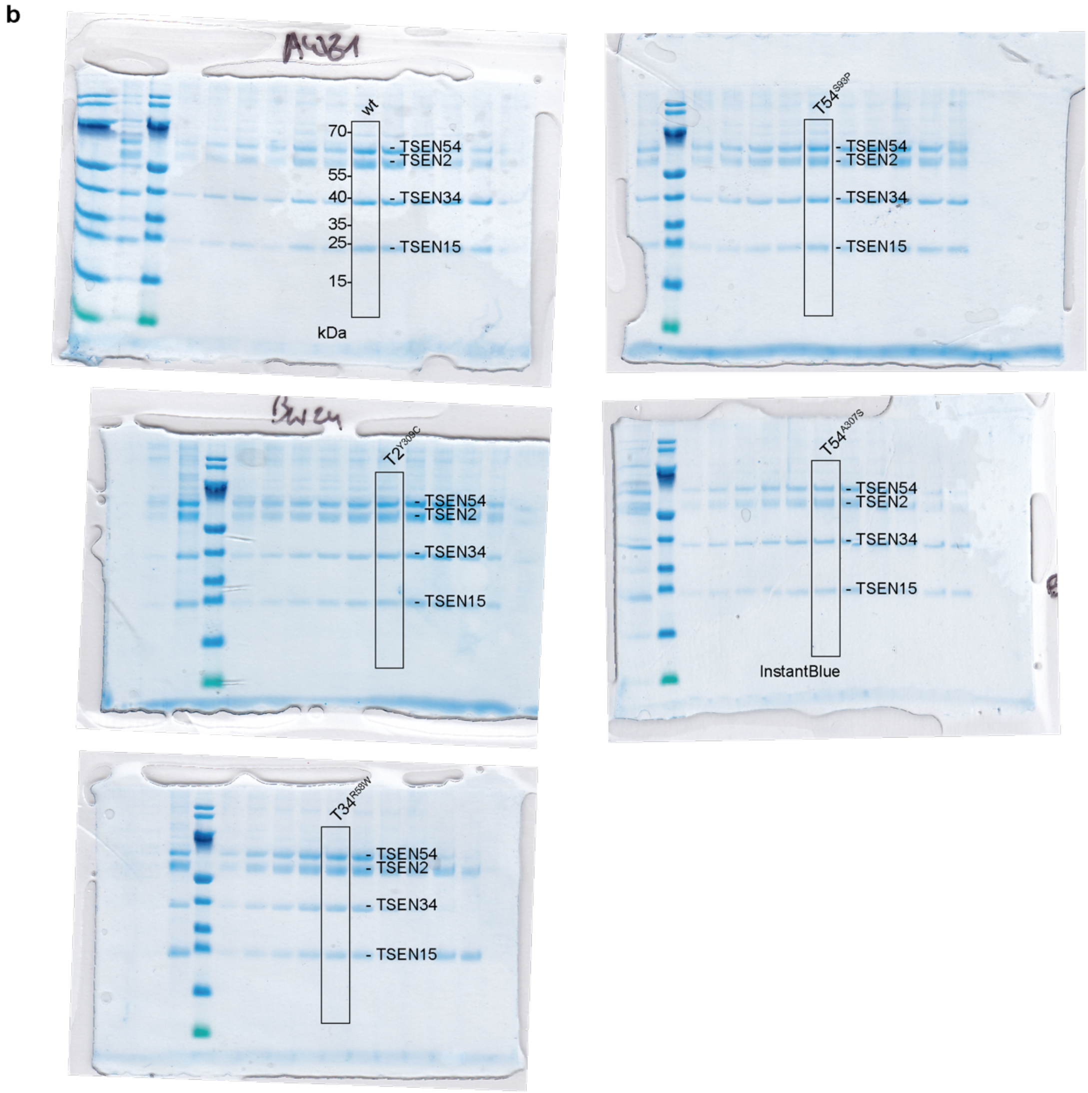
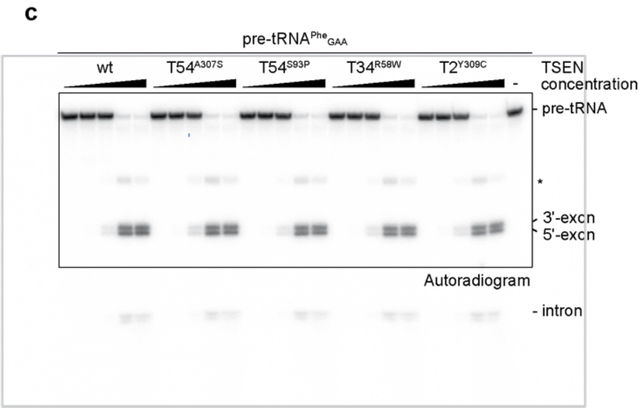


Source Data 8 | Uncropped images as shown in Fig. 4b,c.





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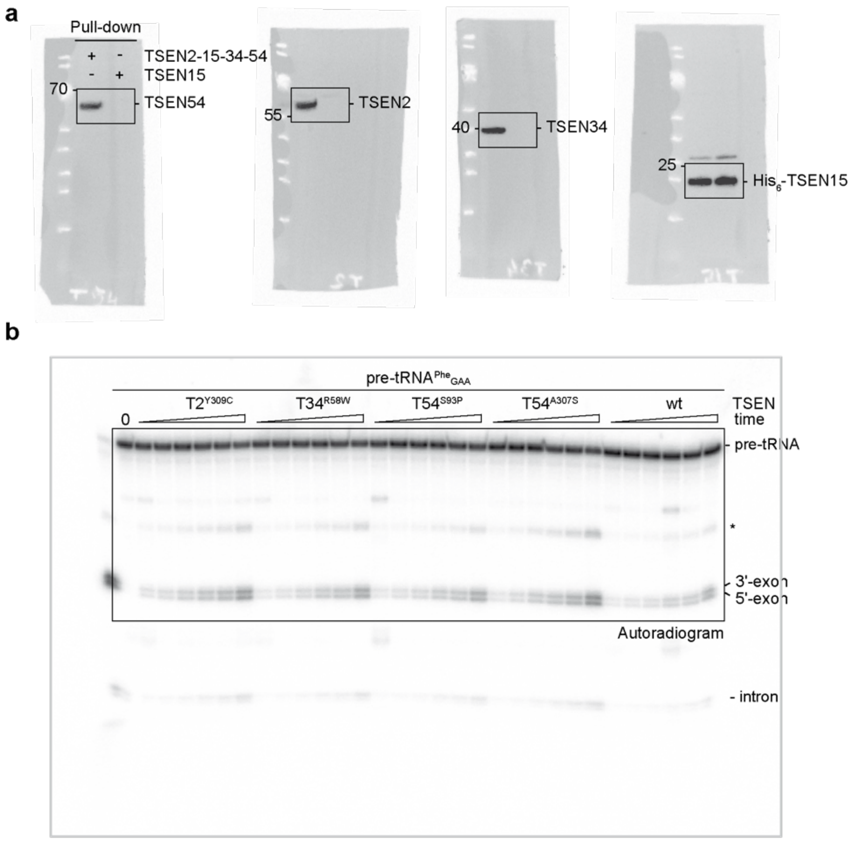

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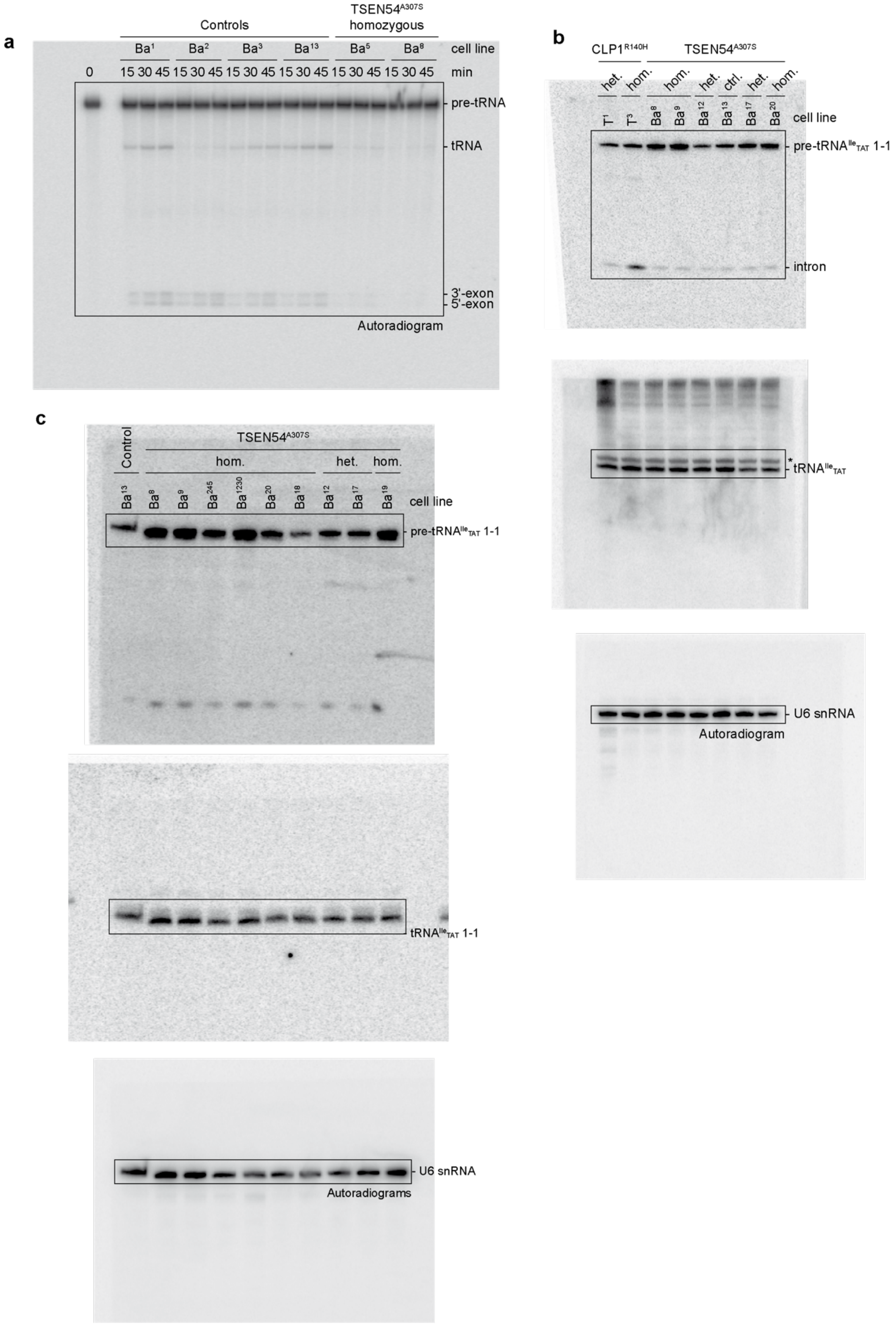
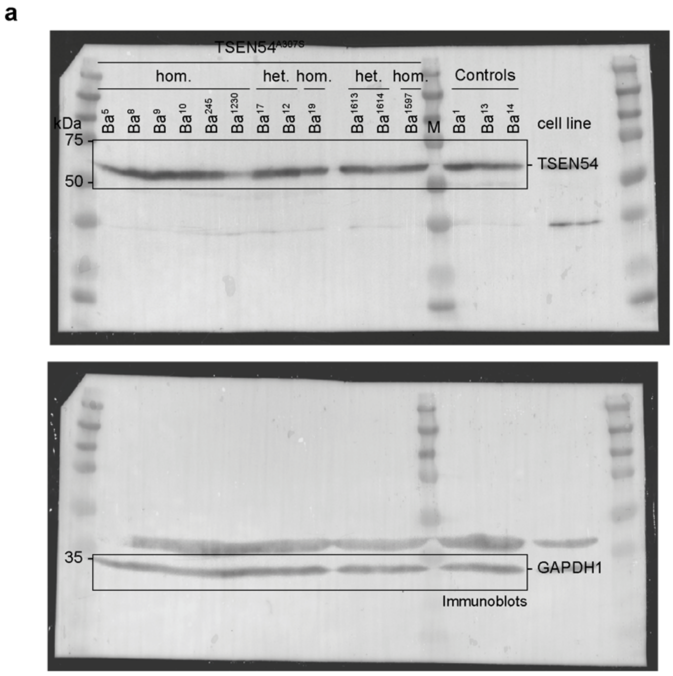


Source Data 11 | Uncropped images as shown in Fig. 6a.



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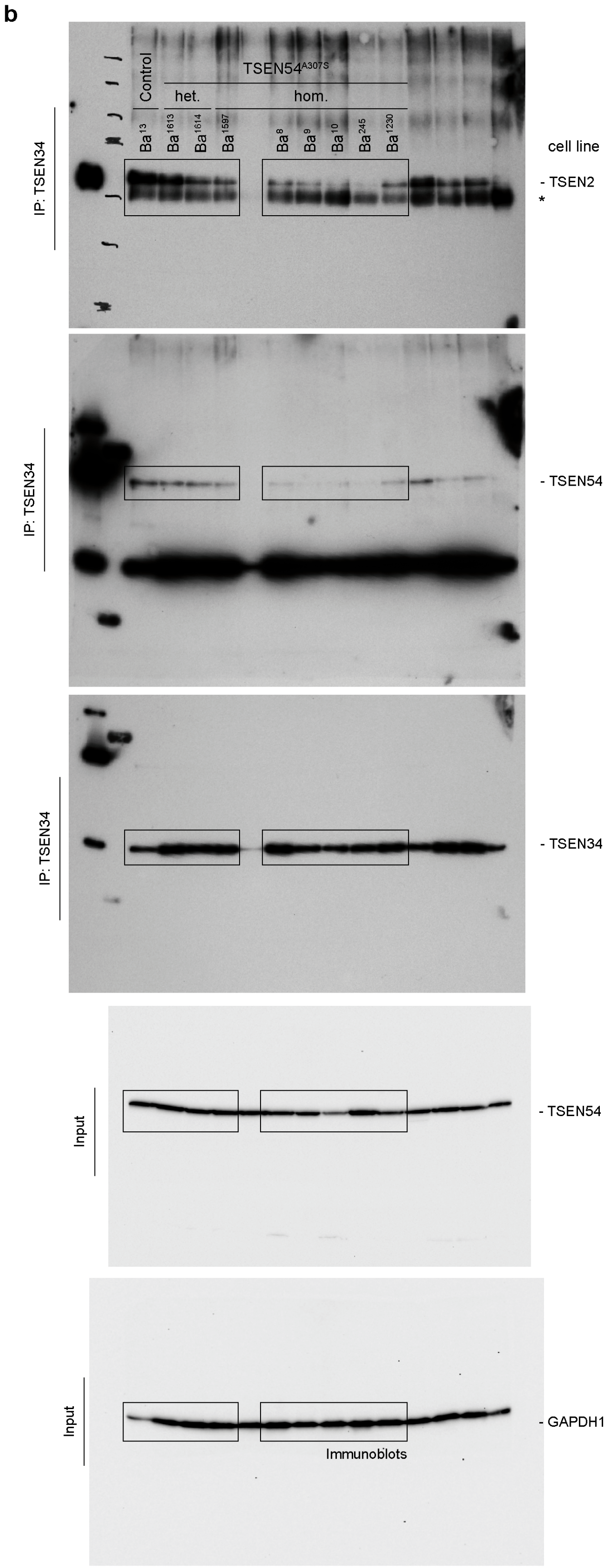

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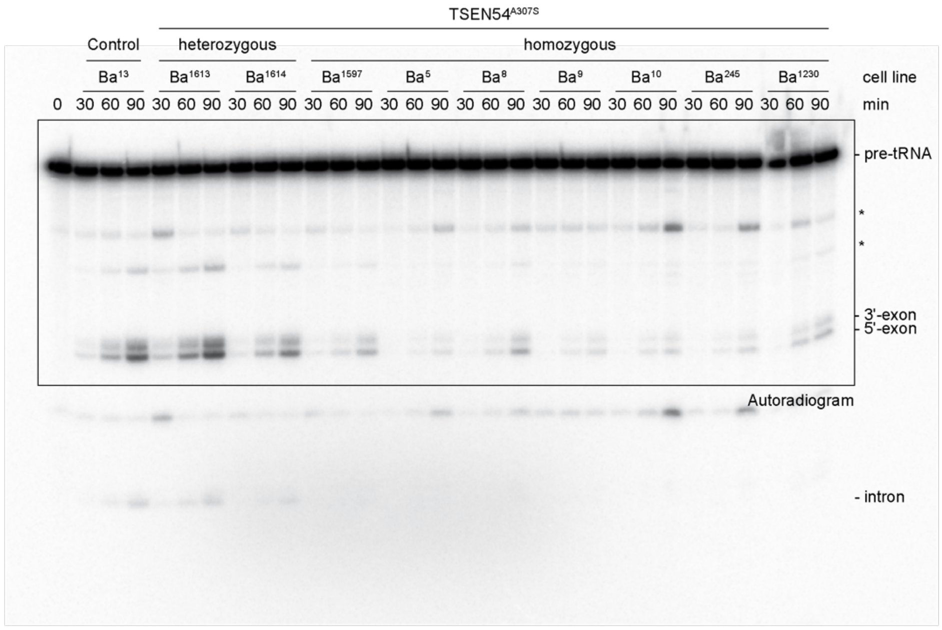
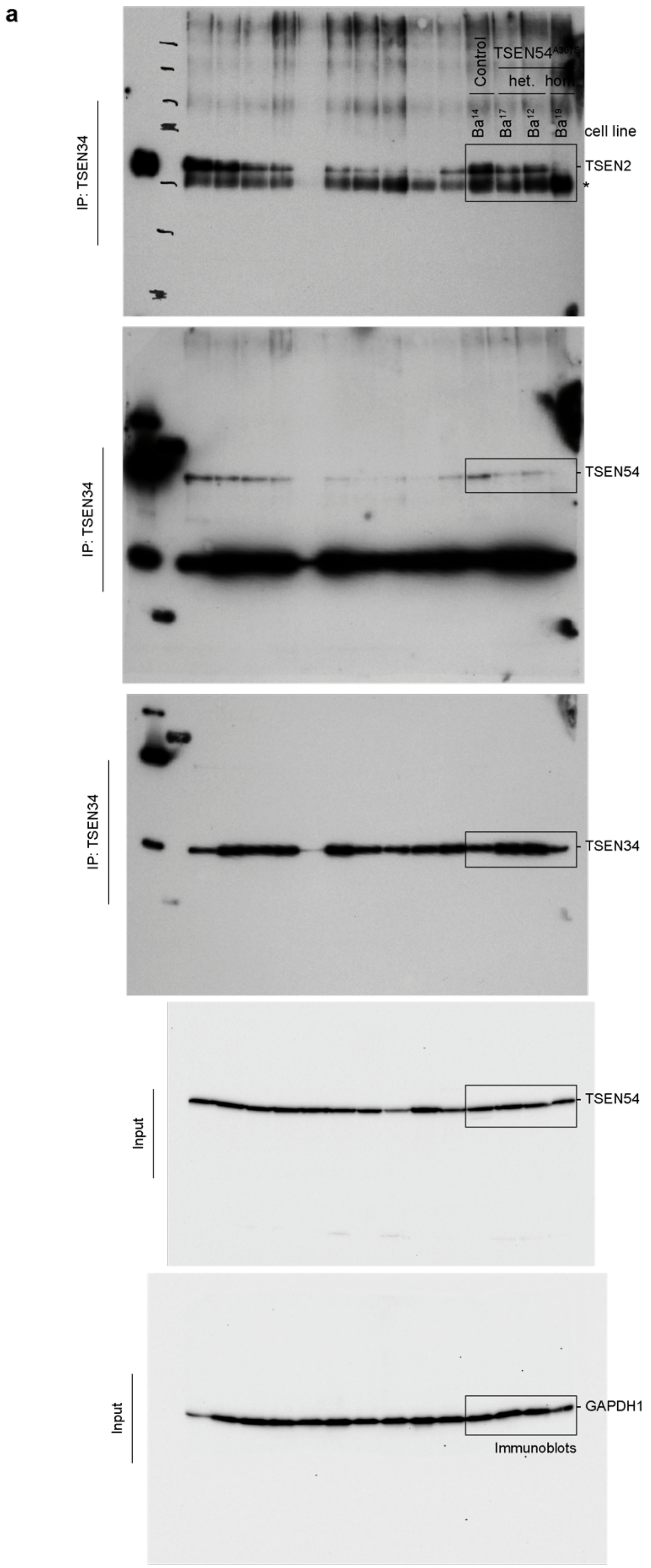
c



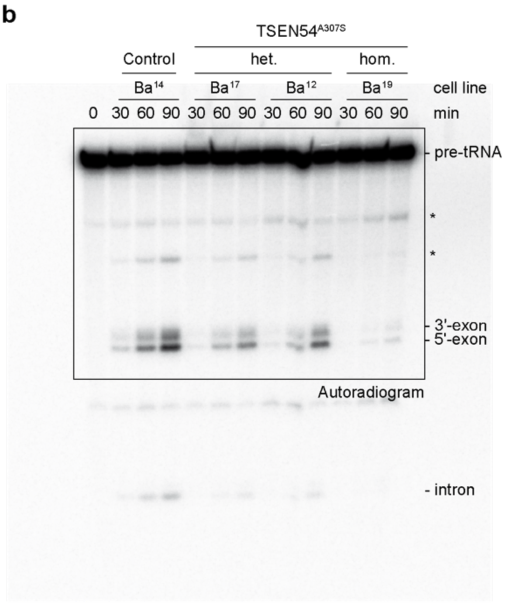
Gamma-corrected image

Source Data 14 | Uncropped images as shown in Extended Data Fig. 6a.



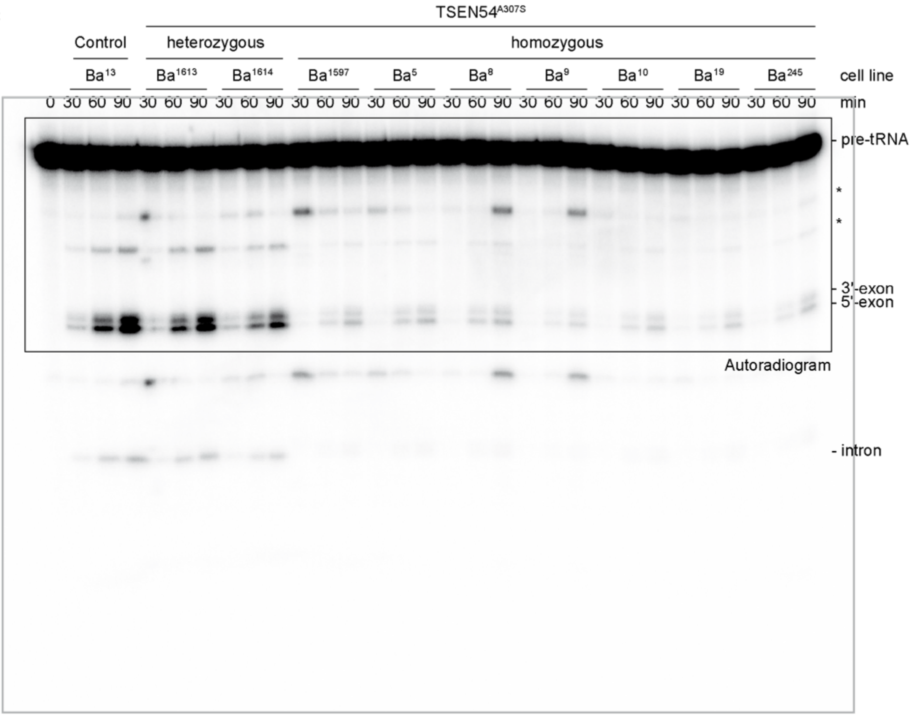
Source Data 15 | Uncropped images as shown in Extended Data Fig. 6b,c.

b



Gamma-corrected image

c



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