

1 **Assembly defects of the human tRNA splicing endonuclease contribute to impaired**
2 **pre-tRNA processing in pontocerebellar hypoplasia**

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29

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31

32 **Abstract**

33 Introns of human transfer RNA precursors (pre-tRNAs) are excised by the tRNA splicing
34 endonuclease TSEN in complex with the RNA kinase CLP1. Mutations in TSEN/CLP1 occur in
35 patients with pontocerebellar hypoplasia (PCH), however, their role in the disease is unclear. Here, we
36 show that intron excision is catalyzed by tetrameric TSEN assembled from inactive heterodimers
37 independently of CLP1. Splice site recognition involves the mature domain and the anticodon-intron
38 base pair of pre-tRNAs. The 2.1-Å resolution X-ray crystal structure of a TSEN15–34 heterodimer and
39 differential scanning fluorimetry analyses show that PCH mutations cause thermal destabilization.
40 While endonuclease activity in recombinant mutant TSEN is unaltered, we observe assembly defects
41 and reduced pre-tRNA cleavage activity resulting in an imbalanced pre-tRNA pool in PCH patient-
42 derived fibroblasts. Our work defines the molecular principles of intron excision in humans and
43 provides evidence that modulation of TSEN stability may contribute to PCH phenotypes.

44

45 Main text

46 All nuclear-encoded transfer RNAs (tRNAs) are processed and modified during trafficking to the
47 cytoplasm, to create functional, aminoacylated tRNAs¹. In humans, 28 out of 429 predicted high
48 confidence tRNA genes contain introns that must be removed from precursor tRNAs (pre-tRNAs) by
49 splicing^{2,3} (<http://gtrnadb.ucsc.edu/>). Some isodecoders, e.g. tRNA^{Tyr}_{GTA}, tRNA^{Ile}_{TAT}, and tRNA^{Leu}_{CAA},
50 are only encoded as intron-containing precursors, for which splicing is essential for their production⁴.
51 Intron excision and ligation of the 5' and 3' tRNA exons is catalyzed by two multiprotein assemblies:
52 the tRNA splicing endonuclease (TSEN)⁵ and the tRNA ligase complex⁶, respectively.

53
54 The human TSEN complex consists of two catalytic subunits, TSEN2 and TSEN34, and two structural
55 subunits, TSEN15 and TSEN54, all expressed at very low copy numbers of ~ 100 molecules per cell
56^{5,7}. TSEN2–54 and TSEN15–34 are inferred to form distinct heterodimers from yeast-two-hybrid
57 experiments, however a solution NMR structure has challenged the proposed model of TSEN
58 assembly^{8,9}. Based on their quaternary structure, archaeal tRNA endonucleases have been classified
59 into four types, α_4 , α'_2 , $(\alpha\beta)_2$, and ε_2 ^{10,11}, whereas the eukaryotic tRNA endonucleases adapt a
60 heterotetrameric $\alpha\beta\gamma\delta$ arrangement^{5,8}. Homotetramer formation in archaeal α_4 -type endonucleases is
61 mediated by a hydrophobic domain interface involving antiparallel β strands of two neighboring α
62 subunits and interactions between a negatively charged L10 loop of one α subunit with a positively
63 charged pocket of an opposing α subunit. These interactions are conserved in all four types of
64 archaeal endonucleases and were also suggested to occur in eukaryotic endonucleases. In humans,
65 TSEN2 and TSEN34 are each predicted to harbor a catalytic triad, composed of Tyr³⁶⁹/His³⁷⁷/Lys⁴¹⁶ in
66 TSEN2 and Tyr²⁴⁷/His²⁵⁵/Lys²⁸⁶ in TSEN34, responsible for cleavage at the 5' and 3' splice sites,
67 respectively^{5,8,12}. His³⁷⁷ and His²⁵⁵ are supposed to act as general acids at the scissile phosphates of
68 the exon-intron junctions of pre-tRNAs^{5,12,13}. Furthermore, TSEN54 was suggested to function as a
69 'molecular ruler' measuring the distance from the mature domain of the tRNA to define the 5' splice
70 site^{8,13-16}.

71
72 The intron in pre-tRNAs is suggested to allow the formation of a double helix that extends the
73 anticodon stem in the conventional tRNA cloverleaf structure and presents the 5' and 3' splice sites in
74 single-stranded regions accessible for cleavage^{17,18}. Such a bulge-helix-bulge (BHB) conformation
75 was postulated to act as a universal recognition motif in archaeal pre-tRNA splicing allowing for intron

76 recognition at various positions in pre-tRNAs¹⁹. In contrast, eukaryotic introns strictly locate one
77 nucleotide 3' to the anticodon triplet in the anticodon loop with varying sequence and length^{3,14}.
78 Experiments using yeast and *Xenopus* tRNA endonucleases showed that cleavage at the exon-intron
79 boundaries requires the presence of an anticodon–intron (A–I) base pair that controls cleavage at the
80 3' splice site besides positioning of the 5' splice site via the mature domain of the pre-tRNA^{14,20,21}. The
81 X-ray crystal structure of an archaeal endonuclease with a BHB-substrate showed that two arginine
82 residues at each active site form a cation- π sandwich with a flipped-out purine base of the pre-tRNA
83 and thereby fixing the substrate for an S_N2-type in line-attack^{12,13,16}. However, biochemical
84 experiments using the yeast endonuclease showed that the cation- π sandwich is only required for
85 cleavage at the 5' splice site, whereas it is dispensable for catalysis at the 3' splice site¹².
86 Specific to mammals is the association of the tRNA splicing endonuclease with the RNA kinase CLP1
87^{5,22}. Mutations in CLP1 were shown to impair tRNA splicing *in vitro* and to cause neuropathologies
88 involving the central and peripheral nervous system²³⁻²⁵. Mutations in all four subunits of the TSEN
89 complex have been associated with the development of pontocerebellar hypoplasia (PCH), a
90 heterogeneous group of inherited neurodegenerative disorders with prenatal onset characterized by
91 cerebellar hypoplasia and microcephaly²⁶⁻³¹.
92
93 High expression of TSEN54 in neurons of the pons, cerebellar dentate, and olivary nuclei suggested
94 that a functional endonuclease complex is essential for the development of these regions²⁶. The most
95 common mutation causing a type 2 PCH phenotype is a homozygous *TSEN54* c.919G>T mutation
96 that leads to an A³⁰⁷S substitution in TSEN54^{26,29}. Other substitutions, e.g. S⁹³P in TSEN54, R⁵⁸W in
97 TSEN34, Y³⁰⁹C in TSEN2, and H¹¹⁶Y in TSEN15, have also been identified as causative for PCH^{26,27}.
98 None of the described disease mutations are located in or in close proximity to the predicted catalytic
99 sites of human TSEN, or in other highly conserved regions of the proteins, and how they contribute to
100 disease development remains enigmatic. Here we present the biochemical and structural
101 characterization of recombinant human TSEN. We analyze PCH-associated mutations at the structural
102 and biochemical levels in reconstitution experiments and reveal biochemical features of the TSEN
103 complex in PCH patient-derived cells.
104

105 **Results**

106 **Assembly of recombinant human tRNA splicing endonuclease**

107 To gain functional insights into human TSEN/CLP1, we designed an expression vector series based
108 on the MultiBac system ³² that allows combinatorial protein complex production in insect and
109 mammalian cells by utilizing a CMV/p10 dual promoter ³³ (Fig. 1a,b and Extended Data Fig. 1a). Using
110 this system, we were able to assemble and purify functional heterotetrameric TSEN and a
111 heteropentameric complex including the RNA kinase CLP1 from infected insect cells (Fig. 1b,c and
112 Extended Data Fig. 1b). The structural integrity of the purified complexes was verified by native mass
113 spectrometry (MS), showing a stoichiometric TSEN2–15–34–54 heterotetramer (165.6 kDa) and a
114 TSEN/CLP1 heteropentamer (213.0 kDa) (Fig. 1c, Extended Data Fig. 1b and Supplementary
115 Tables 1,2). These data are in line with a recent study showing reconstitution of TSEN/CLP1 from a
116 bacterial expression host ³⁴. We also identified TSEN/CLP1 complexes harboring two CLP1 molecules
117 (Extended Data Fig. 1b). Recombinant TSEN54 showed a high degree of phosphorylation as reported
118 for the endogenous protein (Extended Data Fig. 1c) ³⁵.

119
120 Endonuclease activity of tetrameric TSEN was observed in a pre-tRNA cleavage assay using
121 *Saccharomyces cerevisiae* (*S.c.*) pre-tRNA^{Phe}_{GAA} as a substrate, whereas mature *S.c.* tRNA^{Phe}_{GAA}
122 remained unaffected (Fig. 1d). The absence of endonucleolytic activity on mature tRNA confirms the
123 specificity of the complex for its native pre-tRNA substrate. Yeast-two-hybrid experiments with *S.c.*
124 orthologues suggested that strong interactions exist between TSEN15 and TSEN34, as well as
125 between TSEN2 and TSEN54, and that the human endonuclease assembles from preformed dimeric
126 subcomplexes ⁸. Using combinatorial co-expression analyses, we identified the formation of stable
127 TSEN15–34 and TSEN2–54 heterodimers (Fig. 1e). Individual heterodimers did not show
128 endonuclease activity, whereas specific endonucleolytic cleavage was observed after stoichiometric
129 mixing of TSEN15–34 and TSEN2–54 in the absence of ATP (Fig. 1e). Size exclusion
130 chromatography confirmed that a stable tetrameric assembly formed upon mixing the individual
131 heterodimers (Extended Data Fig. 1d,e). These observations indicate that active human TSEN
132 assembles from non-functional, heterodimeric submodules independently of CLP1 and ATP.

133

134 **Human TSEN binds precursor and mature tRNAs with similar affinities**

135 It has been postulated that eukaryotic splicing endonucleases recognize pre-tRNAs via their mature
136 domain^{14,15}. To define tRNA binding parameters of human TSEN, we performed interaction studies
137 using catalytically inactive tetramers (TSEN^{inactive}), in which the active site histidines of TSEN2 (His³⁷⁷)
138 and TSEN34 (His²⁵⁵) were substituted with alanines (Fig. 2 and Extended Data Fig. 2). Alanine
139 substitutions of His³⁷⁷ of TSEN2 and His²⁵⁵ of TSEN34 abolished cleavage at the 5' and 3' splice sites,
140 respectively, and purified TSEN^{inactive} did not cleave pre-tRNA substrates at all (Fig. 2a and Extended
141 Data Fig. 2b).

142
143 To perform fluorescence anisotropy and pull-down experiments, we site-specifically labeled precursor
144 and mature yeast tRNA^{Phe_{GAA}} at the terminal 3' ribose. Despite the inability to cut its native substrate,
145 TSEN^{inactive} interacted stably and specifically with the fluorescently labeled pre-tRNA in a pull-down
146 assay (Fig. 2b). Binding studies using fluorescence anisotropy revealed dissociation constants (K_D) of
147 173±11 nM and 149±20 nM for fluorescently labeled pre-tRNA^{Phe_{GAA}} and mature tRNA^{Phe_{GAA}},
148 respectively (Fig. 2c,d). We determined an inhibition constant (K_i) of 197 nM (95% confidence interval
149 of 168 – 231 nM) in a competition assay confirming the specific interaction, whereas a fluorescent
150 electrophoretic mobility shift assay corroborated a dissociation constant between TSEN and pre-tRNA
151 in the high nanomolar range (Extended Data Fig 2c,d). Our determined K_D values are in good
152 agreement with previously deduced Michaelis constants (K_M) of ~30 nM and 250 nM for intron excision
153 by the yeast and an archaeal tRNA endonuclease, respectively³⁶. Taken together, the results show
154 that substrate recognition by human TSEN is primarily mediated by the mature domain of pre-tRNAs
155 but not their introns and suggests that discrimination between pre- and mature tRNAs might be
156 dictated by kinetic effects.

157

158 **The A–I base pair coordinates cleavage at the 3' splice site in human TSEN**

159 Cleavage of archaeal introns strictly relies on the tRNA BHB motif, whereas the only preserved feature
160 of human tRNA introns is a pyrimidine in the 5' exon at position -6 from the 5' splice site which forms a
161 conserved A–I base pair with a purine base at position -3 from the 3' splice site (Fig. 2e). Studies on
162 the *Xenopus* tRNA endonuclease showed that the A–I base pair is critically involved in the cleavage
163 reaction at the 3' splice site^{20,21,37}. To find out whether the same regulatory principles exist for intron
164 excision in humans, we tested the impact of A–I base pair mutants on endonucleolytic cleavage by
165 tetrameric TSEN (Fig. 2e,f, Extended Data Fig. 2e, and Supplementary Fig. 1). Changing the guanine

166 base G⁵⁴ to cytosine in *S.c.* pre-tRNA^{Phe}_{GAA} produced a pre-tRNA substrate with a disrupted A–I base
167 pair (Fig. 2e,f). Cleavage of this pre-tRNA by wild-type (wt) human TSEN resulted in a 5' exon and an
168 intron-3'-exon intermediate (Fig. 2f). Cleavage at both splice sites was observed when base pairing at
169 the A–I position was restored by mutating cytosine C³² to guanine in the C⁵⁴ background (Fig. 2e,f).
170 The same effect was observed when human pre-tRNA^{Tyr}_{GTA8-1} harboring equivalent mutations was
171 used as substrate (Extended Data Fig. 2e). These findings imply that the presence of an A–I base
172 pair, but not the strict identity of the bases, is essential for cleavage at the 3' splice site by human
173 TSEN^{20,21}.

174

175 **The molecular architecture of TSEN is evolutionarily conserved**

176 Our interaction studies using recombinant proteins showed that active human TSEN assembles from
177 inactive TSEN15–34 and TSEN2–54 heterodimers (Fig. 1e). To gain detailed insights into the
178 molecular architecture of the human TSEN complex, we characterized the TSEN15–34 heterodimer
179 by X-ray crystallography (Fig. 3 and Extended Data Fig. 3). Despite extensive crystallization trials, full-
180 length TSEN15–34 did not crystallize. To define a crystallizable core complex, we subjected the
181 TSEN15–34 complex to limited proteolysis with subsequent size exclusion chromatography and MS
182 analysis (Extended Data Fig. 3a,b and Supplementary Tables 2-4). We observed two comigrating
183 polypeptide species corresponding to residues 23 to 170 of TSEN15 and residues 208 to 310 of
184 TSEN34 covering the predicted conserved nuclease domains (Extended Data Fig. 3b,c and
185 Supplementary Fig. 2)⁸.

186

187 We re-cloned, co-expressed and purified the proteolytically characterized fragments, which readily
188 formed rod-shaped crystals in space group P2₁ and diffracted X-rays to a resolution of 2.1 Å
189 (Extended Data Fig. 3d and Supplementary Table 5). The asymmetric unit is composed of two
190 domain-swapped TSEN34 molecules, each binding one TSEN15 protomer at their C-terminal domains
191 (Extended Data Fig. 3e,f). The domain swap is brought about by a short, structured N-terminal α-
192 helix/β-hairpin element of TSEN34 that is liberated to hook onto the neighboring protomer, presumably
193 due to the truncated N-terminus of the molecule. The domain-swap organization is only found under
194 crystallization conditions, as shown by size exclusion chromatography multi-angle light scattering
195 (SEC-MALS) (Extended Data Fig. 3g). The two TSEN15 and the two TSEN34 molecules in the
196 asymmetric unit are very similar with average overall RMSDs of 0.37 Å and 0.50 Å, respectively. In

197 one TSEN15 protomer, an elongated N-terminal region (residues 162-170) is visible, which is
198 stabilized by crystal contacts ([Extended Data Fig. 3f](#)).

199

200 TSEN15 and TSEN34 display the typical endonuclease fold with the latter harboring the
201 Tyr²⁴⁷/His²⁵⁵/Lys²⁸⁶ catalytic triad as also found in archaeal and eukaryotic endonucleases ([Fig. 3a,b](#)
202 and [Extended Data Fig. 3h](#))¹³. The dimeric TSEN15–34 complex is characterized by an elongated
203 central twisted β -sheet connected by the C-terminal β -strands of TSEN15 and TSEN34 with a buried
204 surface area of $\sim 1980 \text{ \AA}^2$ between the protomers ([Fig. 3a,b](#)). Each face of the individual twisted β -
205 sheets of TSEN15 and TSEN34 is mainly stabilized by hydrophobic interactions to an alpha helix
206 ([Fig. 3a,c,d](#)). In the interface between TSEN15 and TSEN34 two structural water molecules are found,
207 which are coordinated by hydrogen bonds to backbone oxygens or amide groups of Ile¹¹⁰, Ala¹¹², and
208 Leu¹¹⁴ of TSEN15, Ile²⁶⁹, Leu²⁷¹, and Gln²⁷² of TSEN34, and the side chain oxygen of Gln²⁷² ([Fig. 3c](#)).
209 Furthermore, a YY motif in TSEN15 (Tyr¹⁵²/Tyr¹⁵³), which is conserved in eukaryotic endonucleases
210 and archaeal α_4 - and $(\alpha\beta)_2$ -type endonucleases ([Supplementary Fig. 3](#)) both stabilizes TSEN15 by
211 hydrophobic interactions and the dimer interface by hydrogen bonds to the main chain carbonyl
212 oxygen of Leu²⁷⁴ and the side chain oxygen of Ser²⁸³ of TSEN34 ([Fig. 3a,c](#)). In contrast to a previous
213 solution NMR structure of homodimeric TSEN15⁹, our biochemical and structural analyses show that
214 the assembly and architecture of TSEN are conserved and support the hypothesis that tRNA splicing
215 endonucleases arose from a common ancestor through gene duplication and differentiation events³⁸.

216

217 **PCH-causing mutations destabilize recombinant TSEN**

218 A previous genetic study identified a His-to-Tyr substitution at position His¹¹⁶ of TSEN15 in patients
219 with PCH type 2 ([Fig. 3d](#))²⁷. The imidazole group of His¹¹⁶ is central to a hydrogen bond network
220 involving Asn¹¹⁷, Arg¹²⁰, and Asp¹⁵⁷ of TSEN15 and Ser²⁹² and Thr³⁰² of TSEN34 ([Fig. 3d](#)). We tested
221 the impact of this substitution in a pull-down experiment using full-length TSEN15 and TSEN34 and
222 also in a pre-tRNA cleavage assay in the context of the tetrameric assembly ([Fig. 3e,f](#)). We
223 hypothesized that the substitution impairs complex formation and activity due to steric clashes in the
224 dimer interface and loss of the hydrogen bond network. However, TSEN15 carrying the His-to-Tyr
225 mutation engaged in complex formation with TSEN34 similar to the wt protein, and no impairment of
226 catalytic activity was observed ([Fig. 3e,f](#)). We assumed that the large hydrophobic interface
227 compensates for the loss of the hydrogen bond network. To assess the effects of the TSEN15^{H116Y}

228 mutation on the thermal stability of TSEN, we compared the mutant complex to wt by differential
229 scanning fluorimetry (DSF, [Fig. 3g](#))³⁹. This assay reported apparent denaturing temperatures of
230 50.0±0.5 °C and 47.4±0.5 °C for wt and mutant TSEN, respectively ([Fig. 3g](#) and [Extended Data](#)
231 [Fig. 3i](#)). These data suggest that destabilization of TSEN might be a general effect of PCH-causing
232 mutations.

233

234 The molecular basis of PCH mutations on disease development is largely unknown³⁰. It is
235 hypothesized that mutations in TSEN contribute to the disease by interfering with complex assembly,
236 stability, or enzymatic activity. Given that the His-to-Tyr mutation in TSEN15 thermally destabilized the
237 endonuclease complex, we produced heterotetrameric TSEN complexes carrying the PCH-causing
238 mutations TSEN2^{Y309C}, TSEN34^{R58W}, TSEN54^{S93P} and TSEN54^{A307S} in HEK293 cells and performed
239 pull-down experiments to assess complex assembly and integrity ([Fig. 4a](#)). Despite subtle differences
240 in expression levels of the individual subunits, pull-down via TSEN15 co-precipitated TSEN2,
241 TSEN34, and TSEN54, irrespective of the introduced PCH-causing mutation ([Fig. 4a](#)). Control pull-
242 downs from HEK293 cells overexpressing only His-tagged TSEN15 showed that endogenous subunits
243 of TSEN do not associate with overexpressed TSEN15, probably due to their very low copy numbers
244 ([Extended Data Fig. 4a](#)). We produced and purified recombinant heterotetrameric TSEN complexes
245 carrying the pathogenic missense mutations from baculovirus-infected insect cells ([Fig. 4b](#)) and also
246 did not see obvious deleterious effects on subunit composition or pre-tRNA cleavage kinetics
247 ([Fig. 4b,c](#) and [Extended Data Fig. 4b](#)).

248

249 Given the low abundance of TSEN molecules in cells⁸ and that PCH mutations phenotypically affect
250 only cerebellar neurons, we reasoned that expression levels are too high in our reconstitution systems
251 to reveal subtle alterations in TSEN assembly and function. To assess the effects of PCH-causing
252 mutations on complex stability, we used the DSF assay ([Fig. 3g](#) and [Extended Data Fig. 3i](#)). Most
253 PCH-causing mutations led to substantial shifts towards lower denaturation temperatures (e.g. T_d of
254 43.9±0.9 °C for TSEN2^{Y309C} compared to T_d of 50.4±0.5 °C for wt TSEN) when exposed to thermal
255 gradients indicating that mutant TSEN complexes have compromised structural integrity ([Fig. 4d](#) and
256 [Extended Data Fig. 4c](#)). The relative changes in thermostability (T_{Δ}) of the mutant complexes
257 compared to wt TSEN ranged from 6.5 °C for the TSEN2^{Y309C} mutation to 1.2 °C for the TSEN54^{A307S}
258 mutation potentially scaling with the severity of the disease phenotype ([Fig. 4d](#) and [Extended Data](#)

259 [Fig. 4c](#))²⁶. DSF data also revealed two-state unfolding behaviors for all TSEN complexes when
260 analyzed by the ProteoPlex algorithm⁴⁰ suggesting cooperativity of unfolding transitions for the
261 individual subunits ([Supplementary Table 6](#)), thus explaining why mutations in different subunits lead
262 to an overall destabilization of TSEN. Our data suggest that PCH phenotypes in patients potentially
263 develop due to destabilized TSEN complexes.

264

265 **Pre-tRNA processing is impaired in PCH patient cells**

266 To determine if pre-tRNA processing activity is compromised in PCH patients we derived primary skin
267 fibroblasts from PCH patients, their healthy parents, and unrelated controls ([Supplementary Table 7](#)).
268 We chose the common *TSEN54* c.919G>T (*TSEN54*^{A307S}) mutation, which is reported in ~ 90% of
269 recognized TSEN-linked PCH cases³⁰, and for which a large cohort of patient samples are available.
270 The cell lines we created did not show any morphological differences compared to control cells. When
271 we assayed lysates derived from homozygous *TSEN54* c.919G>T cell lines, we observed a reduction
272 in pre-tRNA splicing efficiency compared to control cell lysates ([Fig. 5a](#)). Subtle differences in ligation
273 efficiency, as observed for cell line Ba2, may result from the fibroblasts having different genetic
274 backgrounds.

275

276 This result is reminiscent of observations in patient-derived cell lines carrying a homozygous *CLP1*
277 c.419G>A (*CLP1*^{R140H}) mutation, which leads to severe motor-sensory defects, cortical dysgenesis,
278 and microcephaly^{23,25}. In contrast to homozygous *CLP1*^{R140H} cells, in which introns accumulate, intron
279 accumulation did not occur in either heterozygous or homozygous *TSEN54*^{A307S} backgrounds as
280 judged by northern blot analysis using a probe specific for the intron of pre-tRNA^{lle}_{TAT1-1} ([Fig. 5b](#)).
281 These results suggest an impairment of intron excision rather than a defect in downstream processes
282 of the tRNA splicing reaction, which might lead to the accumulation of pre-tRNAs in patient cells.

283

284 To test this hypothesis, we compared levels of intron-containing pre-tRNAs to their corresponding
285 mature tRNAs in cell lines carrying the homozygous *TSEN54* c.919G>T mutation to heterozygous cell
286 lines and controls by hydro-tRNAseq⁴ and northern blotting ([Fig. 5c,d](#), [Extended Data Fig. 5](#), and
287 [Supplementary Table 8](#)). We observed an accumulation (~2-6 fold) of intron-containing pre-tRNAs in
288 homozygous *TSEN54* c.919G>T cell lines compared to control cell lines, albeit global levels of mature
289 tRNAs remained largely unaffected ([Fig. 5c,d](#) and [Extended Data Fig. 5](#)). The distributions of the ratios

290 of precursor over mature tRNA reads showed that there was no bias for an enrichment of a specific
291 precursor tRNA among samples ([Extended Data Fig. 5a](#) and [Supplementary Table 8](#)). These findings
292 are consistent with the observation that homozygote patient samples exhibited a relative increase in
293 precursor tRNA reads, compared to wild-type controls ([Fig. 5d](#) and [Extended Data Fig. 5b](#)). Therefore,
294 we conclude that in our experimental setup, TSEN54 A³⁰⁷S results in an increase of the steady-state
295 levels of intron-containing tRNAs. Consistently, northern blot analyses showed similar differences in
296 levels of pre-tRNA^{lle}_{TAT1-1} over mature tRNA^{lle}_{TAT1-1} ([Fig. 5c](#)). Taken together, our data show defects
297 in pre-tRNA processing uncoupled from CLP1 function leading to the accumulation of pre-tRNAs in
298 PCH patient-derived cell lines.

299

300 **Pre-tRNA processing defects are linked to altered TSEN composition**

301 To investigate whether the reduction of pre-tRNA processing in cell extracts of homozygous *TSEN54*
302 c.919G>T patients was due to altered TSEN assembly or stability we used rabbit polyclonal antibodies
303 against peptides of TSEN2, TSEN34, and TSEN54³⁵, to assess changes in TSEN subunit abundance
304 and to perform co-immunoprecipitation experiments of endogenous TSEN. Immunoblot analyses
305 showed that the homozygous *TSEN54* c.919G>T mutation does not impact steady-state levels of
306 TSEN54, suggesting that no changes in either mRNA stability, transcription rate or protein turnover
307 occur ([Fig. 6a](#)).

308

309 To evaluate TSEN complex composition and pre-tRNA cleavage activity we performed
310 immunoprecipitation experiments from patient-derived and control fibroblasts using α -TSEN2 or α -
311 TSEN34 antibodies ([Fig. 6b,c](#) and [Extended Data Fig. 6](#)). Immunoblot analyses showed a substantial
312 reduction of co-immunoprecipitated TSEN2 and TSEN54 from patient cell lines using an α -TSEN34
313 antibody, while at the same time, pre-tRNA cleavage activity was strongly diminished in α -TSEN2 and
314 α -TSEN34 immunoprecipitates ([Fig. 6b,c](#) and [Extended Data Fig. 6](#)). These results indicate that TSEN
315 assembly defects lead to reduced pre-tRNA cleavage in PCH patient cells. Since the association of
316 TSEN2 and TSEN54 is likewise affected but steady-state levels of the individual proteins are not, we
317 conclude that impaired TSEN activity is caused by an altered propensity for the formation of the active
318 tetrameric assembly in patient cells.

319

320

321 Discussion

322 Here we report the recombinant expression, purification, and assembly of functional human
323 TSEN/CLP1 complex. We show that heterotetrameric TSEN is assembled from heterodimeric
324 TSEN15–34 and TSEN2–54 subcomplexes, which combine to form the composite active sites for
325 catalysis (Fig. 1e). The nuclease fold seen in our TSEN15–34 X-ray crystal structure is conserved with
326 the archaeal tRNA endonucleases (Fig. 3b)^{13,16} suggesting that the TSEN2–54 heterodimer — and
327 entire TSEN complex — likely forms through interactions similar to those seen in the TSEN15–34
328 heterodimer, as well as related interactions previously observed in archaeal tRNA endonucleases. Our
329 interaction studies with catalytically inactive TSEN mutants show that substrate recognition occurs
330 through interactions with the mature tRNA fold, including the aminoacyl acceptor stem, the D-loop,
331 and the Ψ-loop, and support the ‘ruler model’ of substrate recognition (Fig. 2c,d)⁸. The similar
332 affinities TSEN shows for pre-tRNAs and tRNAs suggest thermodynamic effects are unlikely to play a
333 role in substrate selection (Fig. 2c,d). Instead, we speculate that different binding kinetics should
334 contribute to the selection of pre-tRNAs over mature tRNAs, thereby guaranteeing efficient scanning
335 and processing of the large pre-tRNA pool.

336
337 The tRNA splicing machinery is involved in processing of other RNA species⁴¹⁻⁴⁴. Eukaryotic tRNA
338 endonucleases are involved in processing of mRNAs and rRNAs^{5,44,45}. TSEN is a key factor in the
339 generation of tRNA intronic circular (tric) RNAs, a poorly uncharacterized class of short non-coding
340 RNAs in *Drosophila* and humans⁴¹. Archaeal tRNA endonucleases are capable of binding and cutting
341 any RNA fragment that adopts a BHB motif¹⁹. tRNA splicing in *Xenopus* necessitates a
342 purine/pyrimidine base pair at the A–I base pair positions for 3' splice site recognition and cleavage.
343 Our data show that requirements for cleavage at the 3' splice site by human TSEN are more relaxed
344 and only need the A–I base pair, whereas the purine/pyrimidine identities of the bases are negligible
345 (Fig. 2f and Extended Data Fig. 2e). The relaxed specificity may facilitate recognition and cleavage of
346 non-canonical substrates. However, structures of human tRNA endonucleases with bound pre-tRNA
347 substrate confirming this hypothesis are still missing. Nonetheless, our data suggest that substrate
348 recognition and cleavage by human TSEN are two distinct processes with different structural
349 requirements regarding the RNA.

350

351 While we show that assembly and enzymatic function of recombinant human TSEN complexes are
352 immune to PCH-associated mutations, these mutations cause thermal destabilization with apparent
353 deleterious effects on complex assembly and activity in patient cells (Fig. 3e,f,g, Fig. 4, Extended Data
354 Fig. 4, Fig. 5, Extended Data Fig. 5, Fig. 6, Extended Data Fig. 6). Structural studies on archaeal tRNA
355 endonucleases show that there are two major interaction interfaces: The β - β -interaction, mainly driven
356 by hydrophobic interactions, and the L10 loop, involving hydrogen bonds and salt bridges. The
357 hydrophobic interface has a higher degree of plasticity and thereby could accommodate mutations to a
358 certain extent, whereas interactions within the hydrophilic interface are less tolerant of changes. Since
359 TSEN is low-abundant (~ 100 molecules per cell)⁷, destabilization by PCH-associated mutations may,
360 therefore, have a strong effect on the assembly of the heterotetramer, whereas the individual
361 heterodimers are sufficiently stable to escape protein degradation. In line with this hypothesis, we find
362 decreased levels of TSEN2 and TSEN54 in α -TSEN34 immunoprecipitates from PCH patient cells
363 (Fig. 6b and Extended Data Fig. 6a).

364

365 The question remains why TSEN mutations lead to a disease phenotype only in a subset of neurons,
366 resulting in selective degeneration of cerebellar and, to a variable extent, anterior cortical structures
367 ^{29,46}. Neuropathologies caused by ablation of TSEN54 are not restricted to humans since knockdown
368 of TSEN54 leads to brain hypoplasia in zebrafish ⁴⁶ and a causative mutation in TSEN54 was
369 identified in standard Schnauzers dogs with leukodystrophy ⁴⁷.

370

371 Defective tRNA processing has been linked to various neuronal diseases ⁴⁸. Although adequate
372 supply of faithfully spliced tRNAs is expected to be essential for protein biosynthesis in all cell types ⁴⁹,
373 neurons may be particularly susceptible to subtle translation defects and, consequently, defects in
374 proteostasis ⁵⁰. Such delicate fine-tuning of translation is highly sensitive to changes in tRNA levels,
375 which may influence the local speed of mRNA translation in a tissue-specific manner depending on
376 the availability of cognate tRNAs ⁵¹. Balanced kinetics of tRNA accumulation could be crucial in
377 tissues or cell sub-populations with a high metabolism, so that an otherwise modest defect in
378 production rate might be deleterious where there is a high demand. Neurons require rapid and local
379 protein synthesis for synaptic plasticity, which needs coordinated transport of the translational
380 machinery, mRNAs, and tRNAs themselves. In line with these notions, missense mutations in subunits
381 of the catalytic core of Pol III have been shown to affect assembly of the polymerase and have been

382 linked to leukodystrophies⁵². A large number of human neurodegenerative disorders have been linked
383 to mutations in components of the general translational machinery and to numerous genes involved in
384 tRNA expression and processing^{1,53}. With CLP1²³⁻²⁵ and arginyl tRNA synthetase⁵⁴ at least two other
385 tRNA processing factors are linked to PCH²⁹ with only mild biochemical effects. Our data suggest that
386 tRNA processing defects caused by TSEN or CLP1 mutations are distinct from one another, acting at
387 different steps of splicing³⁴.

388

389 Impaired TSEN function may selectively impact the processing of cerebellum-specific pre-tRNAs. In
390 mammals, expression of tRNA isoacceptor families (tRNAs with the same anticodon) varies between
391 tissues and during development, and can be altered under disease conditions⁵⁵⁻⁵⁷. Changes in tRNA
392 repertoires have been hypothesized to correlate with the codon usage of genes associated with
393 cellular differentiation states to fine-tune their translation⁵⁵⁻⁵⁷. A mutation in a tRNA gene specifically
394 expressed in the central nervous system has been shown to exhibit a synthetic effect with the loss of a
395 ribosome recycling factor, selectively inducing cerebellar neurodegeneration in mice⁵⁸. In a similar
396 scenario, neuron-specific isodecoders could be critically reduced in PCH patients, as a result of TSEN
397 failure to cleave specific precursors.

398

399 tRNAs also function as signaling molecules in the regulation of numerous metabolic and cellular
400 processes, or as stress sensors and in tRNA-dependent biosynthetic pathways⁵⁹. Transfer RNA-
401 derived fragments (tRFs) have been identified as small non-coding RNAs contributing to translational
402 control, gene regulation and silencing, as well as progressive motor neuron loss⁶⁰. Therefore,
403 impaired TSEN activity could potentially result in unbalanced tRF levels with deleterious cellular
404 effects.

405

406 While our data link a pre-tRNA splicing defect to PCH, additional factors and cellular mechanisms
407 could be involved in the disease. Altered complex stability might affect interactions between TSEN and
408 other cellular components. In yeast, TSEN activity has been linked to pre-rRNA and mRNA processing
409^{43,45}, thus certain neuron-specific mRNA transcripts might require some thus far uncharacterized
410 activity of TSEN, which is impaired by the disease mutations. Clearly, future studies will be needed to
411 address these questions *in vivo* and to build disease models.

412

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598

599 **Materials and Methods**

600 **Plasmid constructs.** To enable recombinant protein production in insect and mammalian cells using
601 a single set of transfer vectors, we modified the MultiBac expression vector suite^{32,61} by replacing
602 existing promoters with a dual CMV-p10 promoter³³ to derive the acceptor vector pAMI, and the three
603 donor vectors pMIDC, pMIDK, and pMIDS ([Extended Data Fig. 1](#)). Open reading frames encoding the
604 TSEN subunits TSEN2 (UniProtKB Q8NCE0), TSEN15 (UniProtKB Q8WW01), TSEN34 (UniProtKB
605 Q9BSV6), TSEN54 (UniProtKB Q7Z6J9), and CLP1 (UniProtKB Q92989) were amplified by
606 polymerase chain reaction (PCR) and cloned into the modified MultiBac vectors leading to
607 pAMI_CLP1, pAMI_TSEN2, pMIDC_TSEN54, pMIDK_TSEN15, and pMIDS_TSEN34. An N-terminal
608 His₆-tag followed by a Tobacco Etch Virus (TEV) protease cleavage site was engineered in vectors
609 encoding CLP1, TSEN2, and TSEN15, leading to pAMI_His₆-TEV-CLP1, pAMI_His₆-TEV-TSEN2, and
610 pMIDK_His₆-TEV-TSEN15, respectively. Furthermore, a pMIDK plasmid encoding TSEN15 with an N-
611 terminal TEV protease-cleavable Strepavidin-binding peptide (SBP) tag was generated (pMIDK_SBP-
612 TEV-TSEN15). The PCH-causing mutations Tyr³⁰⁹Cys (TSEN2), His¹¹⁶Tyr (TSEN15), Arg⁵⁸Trp
613 (TSEN34), Ser⁹³Pro (TSEN54), and Ala³⁰⁷Ser (TSEN54), and the active site mutations His²⁵⁵Ala
614 (TSEN34), and His³⁷⁷Ala (TSEN2) were introduced via QuikChange mutagenesis. For crystallographic
615 purposes, the coding sequences of TSEN34 (residues 208-310) and TSEN15 (residues 23-170) were
616 cloned into pAMI and pMIDK, respectively, attaching an N-terminal His₁₀-tag followed by a TEV
617 protease cleavage site to TSEN15. Prior to integration into the EMBAcY baculoviral genome via Tn7
618 transposition⁶¹, acceptor and donor vectors were concatenated by Cre-mediated recombination
619 utilizing the LoxP sites present on each vector. For co-expression of the TSEN15–34 heterodimer, the
620 vectors pMIDK_His₆-TEV-TSEN15 and pMIDS_TSEN34 were concatenated with the vector
621 pADummy, which was generated by removing the CMV-p10-SV40 expression cassette from pAMI by
622 cleavage with *AvrII* and *SpeI* restriction enzymes and re-ligation of the backbone.

623 For two-color pre-tRNA cleavage assays, TSEN/CLP1-FLAG and TSEN-STREP wt complexes were
624 cloned into pBIG2ab and pBIG1a expression vectors, respectively, using the biGBac cloning system
625 ⁶². TSEN2^{H377A} and TSEN34^{H255A} point mutants were generated using the Q5 site-directed
626 mutagenesis kit (New England Biolabs) prior to assembly into biGBac vectors, generating both the
627 TSEN/CLP1-FLAG (TSEN2^{H377A}) and TSEN/CLP1-FLAG (TSEN34^{H255A}) pBIG2ab constructs.

628

629 Yeast and human pre-tRNA genes were amplified by PCR from genomic DNA of *Saccharomyces*
630 *cerevisiae* strain S288C and human embryonic kidney (HEK293) cells, respectively. Pre-tRNA
631 sequences were optimized for *in vitro* transcription (GG at the starting position, CC at pairing position
632 in acceptor stem) and flanked by a preceding T7 promoter sequence and a *Bst*NI cleavage site at the
633 3' end of each pre-tRNA. DNA fragments were cloned into a pUC19 vector via sticky end ligation using
634 *Bam*HI and *Hind*III restriction sites. Mature tRNA sequences were obtained by deleting the intron
635 sequence using the Q5 Site-Directed Mutagenesis kit (New England Biolabs). All constructs in this
636 study were verified by Sanger sequencing.

637

638 **Production and purification of human TSEN complexes.** Recombinant human TSEN complexes
639 were overexpressed in *Spodoptera frugiperda* (*Sf*) 21 cells essentially as described ^{32,61,63}. In brief,
640 transfer plasmids encoding TSEN subunits were created by Cre-mediated recombination and
641 recombinant baculoviral BACs were generated by Tn7 transposition in *Escherichia coli* DH10EMBacY
642 cells (Geneva Biotech). *Sf*21 cells were grown in Sf-900 II SFM medium (Thermo Fischer Scientific),
643 transfected with recombinant EMBacY BACs using X-tremeGENE DNA Transfection Reagent
644 (Roche), and incubated for 72 h at 28 °C. Recombinant initial baculoviruses (V_0) were harvested from
645 cell supernatants and used for production of amplified baculovirus (V_1) in *Sf*21 suspension cultures at
646 a multiplicity of infection (MOI) < 1. Typically, TSEN complexes were produced in 1.6 liters of *Sf*21
647 suspension culture at a cell density of 1×10^6 cells ml⁻¹ by infection with 0.5-1% (v/v) of V_1 baculovirus
648 supernatant. 72 h post cell proliferation arrest, insect cells were harvested by centrifugation at 800g for
649 5 min. Cell pellets were flash-frozen in liquid nitrogen and stored at -80 °C until further use.

650 Insect cell pellets were resuspended in 10 ml of lysis buffer comprising 50 mM HEPES-NaOH, pH 7.4,
651 400 mM NaCl, 10 mM imidazole, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine,
652 per 100 ml expression volume and lysed by sonication. Lysates were cleared by centrifugation at
653 37,000 rpm for 40 min in a Type 45 Ti fixed-angle rotor (Beckman Coulter). Pre-equilibrated Ni²⁺-

654 nitrilotriacetic acid (NTA) agarose resin (Thermo Fisher Scientific) was added to the soluble fraction
655 and incubated for 45 min at 4 °C under agitation. Agarose resin was recovered by centrifugation and
656 washed extensively in lysis buffer without protease inhibitors. Bound proteins were eluted in 50 mM
657 HEPES-NaOH, pH 7.4, 400 mM NaCl, 250 mM imidazole. Eluates of immobilized metal ion affinity
658 chromatography (IMAC) were diluted to 150 mM NaCl and loaded onto a HiTrap Heparin HP column
659 (GE Healthcare). Protein complexes were eluted by a linear salt gradient from 150 mM to 2 M NaCl.
660 TSEN complexes were subjected to TEV protease cleavage (1:50 protease to protein mass ratio) at
661 4 °C to remove the His-tag, concentrated by ultrafiltration using Amicon Ultra centrifugal filters (Merck)
662 with a molecular weight cut-off (MWCO) of 30 kDa and polished by size exclusion chromatography on
663 a Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated in 50 mM HEPES-NaOH,
664 pH 7.4, 400 mM NaCl. Peak fractions were pooled, concentrated by ultrafiltration, and flash-frozen in
665 liquid nitrogen after supplementation with 15% (v/v) glycerol.

666 TSEN15–34 was typically purified from 1.6 liters of infected *Sf21* suspension culture as stated above
667 but leaving out the heparin chromatography step. IMAC eluates were buffer exchanged into 25 mM
668 HEPES-NaOH, pH 7.4, 400 mM NaCl on a PD-10 desalting column (GE Healthcare), supplemented
669 with TEV protease (1:50 protein to protease mass ratio), concentrated by ultrafiltration using Amicon
670 Ultra (10 kDa MWCO) centrifugal filters (Merck) and polished on a Superdex 200 Increase 10/300 GL
671 column (GE Healthcare) in 25 mM HEPES-NaOH, pH 7.4, 500 mM NaCl. Peak fractions were pooled,
672 concentrated at room temperature to 25 mg ml⁻¹ by ultrafiltration, and diluted to 250 mM NaCl and a
673 final protein concentration of 12 mg ml⁻¹ for crystallization trials.

674 For two-color pre-tRNA cleavage assays, viral bacmids encoding wt TSEN-STREP, wt TSEN/CLP1-
675 FLAG, TSEN/CLP1-FLAG (TSEN2^{H377A}) and TSEN/CLP1-FLAG (TSEN34^{H255A}) pBIG2ab constructs
676 were generated using the Tn7 transposition system in DH10EMBacY cells. The resulting bacmids
677 were transfected into *Sf9* insect cells using cellfectin II (Gibco). Virus was harvested after 3 days and
678 used to further amplify the viral concentration in a larger *Sf9* cell culture. Following amplification,
679 protein complexes were expressed in High Five cells for 72 hours at 28 °C and 130 rpm which were
680 subsequently harvested by centrifugation at 1,000 x g. Cell pellets were resuspended in purification
681 buffer comprising 20 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM MgCl₂ and lysed using multiple passes
682 through a dounce homogenizer followed by sonication. Lysate was cleared via centrifugation at 28,000
683 x g for 40 min at 4 °C followed by filtration through a 0.45 µm filter. Purification of TSEN/CLP1-FLAG,
684 TSEN/CLP1-FLAG (TSEN2^{H377A}) and TSEN/CLP1-FLAG (TSEN34^{H255A}) constructs was carried out via

685 FLAG purification, using the FLAG tag carried by the CLP1 subunit. Lysate was incubated with anti-
686 DYKDDDDK G1 affinity beads (Genscript) for 3 hours at 4 °C and washed with 20 column volumes of
687 purification buffer. Recombinant protein was eluted using 20 column columns purification buffer
688 supplemented with 1 µM DYKDDDDK FLAG peptide (Genscript). Affinity purification of the TSEN-
689 STREP construct was carried out using the STREP tag carried by the TSEN2 subunit. Cleared lysate
690 was loaded onto a StrepTrap HP column (GE Healthcare) pre-equilibrated with purification buffer.
691 Protein was eluted using purification buffer supplemented with 5 mM D-desthiobiotin (Sigma).
692 Following affinity purification, protein-containing fractions were pooled and loaded onto a HiTrap Q
693 column. Protein complexes were eluted in a linear gradient from 150 mM to 2 M NaCl in 20 mM
694 HEPES, pH 8.0, 1 mM MgCl₂. TSEN-containing fractions were pooled and loaded onto a Superose 6
695 Increase 10/300 GL column (GE Healthcare) pre-equilibrated in purification buffer. Purified TSEN
696 complexes were analyzed by SDS-PAGE and western blotting.

697 For overproduction of heterotetrameric TSEN-SBP and TSEN-SBP (TSEN15^{H116Y}), adherent human
698 embryonic kidney (HEK) 293T cells were transfected with the expression plasmids with branched
699 polyethyleneimine (PEI, Sigma-Aldrich). In detail, 4 x 10⁶ HEK293T cells were seeded the day before
700 transfection in 100 mm dishes in DMEM medium (Gibco Life Technologies) with 10% fetal bovine
701 serum (FBS, Capricorn Scientific) and incubated at 37 °C, 5% CO₂ and 90% humidity. After 24 h, cells
702 were transfected with 13 µg of DNA and a 1:4 ratio of PEI per 100 mm dish. Transfected cells were
703 further incubated for 48 h, detached by addition of Trypsin-EDTA (Sigma Aldrich) and harvested by
704 centrifugation at 500 x g for 5 min. The cell pellets were flash frozen in liquid nitrogen and stored at -
705 80 °C until further use. Frozen cell pellets were thawed and resuspended in 1 ml of lysis buffer
706 containing 50 mM HEPES-NaOH, pH 7.4, 400 mM NaCl, 0.5 mM PMSF, 1.25 mM benzamidine, per
707 100 mm dish and lysed by sonication. Lysates were cleared by centrifugation at 20,817 x g for 1 h.
708 Pre-equilibrated High Capacity Streptavidin agarose resin (Pierce) was added to the soluble fraction
709 and incubated for 1 h at 4 °C under agitation. Agarose resin was recovered by centrifugation and
710 washed extensively in lysis buffer without protease inhibitors. Bound proteins were eluted in 50 mM
711 HEPES-NaOH, pH 7.4, 400 mM NaCl, 2.5 mM biotin. TSEN complex eluates were subjected to TEV
712 protease cleavage (1:20 protease to protein mass ratio) at 4 °C to remove the SBP-tag and polished
713 by size exclusion chromatography on a Superdex 200 Increase 3.2/300 column (GE Healthcare)
714 equilibrated in 50 mM HEPES-NaOH, pH 7.4, 400 mM NaCl. Peak fractions were pooled and
715 subjected to pre-tRNA cleavage assays and differential scanning fluorimetry.

716

717 **Native mass spectrometry.** The buffer of purified TSEN complexes (50 μl at 1.09 mg ml^{-1} for wt
718 TSEN and 1.88 mg ml^{-1} for wt TSEN/CLP1) was exchanged for 200 mM ammonium acetate buffer, pH
719 7.5, using 30 kDa MWCO centrifugal filter devices (Vivaspin, Sartorius). Native MS experiments were
720 performed on a Quadrupole Time-of-flight (Q-ToF) Ultima mass spectrometer modified for
721 transmission of high mass complexes (Waters, Manchester, UK)⁶⁴. For data acquisition, 3-4 μl of the
722 sample were loaded into gold-coated capillary needles prepared in-house⁶⁵. Mass spectrometric
723 conditions were capillary voltage, 1.7 kV; cone voltage, 80 V; RF lens voltage, 80 V; collision energy,
724 20 V; Aperature3, 13.6. Mass spectra were processed using MassLynx 4.1. At least 100 scans were
725 combined. Acquired mass spectra were calibrated externally using 100 mg ml^{-1} cesium iodide solution.
726 At least 100 scans were combined. Mass spectra were processed in MassLynx and analyzed using
727 Massign⁶⁶.

728

729 **Phosphoprotein analysis.** To analyze the phosphorylation state of TSEN subunits, 50 μg of purified
730 protein complexes were treated with 2,000 U of Lambda Protein Phosphatase (New England Biolabs)
731 in 200 μl dephosphorylation buffer (50 mM HEPES-NaOH, pH 7.4, 400 mM NaCl, 1 mM DTT, 1 mM
732 MnCl_2) for 2 h at 30 °C. Untreated and dephosphorylated complexes were analyzed via SDS-PAGE.
733 Gels were stained with ProQ Diamond Phosphoprotein Gel Stain (Thermo Fisher Scientific) according
734 to the manufacturer's instructions and imaged on a Typhoon Bioimager (GE Healthcare) at excitation
735 and emission wavelengths of 532 nm and 560 nm, respectively. Imaged gels were subsequently
736 stained with InstantBlue Coomassie (Expedeon).

737

738 **Nuclear Extracts.** To assay pre-tRNA splicing using patient fibroblasts, we prepared nuclear extracts.
739 Cells from at least four confluent 15 cm dishes were trypsinized, the cell pellet washed once with PBS
740 and spun for 2 min at 1,200 rpm. The pellet was re-suspended in 1 ml 1 x PBS and transferred to a
741 1.5 ml tube. The tubes were centrifuged for 5 min at 1,200 rpm. The pellet was re-suspended in one
742 volume Buffer A (10 mM HEPES-KOH pH 8.0, 1 mM MgCl_2 , 10 mM KCl, 1 mM DTT) and incubated for
743 15 min on ice. A 1-ml syringe (fitted with a 0.5 mm x 16 mm needle) was filled with Buffer A and
744 thereafter fully displaced by the plunger to remove all the remaining air within the syringe. Cells were
745 lysed by slowly drawing the suspension into the syringe followed by rapidly ejecting against the tube
746 wall. This step was repeated five times for complete lysis to occur. The sample was then spun for 20 s

747 at 13,000 rpm. The pellet was re-suspended in two-thirds of one packed cell volume in Buffer C (20
748 mM HEPES-KOH, pH 8.0, 1.5 mM MgCl₂, 25 % (v/v) glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.1 mM
749 PMSF, 1 mM DTT) and incubated on ice with stirring for 30 min. The suspension was centrifuged for 5
750 min at 12,000 rpm. The supernatant (corresponding to nuclear extracts) was dialyzed for 1 h against
751 30 mM HEPES-KOH, pH 7.4, 100 mM KCl, 5 mM MgCl₂, 10 % (v/v) glycerol, 1 mM DTT, 0.1 mM
752 AEBSF using dialysis membranes (Millipore 'V' series membrane). Afterwards, protein concentrations
753 were determined (BioRad Bradford reagent), normalized using dialysis buffer and immediately used
754 for enzymatic assays or snap-frozen and stored at -80 °C.

755

756 **Pre-tRNA cleavage assays.** For non-radioactive assays, pUC19 vectors encoding S.c. pre-
757 tRNA^{Phe}_{GAA}2-2, human pre-tRNA^{Tyr}_{GTA8}-1, S.c. tRNA^{Phe}_{GAA}2-2 and human tRNA^{Tyr}_{GTA8}-1 were
758 linearized using *Bst*NI and template DNA was isolated by agarose gel electrophoresis. RNA
759 substrates were produced by run-off *in vitro* transcription using T7 RNA polymerase (New England
760 Biolabs) and purified via anion exchange chromatography as described before with slight
761 modifications^{36,67}. Briefly, 1 µg ml⁻¹ of template DNA was mixed with 1000 U ml⁻¹ of T7 polymerase
762 and 1.5 mM of each rNTP (New England Biolabs) in 40 mM Tris-HCl, pH 7.9, 9 mM MgCl₂, 2 mM
763 spermidine, 1 mM DTT, and incubated for 4 h at 37 °C. To isolate transcribed RNAs, the reaction
764 mixture was diluted in a 1:1 ratio (v/v) with AEX buffer comprising 50 mM sodium phosphate, pH 6.5,
765 0.2 mM EDTA, and loaded onto a HiTrap DEAE FF column (GE Healthcare) equilibrated in AEX buffer
766 and eluted by a linear gradient from 0 to 700 mM NaCl. RNA containing fractions were analyzed via
767 denaturing Urea-PAGE with subsequent toluidine blue staining. RNAs were concentrated by
768 ultrafiltration using Amicon Ultra 3 MWCO centrifugal filters (Merck) and stored at -20 °C. 1 µg TSEN
769 complexes were mixed with the respective RNA in a 1:5 molar ratio in 50 mM HEPES-NaOH, pH 7.4,
770 100 mM NaCl, 2 mM MgCl₂, 1 mM DTT in a 20 µl reaction volume and incubated at 37 °C for 45 min.
771 Reactions were stopped by the addition of a 2x RNA loading buffer (95% formamide, 0.02% SDS,
772 1 mM EDTA) and incubation at 70 °C for 10 min. Reaction products were separated by denaturing
773 Urea-PAGE and visualized by toluidine blue staining.

774 For pre-tRNA cleavage assays using radioactive probes, S.c. pre-tRNA^{Phe}_{GAA} (plasmid kindly provided
775 by C. Trotta) was transcribed *in vitro* using the T7 MEGAscript kit (Ambion) including 1.5 MBq
776 [α ³²P]-guanosine-5'-triphosphate (111 TBq/mmol, Hartmann Analytic) per reaction. The pre-tRNA was
777 resolved in a 10% denaturing polyacrylamide gel, visualized by autoradiography and passively eluted

778 from gel slices overnight in 0.3 M NaCl. RNA was precipitated by the addition of three volumes of
779 ethanol and dissolved at 0.1 μ M in buffer containing 30 mM HEPES-KOH, pH 7.3, 2 mM MgCl₂, 100
780 mM KCl. To assess pre-tRNA splicing, one volume of 0.1 μ M body labeled *S. cerevisiae* pre-tRNA^{Phe},
781 pre-heated at 95°C for 60 sec and incubated for 20 min at room temperature, was mixed with four
782 volumes of reaction buffer (100 mM KCl, 5.75 mM MgCl₂, 2.5 mM DTT, 5 mM ATP, 6.1 mM
783 Spermidine-HCl pH 8.0 (Sigma), 100 U ml⁻¹ RNasin RNase inhibitor (Promega)). Equal volumes of this
784 reaction mixture and cell extracts with a total protein concentration of 6 mg ml⁻¹ were mixed and
785 incubated at 30°C. At given time points, 5 μ l of the mix were deproteinized with proteinase K, followed
786 by phenol/chloroform extraction and ethanol precipitation. Reaction products were separated on a
787 10% denaturing Urea-polyacrylamide gel, and tRNA exon formation was monitored by
788 phosphorimaging. Quantification of band intensities was performed using ImageQuant software.

789 For two-colored pre-tRNA cleavage assays, 5'-cyanine5 (Cy5) and 3'-Fluorescin (FITC) labelled
790 human pre-tRNA^{Tyr}_{GTA} 3-1 was purchased from Dharmacon. Pre-tRNA was resuspended in nuclease-
791 free water (New England Biolabs) at 100 μ M stock concentration. Prior to use, pre-tRNA stock was
792 diluted 1 in 2 into RNA loading buffer (New England Biolabs) and separated on a 10% acrylamide
793 urea-TBE denaturing gel, with the band corresponding to pre-tRNA excised. The excised bands were
794 crushed using a pipette tip in a 1.5 ml Eppendorf tube and incubated in 300 μ l of 20 mM Tris-HCl, pH
795 8.0, 250 mM KCl, overnight at room temperature. Gel fragments were removed by centrifugation at
796 17,000 \times g. Supernatant was transferred to a fresh Eppendorf tube and tRNA precipitated through
797 addition of 4 μ l RNA-grade glycogen (Thermo Fisher Scientific) and 1 ml of 100% isopropanol.
798 Precipitate was collected through centrifugation at 17,000 \times g and the pellet washed in 75% ethanol.
799 The resulting pellet was resuspended in nuclease free water (New England Biolabs) and RNA
800 quantified through measurement of A₂₆₀ prior to storage at -80 °C. Purified pre-tRNA was diluted 1:10
801 in cleavage buffer (20 mM HEPES, pH 8.0, 100 mM KCl, 2.5 mM Dithiothreitol, 5 mM Spermidine-HCl,
802 5 mM MgCl₂). Pre-tRNA was incubated at 90 °C for 1 min and cooled to room temperature for 20 min
803 to ensure folding. 20 pmols of folded pre-tRNA substrate was incubated with a final concentration of 5
804 U ml⁻¹ RNasin plus inhibitor (Promega), 5 mM ATP and 8 pmols of TSEN complex in a final reaction
805 volume of 20 μ l for 1 h at 30 °C. RNA was extracted through addition of 150 μ l of cleavage buffer
806 followed by 150 μ l of 25:24:1 phenol:chloroform:isoamyl alcohol solution (Thermo Fisher Scientific).
807 Samples were mixed and centrifuged at 17,000 \times g for separation of RNA and protein layers. The top
808 layer was transferred to a fresh Eppendorf tube and RNA precipitated through addition of 4 μ l RNA-

809 grade glycogen (Thermo Fisher Scientific) and 1 ml of 100% isopropanol. Precipitated RNA was
810 centrifuged at 17,000 x g and the pellet washed in 75% ethanol solution. RNA was resuspended in 10
811 μ l of nuclease free water (New England Biolabs). 5 μ l of RNA solution was suspended in 5 μ l of RNA
812 loading buffer (95% (v/v) formamide, 10 mM EDTA). Samples were boiled at 95 °C for 10 min prior to
813 loading on a 10% acrylamide urea-TBE denaturing gel. Results were visualized using a Typhoon FLA
814 9000 (GE Healthcare).

815

816 **Fluorescent 3' end labeling of RNA.** RNAs were labeled site-specifically at their 3' ends using
817 periodate chemistry and a hydrazide derivate of cyanine5 (Cy5) fluorophore (Lumiprobe) as described
818 previously⁶⁸. Typically, 5 μ M of RNA were mixed with 2.5 μ l of 400 mM NaIO₄, 13.33 μ l of 3 M KOAc,
819 pH 5.2, in a total volume of 400 μ l and incubated for 50 min on ice to oxidize the 2'-3' diols of the
820 terminal ribose to aldehydes. Oxidized RNAs were ethanol precipitated and resuspended in 400 μ l of
821 diethylpyrocarbonate (DEPC)-treated water containing 1 mM of Cy5-hydrazide and 13.33 μ l of 3 M
822 KOAc, pH 5.2. After incubation at 4 °C overnight in the dark under agitation, RNA was ethanol
823 precipitated and buffer exchanged to fresh DEPC-treated water using a Zeba Spin desalting column
824 (Thermo Fisher Scientific) to remove the unreacted dye. The optical density at wavelengths of 260 nm
825 and 650 nm was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) to
826 determine the frequency of incorporation (FOI; the number of incorporated fluorophores per 1000
827 nucleotides) and labeling efficiency.

828

829 **tRNA pull-down assays.** 25 μ l of the monoclonal α -His antibody (Catalog-ID H1029, Sigma-Aldrich)
830 were mixed with 100 μ l buffer comprising 50 mM HEPES-NaOH, pH 7.4, 400 mM NaCl (HS buffer)
831 and coupled to 25 μ l of Protein G Agarose (Thermo Fischer Scientific) for 30 min at 4 °C under
832 agitation. Beads were washed twice with 1 ml HS buffer (1500x g, 3 min) and incubated with 8 μ g of
833 inactive His-tagged tetrameric TSEN complex (His₆-tag on TSEN15) in a total volume of 100 μ l for 1h
834 at 4 °C. After washing three times with 150 μ l buffer comprising 50 mM HEPES-NaOH, pH 7.4, 100
835 mM NaCl (LS buffer), 100 ng of Cy5-labeled RNA were added to the beads and incubated for 1h at 4
836 °C under agitation. After binding, beads were washed again 3x in 150 μ l LS buffer and bound
837 macromolecules were eluted by addition of 5 μ l 4x SDS loading buffer plus 20 μ l LS buffer and
838 incubation at 70 °C for 3 min. Eluted components were separated by SDS-PAGE and visualized by in-
839 gel fluorescence on an ImageQuant LAS 4000 system and immunoblotting. As positive and negative

840 controls, the pull-down assay was performed without the addition of inactive tetrameric TSEN to the
841 antibody-coupled beads or in the presence of an excessive amount (2 μg) of unlabeled RNA,
842 respectively.

843

844 **Electrophoretic mobility shift assays.** 3'-Cy5-labelled pre-tRNA substrates (10 nM final) were mixed
845 with increasing amounts of inactive tetrameric TSEN complexes (typically 10 nM up to 1 μM) in a total
846 volume of 20 μl EMSA buffer comprising 50 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 1 mM DTT,
847 4% (v/v) glycerol in DEPC-treated water. After incubation for 30 min on ice in the dark, samples were
848 loaded onto a 4% Tris-Borate-EDTA native polyacrylamide gel, which had been pre-run for 15 min at
849 180V in 0.5x TBE buffer. Free and complexed RNAs were separated for 1 h at 180V at 4 $^{\circ}\text{C}$ in the
850 dark. In-gel fluorescence was detected on an ImageQuant LAS4000 or Typhoon 9400 device (GE
851 Healthcare) to visualize labeled RNA.

852

853 **Fluorescence anisotropy measurements.** Fluorescence anisotropy measurements were conducted
854 on a Fluorolog-3 spectrofluorometer (Horiba) equipped with automated polarization filters at a
855 controlled temperature of 22 $^{\circ}\text{C}$. 120 μl of Cy5-labeled RNA with a concentration of 70 nM in 50 mM
856 HEPES-NaOH, pH 7.4, 100 mM NaCl were titrated with TSEN complexes (1.5 μM stock) in a micro
857 fluorescence cuvette. To avoid dilution effects, the titrant solution contained identical concentrations of
858 the labeled RNAs. After each titration step, the solution was mixed carefully and fluorescence
859 anisotropy was continuously assessed in 15 s increments over a period of 450 s. Anisotropy values of
860 each data point were averaged, plotted in dependency of the protein concentration, and dissociation
861 constants (K_D) were obtained by non-linear curve fitting according to a quadratic equation in Prism 5
862 (GraphPad Software) to compensate for non-negligible receptor concentrations. Experiments were
863 performed in at least biological duplicates.

864

865 **Differential scanning fluorimetry.** TSEN complexes were mixed to a final concentration of 1 or 3 μM
866 with 4x SYPRO Orange (Merck) stock in 50 mM HEPES-NaOH, pH 7.4, 400 mM NaCl. Protein
867 unfolding was assessed on a PikoReal96 thermocycler (Thermo Fisher) by measuring SYPRO
868 Orange fluorescence over a temperature gradient from 20 – 95 $^{\circ}\text{C}$ (temperature increment 0.2 $^{\circ}\text{C}$,
869 hold time 10 s) in a 96-well plate format. Values of technical triplicates were averaged, blank
870 corrected, and apparent unfolding temperatures were determined as the half maximum of a sigmoidal

871 Boltzmann fit in Prism 8 (GraphPad Software). Unfolding temperatures of PCH mutants were
872 compared to wt TSEN complex in technical triplicates to assess their impact on stability and are
873 representative of biological duplicates.

874

875 **Size exclusion chromatography multi-angle light scattering.** Multi-angle light scattering coupled
876 with size exclusion chromatography (SEC-MALS) was done using a Superdex200 Increase 10/300 GL
877 column (GE Healthcare) at a flow rate of 0.5 ml min⁻¹ on an HPLC system composed of PU-2080
878 pumps, PU-2075 UV detector and degaser (JASCO) connected to a 3-angle miniDAWN TREOS light
879 scattering detector (Wyatt Technology Corporation) and an Optilab T-rEX refractive index detector
880 (Wyatt Technology Corporation). A BSA sample (400 µg) for calibration and 330 µg of TSEN15–34
881 complex at a concentration of 1.65 mg ml⁻¹ were run on a pre-equilibrated column in 25 mM HEPES-
882 NaOH, pH 7.5, 250 mM NaCl filtered through a 0.1 µm pore size VVLP filter (Millipore). The refractive
883 index increment (dn/dc) of the TSEN15–34 complex was predicted to 0.188 ml g⁻¹ using its amino acid
884 composition⁶⁹. The extinction coefficient of the TSEN15–34 complex at 280 nm was calculated using
885 the ProtParam server (<https://web.expasy.org>). Data analysis was accomplished using the ASTRA
886 software package (Wyatt Technology Corporation) across individual peaks using the Zimm's model for
887 data fitting⁷⁰.

888

889 **Limited proteolysis.** Purified, full-length TSEN15–34 complex (0.9 mg ml⁻¹) was incubated with
890 trypsin (15 µg ml⁻¹) in 50 mM HEPES-NaOH, pH 7.4, 400 mM NaCl for 1h at room temperature. The
891 reaction was stopped by the addition of 1 mM PMSF and the proteolyzed complex was applied to a
892 Superdex 200 Increase 10/300 GL column (GE Healthcare) equilibrated in 50 mM HEPES-NaOH, pH
893 7.4, 400 mM NaCl. Peak fractions were run out on denaturing 11% SDS-PAGE and visualized by
894 staining with InstantBlue Coomassie (Expedeon).

895

896 **Denaturing mass spectrometry.** The buffer of TSEN15–34 complexes (10 µl at 1.05 mg ml⁻¹ in 10
897 mM HEPES, pH 7.4, 400 mM NaCl, 0.3x Protease Inhibitor) derived from limited proteolysis was
898 exchanged for 200 mM ammonium acetate, pH 7.5, using 3 kDa MWCO Amicon centrifugal filters
899 (Merck Millipore). For protein denaturation, isopropanol was added to a final concentration of 1% (v/v).
900 Subsequently, the sample was analyzed by direct infusion on a Q Exactive Plus Hybrid Quadrupole-
901 Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a Nanospray Flex ion source

902 (Thermo Fisher Scientific). For this, 2–3 μ l were loaded into gold-coated capillary needles prepared in-
903 house. MS spectra were recorded in positive ion mode using the following settings: capillary voltage, 2
904 kV; capillary temperature, 250 °C; resolution, 70,000; S-lens RF level, 50; max injection time, 50 ms;
905 automated gain control, $1 \cdot 10^6$; MS scan range 1000 – 6000 m/z . Approximately 300 scans were
906 combined and the peaks were assigned manually.

907

908 **Identification of proteins and protein fragments.** Gel electrophoresis was performed using 4-12%
909 NuPAGE Bis-Tris gels according to manufacturer's protocols (NuPAGE system, Thermo Fisher
910 Scientific). Protein gel bands were excised, and the proteins were hydrolyzed as described previously
911 ⁷¹. Briefly, proteins were reduced with 10 mM dithiothreitol, alkylated with 55 mM iodoacetamide, and
912 hydrolyzed with Trypsin (Roche). Extracted peptides were dissolved in 2% (v/v) acetonitrile, 0.1% (v/v)
913 formic acid and separated using a DionexUltiMate 3000 RSLCnano System (Thermo Fisher
914 Scientific). For this, the peptides were first loaded onto a reversed-phase C18 pre-column (μ -
915 Precolumn C18 PepMap 100, C18, 300 μ m I.D., particle size 5 μ m pore size; Thermo Fisher
916 Scientific). 0.1% formic acid (v/v) was used as mobile phase A and 80% (v/v) acetonitrile, 0.1% (v/v)
917 formic acid, as mobile phase B. The peptides were then separated on a reversed-phase C18
918 analytical column (HPLC column Acclaim® PepMap 100, 75 μ m I.D., 50 cm, 3 μ m pore size; Thermo
919 Fisher Scientific) with a gradient of 4 - 90% B over 70 min at a flow rate of 300 nl min^{-1} . Peptides were
920 directly eluted into a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher
921 Scientific). Data acquisition was performed in data-dependent and positive ion modes. Mass
922 spectrometric conditions were: capillary voltage, 2.8 kV; capillary temperature, 275 °C; normalized
923 collision energy, 30%; MS scan range in the Orbitrap, m/z 350–1600; MS resolution, 70,000;
924 automatic gain control (AGC) target, $3e6$. The 20 most intense peaks were selected for fragmentation
925 in the HCD cell at an AGC target of $1e5$. MS/MS resolution, 17,500. Previously selected ions were
926 dynamically excluded for 30 s and singly charged ions and ions with unrecognized charge states were
927 also excluded. Internal calibration of the Orbitrap was performed using the lock mass m/z 445.120025
928 ⁷². Obtained raw data were converted to .mgf files and were searched against the SwissProt database
929 using the Mascot search engine 2.5.1 (Matrix Science).

930

931 **Crystallization, structure determination, and validation of a minimal TSEN15-34 complex.**
932 Crystals of truncated TSEN15–34 complex (TSEN15 residues 23-170 and TSEN34 residues 208-310)

933 were refined manually at 18°C by mixing equal volumes of protein solution containing 12–15 mg ml⁻¹
934 TSEN15–34 in 25 mM HEPES-NaOH, pH 7.4, 250 mM NaCl, and crystallization solution containing
935 0.1 M Imidazole/MES, pH 6.5, 20% PEG3350, and 0.2 M MgCl₂ in a vapor diffusion setup. Crystals
936 were cryoprotected by adding 20% (v/v) glycerol to the reservoir solution and flash-frozen in liquid
937 nitrogen. Diffraction data were collected at 100 K to a resolution of 2.1 Å on beamline P14 of the
938 Deutsches Elektronen-Synchrotron (DESY) and were processed and scaled using the X-ray Detector
939 Software (XDS) package ⁷³. Crystals belong to the monoclinic space group P2₁ with two complexes in
940 the asymmetric unit. The structure of TSEN15-34 was solved by molecular replacement with Phaser ⁷⁴
941 within the Phenix software package ⁷⁵ using a truncated poly-Ala model of the *Aeropyrum pernix*
942 endonuclease (residues 83-169 of the I chain and residues 93-168 of the J chain) (PDB 3P1Z) ⁷⁶ as a
943 search model. The structures of the two domain-swapped TSEN15-34 dimers were manually built with
944 Coot ⁷⁷ and refined with Phenix ⁷⁸ with good stereochemistry. Statistical quality of the final model was
945 assessed using the program Molprobit ⁷⁹. Structure figures were prepared using PyMOL.

946

947 **Cell Culture.** Human fibroblasts were cultured at 37 °C, 5% CO₂ in Dulbecco's modified Eagle's
948 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 100 U ml⁻¹ penicillin and 100 µg
949 ml⁻¹ streptoMycin sulfate (Lonza). Cells were split and/or harvested at 80-90% confluency using 0.05%
950 Trypsin–EDTA.

951

952 **Northern blotting.** Isolation of total RNA from cell lines was performed using the Trizol Reagent
953 (Invitrogen) according to the manufacturer's instructions. Typically, 4-5 µg of RNA was separated in a
954 10% denaturing Urea-polyacrylamide gel (20 x 25 cm; Sequagel, National Diagnostics). The RNA was
955 blotted on Hybond-N+ membranes (GE Healthcare) and fixed by ultraviolet cross-linking. Membranes
956 were pre-hybridized in 5x SSC, 20 mM Na₂HPO₄, pH 7.2, 7% SDS, and 0.1 mg ml⁻¹ sonicated salmon
957 sperm DNA (Stratagene) for 1h at 80 °C (for DNA/LNA probes) or 50 °C (for DNA probes).
958 Hybridization was performed in the same buffer overnight at 80 °C (for DNA/LNA probes) or 50 °C (for
959 DNA probes) including 100 pmol of the following [5'-³²P]-labeled DNA/LNA probe (Exiqon, Denmark;
960 LNA nucleotides are indicated by “*X”): tRNA^{Ile}_{TAT}1-1 5' exon probe, 5'-TA*T AA*G TA*C CG*C GC*G
961 CT*A AC-3', or the following DNA probe: tRNA^{Ile}_{TAT}1-1 intron probe, 5'-TGC TCC GCT CGC ACT GTC
962 A-3'. Blots were subsequently washed twice at 50°C with 5x SSC, 5% SDS and once with 1x SSC, 1%
963 SDS and analyzed by phosphorimaging. Membranes were re-hybridized at 50 °C using a DNA probe

964 (5'-GCA GGG GCC ATG CTA ATC TTC TCT GTA TCG-3') complementary to U6 snRNA to check for
965 equal loading.

966

967 **Immunoprecipitation of TSEN components.** Antibodies against TSEN2, TSEN34, and TSEN54 ³⁵
968 were affinity-purified, and cross-linked to agarose beads, as described ⁸⁰. Briefly, bead-bound
969 antibodies were incubated in 20 mM dimethylphenol (DMP), 200 mM sodium tetraborate at RT and the
970 reaction was then stopped by transferring the beads to 200 mM Tris-HCl, pH 8.0. After washing 3x
971 with TBS/0.04% Triton-X-100, beads were stored at 4 °C. For immunoprecipitation (IP), total cell
972 lysates were prepared from fresh or frozen cell pellets of primary fibroblasts as described ⁸¹ Upon
973 centrifugation at 16,000 x g, clear lysates were collected, protein concentration was measured, and
974 equal amounts of total protein for each sample were used for the IPs. Upon incubation with cell lysates
975 for 90 min at 4 °C while rotating, TSEN complex-bound beads were washed as described ⁸⁰ and split
976 into two aliquots; one was used for a pre-tRNA splicing assay and the other was boiled in SDS-PAGE
977 loading buffer. Pre-tRNA splicing assay was performed as described above, omitting the proteinase K
978 treatment and the phenol/chloroform extraction and ethanol precipitation steps. Instead, aliquots were
979 collected at indicated time-points in tubes already containing an equal amount of 2 x loading buffer
980 and stored at -20 °C. Protein samples were analyzed by SDS-PAGE and immunoblotting.

981

982 **Hydro-tRNA sequencing.** tRNA sequencing was performed using the hydro-tRNAseq protocol, as
983 described previously ⁴. Briefly, total RNA from human derived fibroblasts was resolved on 12% urea-
984 polyacrylamide gel, followed by recovery of the tRNA fraction within a size window of 60-100 nt. The
985 eluted fraction was subjected to limited alkaline hydrolysis in 10 mM Na₂CO₃ and NaHCO₃ at 60 °C for
986 1 hr. The hydrolyzed RNA was dephosphorylated and rephosphorylated to reconstitute termini
987 amenable for sequential adapter ligation. Fragments of 19-35 nt were converted into barcoded cDNA
988 libraries, as described previously ⁸², and sequenced on an Illumina HiSeq 2500 instrument. Adapters
989 were trimmed using cutadapt (<http://journal.embnet.org/index.php/embnetjournal/article/view/200/458>).
990 Sequence read alignments and analysis was performed as described previously ⁴. Split read counts
991 were used for multimapping tRNA reads. Precursor tRNA reads spanned the junctions between
992 mature sequences and leaders, trailers, or introns.

993

994 **Sequence alignments.** Sequence alignments were done with Clustal Omega⁸³ and visualized using
995 ESPript 3.0⁸⁴. Alignments of pre-tRNAs and tRNAs were manually edited in Jalview⁸⁵.

996

997 **Statistical analysis.** Student's two-tailed nonpaired t tests were carried out to determine the statistical
998 significance of differences between samples. A p value less than 0.05 was considered nominally
999 statistically significant for all tests.

1000

1001 **Patient recruitment and ascertainment.** Patients suspected for PCH were submitted to the pediatric
1002 neurology of the Academic Medical Centre (AMC) for diagnostics. Primary fibroblast cell lines were
1003 generated from skin biopsies taken for diagnostic procedures. As soon as DNA diagnostics became
1004 available, patient DNA was subjected to genetic analyses. DNA sequencing confirmed the diagnosis
1005 and the mutations were confirmed in the fibroblast lines. All procedures were performed with full
1006 consent of the legal representative and approval of the Institutional Review Board (IRB).

1007

1008 **Data availability**

1009 Atomic coordinates and structure factors were deposited to the Protein Data Bank
1010 (<http://www.rcsb.org>) under accession number PDB ID 6Z9U. The mass spectrometry proteomics data
1011 were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the
1012 dataset identifier PXD019034. Hydro-tRNAseq data were deposited with the Gene Expression
1013 Omnibus (GEO) repository under accession code GSE151236. Source data for [Figs. 1-6](#) and
1014 [Extended Data Figs. 1-6](#) are provided with the paper.

1015

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1030

1031 **Author contributions**

1032 S.S., P.D., A.P., and S.T. expressed, purified, and prepared protein complexes from insect and
1033 mammalian cells. S.S., P.D., A.P., and S.P. performed biochemical assays. E.P.R. cloned and purified
1034 FLAG- and STREP-tagged TSEN complexes and performed dual-color pre-tRNA cleavage assays.
1035 S.P. and S.W. performed pre-tRNA splicing assays; Northern blots and IP experiments on human
1036 fibroblasts were performed by S.P.. M.B. and C.S. conducted MS experiments and analyzed the data.
1037 S.S. and S.T. performed crystallography experiments, collected X-ray diffraction data, and built the
1038 atomic model. F.B. generated cell lines of PCH patient-derived fibroblast. T.G. performed hydro-
1039 tRNAseq experiments and bioinformatic analyses under supervision of T.T.. J.M. and S.T conceived
1040 the project, supervised the work, and designed the experiments. S.S., P.D., and S.T. wrote the initial
1041 draft of the manuscript with input from all authors. J.M., and S.T. acquired funding.

1042

1043 **Competing interests**

1044 The authors declare no competing interests.

1045

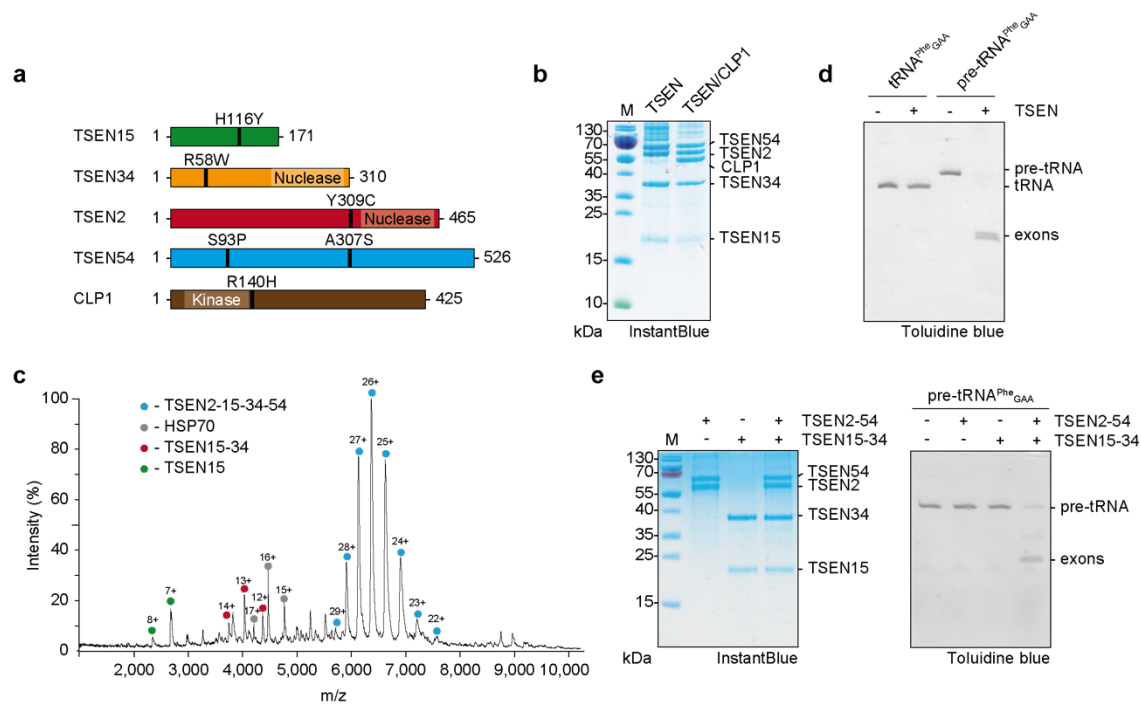
1046 **Additional information**

1047 Correspondence and requests for materials should be addressed to J.M. or S.T.

1048

1049

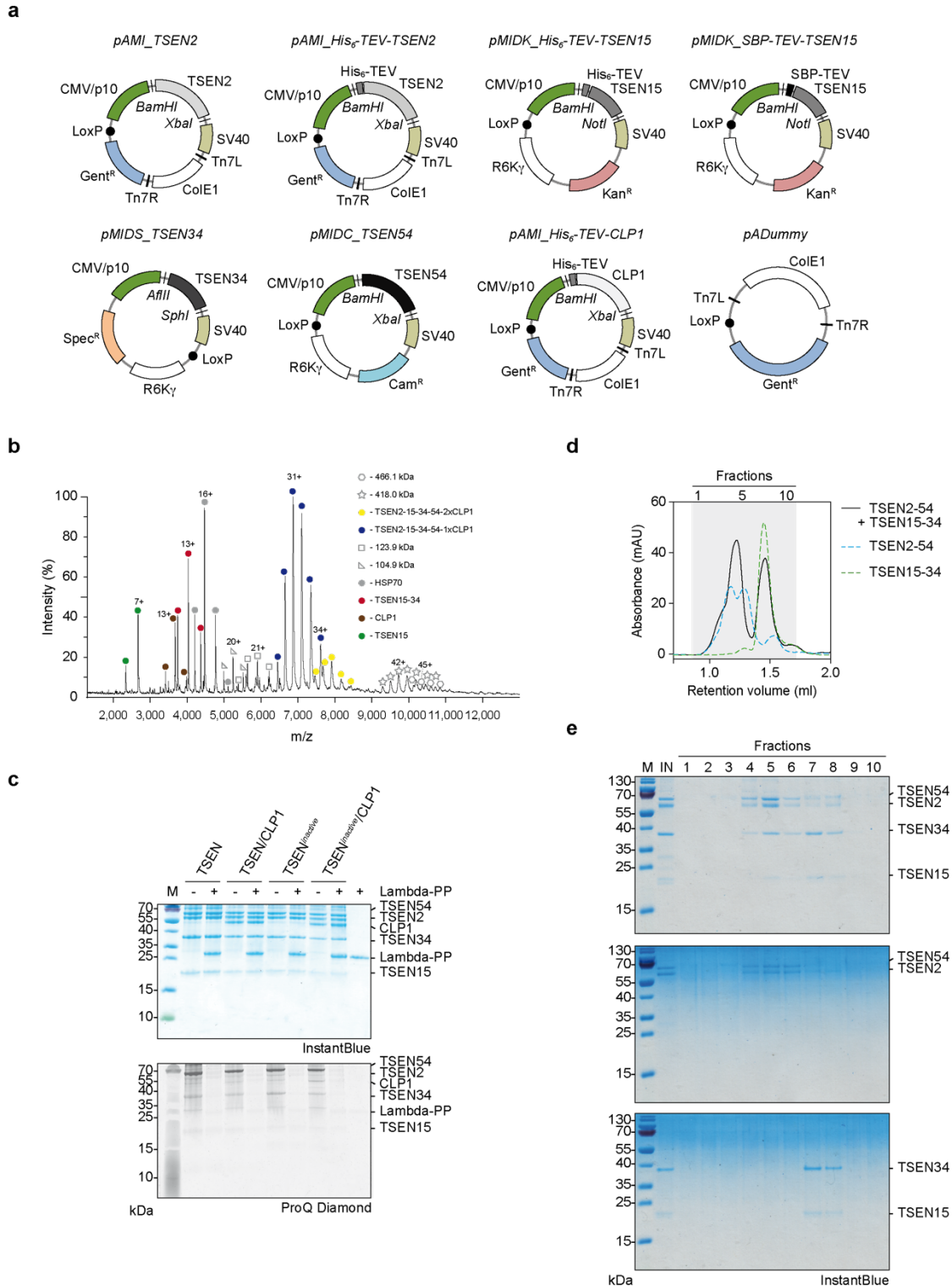
1050 **Figures**



1051

1052 **Figure 1 | Assembly and catalysis of recombinant human TSEN.** **a**, Bar diagrams of TSEN
 1053 subunits and CLP1 depicting positions of PCH mutations, predicted nuclease domains of TSEN2 and
 1054 TSEN34, and the RNA kinase domain for CLP1. Total amino acids of each protein are indicated. **b**,
 1055 SDS-PAGE of purified recombinant TSEN and TSEN/CLP1 complexes visualized by InstantBlue
 1056 staining. Protein identities and size markers are shown. **c**, Native mass spectrum of tetrameric TSEN
 1057 complex from an aqueous ammonium acetate solution. Charge states of the predominant TSEN2–15–
 1058 34–54 heterotetramer (blue circles), Heat Shock Protein (HSP) 70 (grey circles), the heterodimer
 1059 TSEN15–34 (red circles) and TSEN15 (green circles) are indicated. **d**, Pre-tRNA cleavage assay
 1060 using tetrameric TSEN complex with *Saccharomyces cerevisiae* (*S.c.*) pre-tRNA^{Phe}_{GAA} and mature
 1061 tRNA^{Phe}_{GAA}. Input samples and cleavage products were separated via Urea-PAGE and visualized by
 1062 Toluidine blue. RNA denominations are given on the right. **e**, Pre-tRNA cleavage assay with TSEN
 1063 heterodimers and *S.c.* pre-tRNA^{Phe}_{GAA}. SDS-PAGE of the indicated heterodimers and the reconstituted
 1064 TSEN tetramer is shown on the left (InstantBlue stain), Urea-PAGE of the cleavage products on the
 1065 right (Toluidine blue stain). Gels are representative of three independent experiments. Unprocessed
 1066 gels for **b**, **c** and **e** are shown in [Source Data 1](#).

1067



1068

1069 **Extended Data Fig. 1 | Biochemical characterization of recombinant TSEN and TSEN/CLP1**

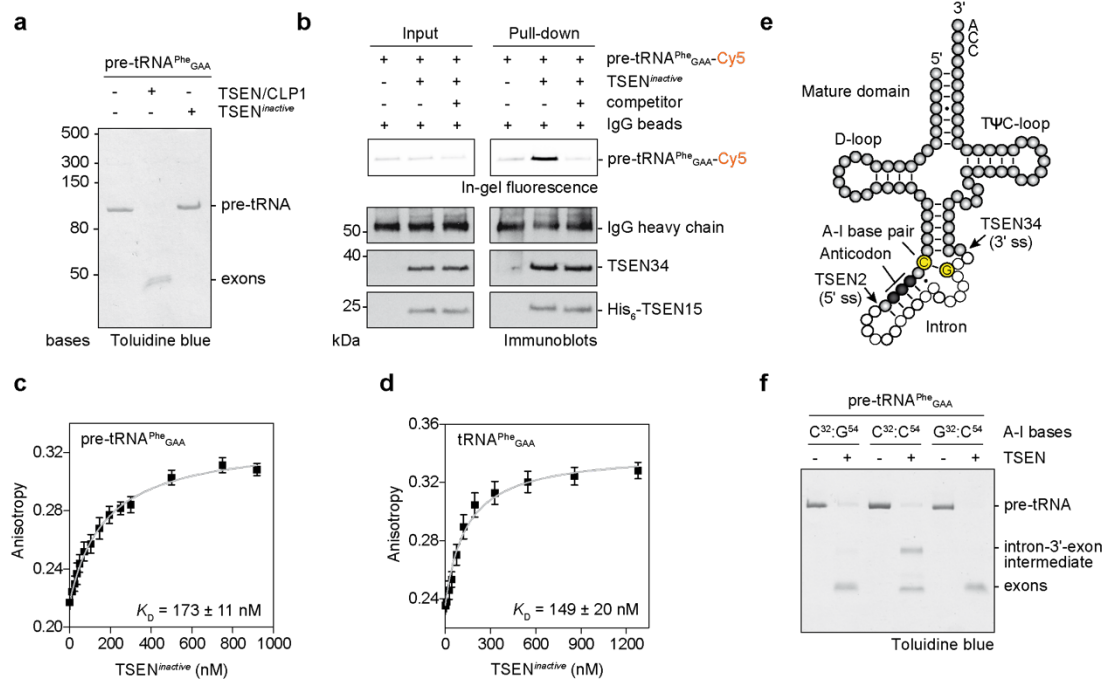
1070 **complexes.** a, Maps of modified MultiBac vectors encoding TSEN/CLP1 complex components. For

1071 expression in mammalian and insect cells, the acceptor vector pAMI and donor vectors pMIDC,

1072 pMIDK, and pMIDS carry the CMV/p10 dual promoter. Transcription is terminated by the SV40 poly-A

1073 late signal (SV40). The transposase elements Tn7L and Tn7R, the LoxP element (black dot) for Cre-

1074 mediated recombination, the origins of replication ColE1 and R6K γ , and the resistance markers for
1075 gentamicin (Gent^R), chloramphenicol (Cam^R), kanamycin, (Kan^R), and spectinomycin (Spec^R) are
1076 shown. Restriction sites, hexahistidine-tags (His₆), the Streptavidin-binding peptide-tag (SBP), and the
1077 TEV cleavage site (TEV) are indicated. **b**, Native mass spectrum of pentameric TSEN/CLP1 complex
1078 from an aqueous ammonium acetate solution. Charge states of the predominant TSEN/CLP1
1079 assembly (blue circles), a minor populated TSEN complex with two CLP1 subunits (yellow circles),
1080 monomeric CLP1 (brown circles), HSP70 (grey circles), the TSEN15–34 heterodimer (red circles) and
1081 TSEN15 (green circles) are indicated. Unidentified protein assemblies are denominated by their
1082 molecular weights. **c**, Analysis of phosphorylation states of TSEN components by the phospho-
1083 specific ProQ Diamond gel stain. The strong band at 59 kDa in the ProQ Diamond stain corresponds
1084 to TSEN54. Lambda-PP, Lambda protein phosphatase. **d**, Assembly assay with TSEN2–54 and
1085 TSEN15–34 heterodimers via size exclusion chromatography (SEC). Absorbance profiles (280 nm) of
1086 reconstituted TSEN complex (black line), and the heterodimers TSEN2–54 (blue dashed line) and
1087 TSEN15–34 (green dashed line) are shown. **e**, SDS-PAGE of SEC fractions (grey area as indicated in
1088 **d**) with subsequent InstantBlue staining. Unprocessed gels for **c** and **e** are shown in [Source Data 2](#).
1089



1090

1091 **Fig. 2 | Active involvement of the A-I base pair in coordinating pre-tRNA cleavage.** **a**, Pre-tRNA

1092 cleavage assay comparing recombinant, inactive TSEN tetramer (TSEN2^{H377A} and TSEN34^{H255A}

1093 double mutant) to the TSEN/CLP1 complex. Cleavage products are visualized by denaturing Urea-

1094 PAGE with subsequent Toluidine blue staining. RNA size markers are indicated on the left of the gel.

1095 **b**, Pull-down assay with fluorescently labeled *S.c.* pre-tRNA^{Phe_{GAA}} and inactive, tetrameric TSEN

1096 captured on protein G agarose functionalized with an α -His-antibody. Protein size markers are

1097 indicated on the left of each immunoblot, protein and RNA identities on the right. Input and co-

1098 precipitated, labeled pre-tRNAs were visualized by in-gel fluorescence, TSEN subunits and the

1099 immunoglobulin G (IgG) heavy chain by immunoblotting. The IgG heavy chain served as loading

1100 control. **c**, Thermodynamic binding parameters of fluorescently labeled *S.c.* pre-tRNA^{Phe_{GAA}} and

1101 inactive, tetrameric TSEN revealed by fluorescence anisotropy. **d**, Thermodynamic binding

1102 parameters of fluorescently labeled tRNA^{Phe_{GAA}} and inactive, tetrameric TSEN revealed by

1103 fluorescence anisotropy. **e**, Schematic depiction of a pre-tRNA molecule showing ribonucleotides

1104 belonging to the mature domain (grey spheres), the intronic region (white spheres), the anticodon

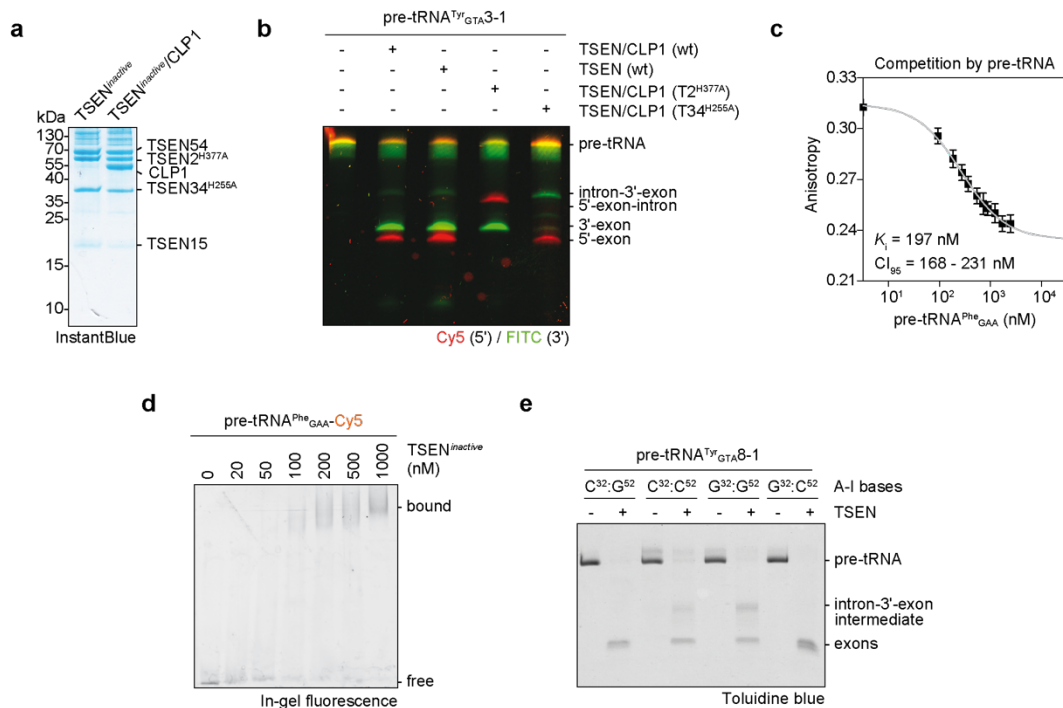
1105 (black spheres), and the A-I base pair (yellow spheres). Proposed 5' and 3' splice sites (ss) are

1106 indicated. **f**, Impact of A-I base pair mutations in *S.c.* pre-tRNA^{Phe_{GAA}} on endonucleolytic activity of

1107 tetrameric TSEN revealed by a pre-tRNA cleavage assay. C³²:G⁵⁴ – canonical A-I base pair, C³²:C⁵⁴ –

1108 disrupted A-I base pair, G³²:C⁵⁴ – inverted A-I base pair. All experiments are representatives of three

1109 independent assays. Unprocessed gels for **a**, **b** and **f** are shown in [Source Data 3](#).



1110

1111 **Extended Data Fig. 2 | Active involvement of the A-I base pair in coordinating pre-tRNA**

1112 **cleavage.** **a**, SDS-PAGE of purified, recombinant inactive TSEN and inactive TSEN/CLP1 complexes

1113 (TSEN2^{H377A} and TSEN34^{H255A} double mutant). Protein size markers and protein identities are

1114 indicated. **b**, Two-colored pre-tRNA cleavage assay with TSEN-STREP and TSEN/CLP1-FLAG wt

1115 complexes and complexes carrying the TSEN2^{H377A} (T2^{H377A}) or TSEN34^{H255A} (T34^{H255A}) substitution.

1116 RNA cleavage products were separated on a denaturing Urea-PAGE and visualized by fluorescence

1117 of cyanine5 (Cy5) and Fluorescein (FITC). **c**, Thermodynamic competition parameters deduced from

1118 fluorescence anisotropy experiments. Inactive, tetrameric TSEN bound to fluorescently labeled pre-

1119 tRNA^{Phe_GAA} was titrated with unlabeled pre-tRNA. **d**, Electrophoretic mobility shift assay with

1120 fluorescently labeled pre-tRNA^{Phe_GAA} and inactive, tetrameric TSEN (TSEN^{inactive}). Free and bound

1121 fractions of pre-tRNA were analyzed via 4% TBE native PAGE with subsequent in-gel fluorescence

1122 measurement. **e**, Impact of A-I base pair mutations in pre-tRNA^{Tyr_GTA8-1} on endonucleolytic activity by

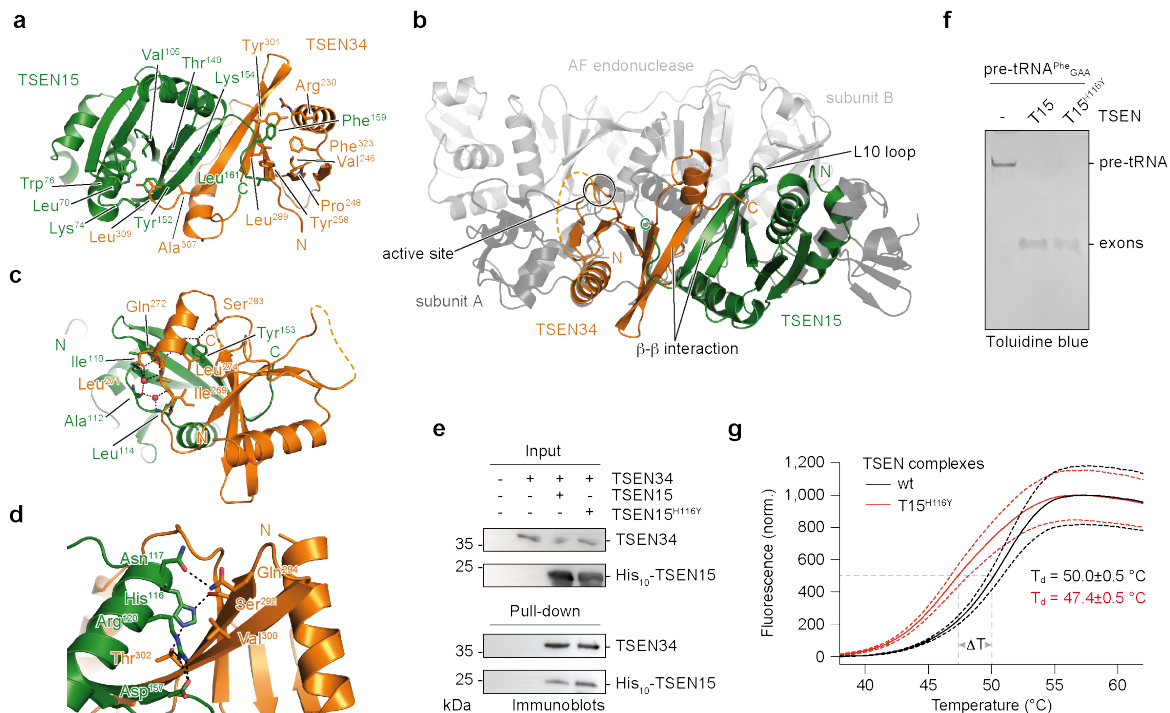
1123 tetrameric TSEN revealed by a pre-tRNA cleavage assay. CI_{95} – 95% confidence interval, C³²:G⁵² –

1124 canonical A-I base pair, C³²:C⁵² and G³²:G⁵² – disrupted A-I base pair, G³²:C⁵² – inverted A-I base pair.

1125 Panels are representatives of three independent experiments. Unprocessed gels for **a**, **b**, **d**, and **e** are

1126 shown in [Source Data 4](#).

1127



1128

1129 **Fig. 3 | Structural and functional details of the TSEN15–34 dimer interface.**

1130 structure of a TSEN15–34 complex derived from limited proteolysis experiments. TSEN15 (green) and

1131 TSEN34 (orange) are shown in cartoon representation. Key amino acids are depicted in stick

1132 representation together with amino- (N) and carboxy (C)-termini. **b**, Superposition of the TSEN15–34

1133 heterodimer and the pre-tRNA endonuclease from *Archaeoglobus fulgidus* (AF) (PDB ID 2GJW) ¹³.

1134 The position of the catalytic triad of TSEN34 (active site), the L10 loop of TSEN15 and the β -strands

1135 involved in the β - β interaction between TSEN15 and TSEN34 are shown. **c**, Cartoon representation of

1136 the dimer interface with amino acid residues in stick representation (color coding as in **a**). Water

1137 molecules and hydrogen bonds are shown as red spheres and black dashed lines, respectively. **d**,

1138 Cartoon representation of the TSEN15–34 interface highlighting histidine 116 (His¹¹⁶) of TSEN15,

1139 mutated in patients with a PCH type 2 phenotype. **e**, Pull-down experiment with TSEN34, wt TSEN15

1140 and TSEN15 carrying the H116Y mutation. Input and co-precipitated proteins were separated by SDS-

1141 PAGE and visualized by immunoblotting. Size markers and protein identities are shown. **f**, Pre-tRNA

1142 cleavage assay with wt, tetrameric TSEN complex and a tetrameric TSEN complex carrying the

1143 TSEN15^{H116Y} (T15^{H116Y}) mutation. Cleavage products were separated by Urea-PAGE and visualized

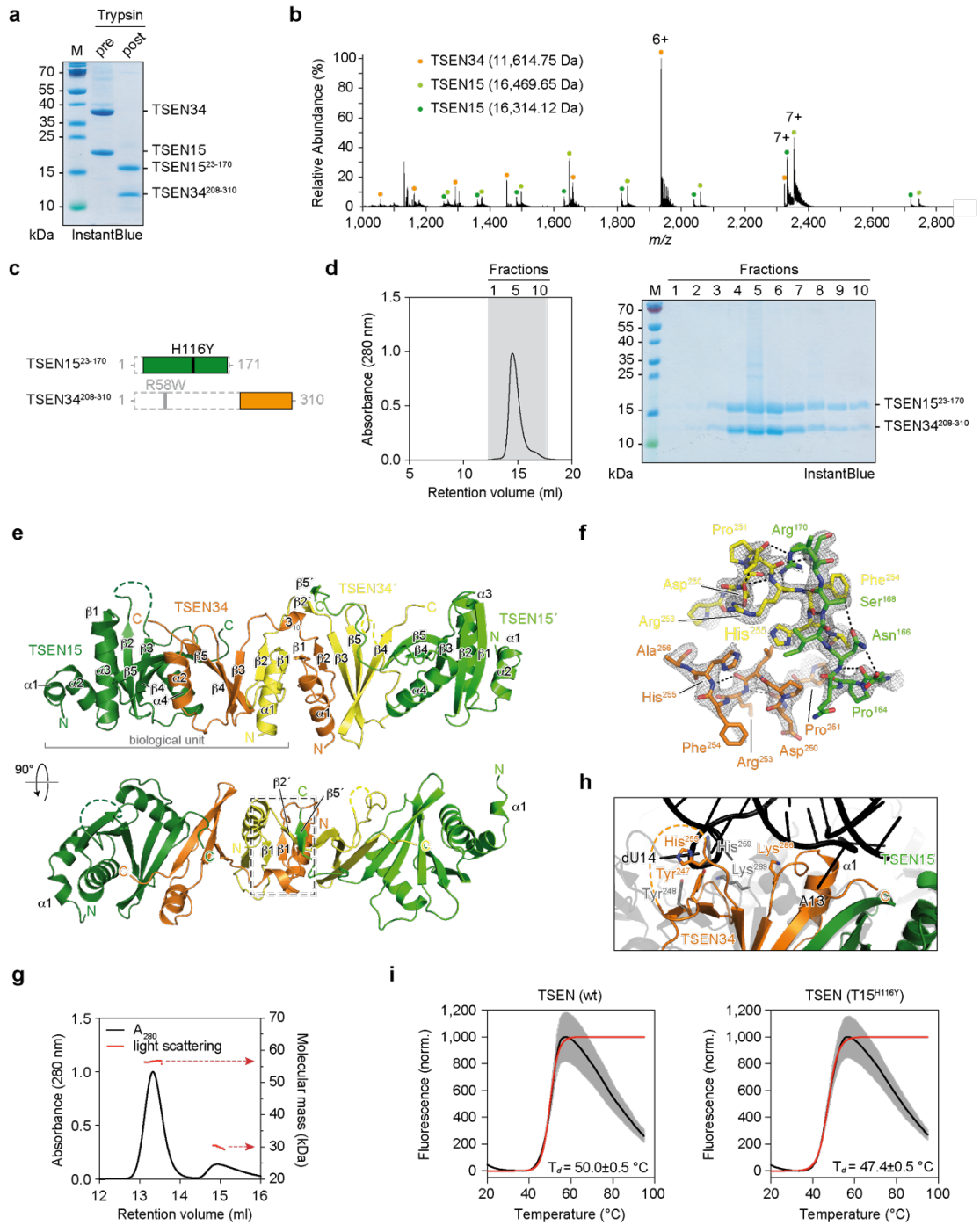
1144 with toluidine blue. **g**, Thermal stability of wt, tetrameric TSEN (black line) and TSEN15^{H116Y} mutant

1145 complex (red line) assessed by differential scanning fluorimetry (DSF). Note that recombinant

1146 complexes were purified from HEK293 cells. Normalized (norm.) fluorescence is plotted against

1147 temperature in degree Celsius (°C). Denaturation temperatures (T_d) of the complexes were derived
1148 from sigmoidal Boltzmann fits (grey dashed lines) with error of fits. Standard deviations (SD) of
1149 technical triplicates are represented by red (T15^{H116Y}) and black (wt) dashed lines. Unprocessed gels
1150 for **e** and **f** are shown in [Source Data 5](#).

1151



1152

1153 **Extended Data Fig. 3 | X-ray crystal structure of a TSEN15–34 heterodimer derived by limited**

1154 **proteolysis. a**, Analysis of the limited tryptic digestion of the TSEN15–34 heterodimer by SDS-PAGE.

1155 **b**, Denaturing mass spectrometry of the proteolytically stable fragments of the TSEN15–34 heterodimer

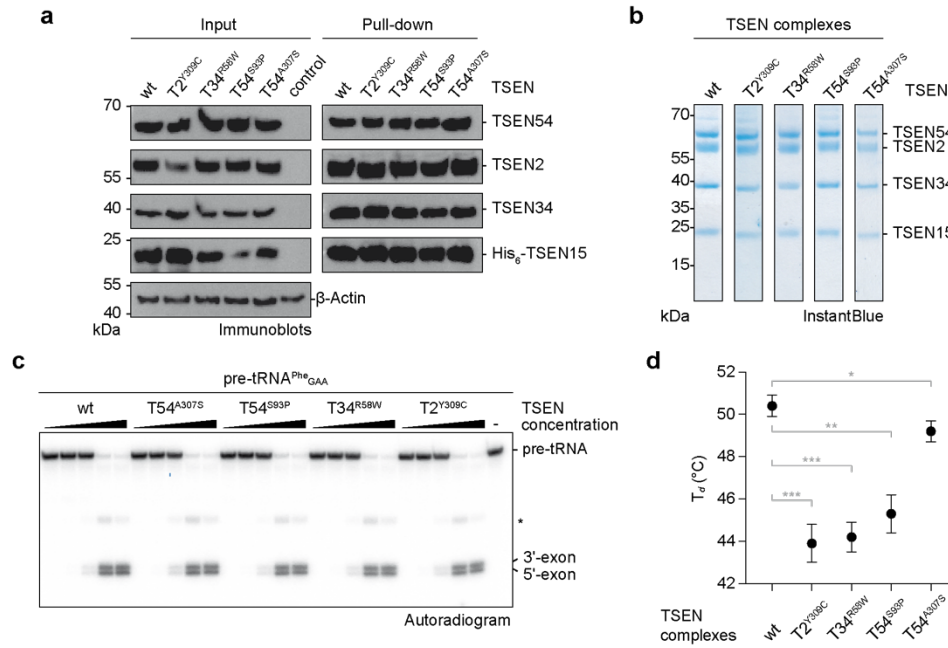
1156 from an aqueous ammonium acetate solution. The mass spectrum shows the presence of a TSEN34

1157 fragment (orange circles), and two TSEN15 fragments (dark and light green circles) differing in mass

1158 only by a C-terminal arginine as revealed by LC-MS/MS. **c**, Bar diagrams of tryptic fragments of

1159 TSEN15 and TSEN34. Proteolyzed regions are indicated by dashed boxes. Positions of PCH

1160 mutations are shown. **d**, Purification of the re-cloned core of the TSEN15–34 heterodimer via SEC.
1161 The absorbance profile at 280 nm is shown. Fractions of the indicated retention range (grey area)
1162 were analyzed by SDS-PAGE. **e**, Asymmetric unit of the TSEN15–34 crystal. The biological unit
1163 (bracket) and the domain-swap area (dashed box) are indicated. α -helices and β -sheets are
1164 numbered for each subunit. **f**, Stick representation of amino acids of the domain-swap area with
1165 electron density ($2F_o - F_c$, 1.5σ). **g**, Molecular mass determination by size exclusion chromatography
1166 multi-angle light scattering (SEC-MALS) of the TSEN15–34 sample used for crystallization. The data
1167 reveal a dominant population of a dimer-of-a-heterodimer (13.2 ml, 56.6 kDa) and a minor populated
1168 heterodimer (14.9 ml, 30.0 kDa). Light scattering is shown as red dots. Mass determination by SEC-
1169 MALS was confirmed by two independent experiments. **h**, Superposition of the TSEN15–34
1170 heterodimer and the pre-tRNA endonuclease from *Archaeoglobus fulgidus* at the interaction sites with
1171 the bulge-helix-bulge RNA (PDB ID 2GJW). Nucleotide positions of the RNA (black) and residues of
1172 the catalytic triads are shown. **i**, Representative thermal denaturation curves as shown in [Fig. 3g](#) of
1173 recombinant wt TSEN and mutant TSEN (T15^{H116Y}) complexes derived from DSF. Sigmoidal
1174 Boltzmann fits are shown as red lines. Grey zones show standard deviations (SD) from technical
1175 triplicates. Denaturation temperature (T_d) is presented with error of fit. Unprocessed gels for **a** and **d**
1176 are shown in [Source Data 6](#).
1177



1178

1179 **Fig. 4 | PCH mutations affect thermal stability but not activity of recombinant TSEN. a,**

1180 Immunoblot analysis of a pull-down assay with wt and mutant TSEN complexes co-expressed in

1181 HEK293 cells. Size markers and protein identities are indicated. **b,** SDS-PAGE of SEC peak fractions

1182 of purified recombinant wt and mutant heterotetrameric TSEN complexes. **c,** Pre-tRNA endonuclease

1183 assay of radioactively labelled *S.c.* pre-tRNA^{Phe}_{GAA} with increasing amounts of recombinant wt or

1184 mutant TSEN complexes revealed by phosphorimaging. **d,** Thermal stability of recombinant wt and

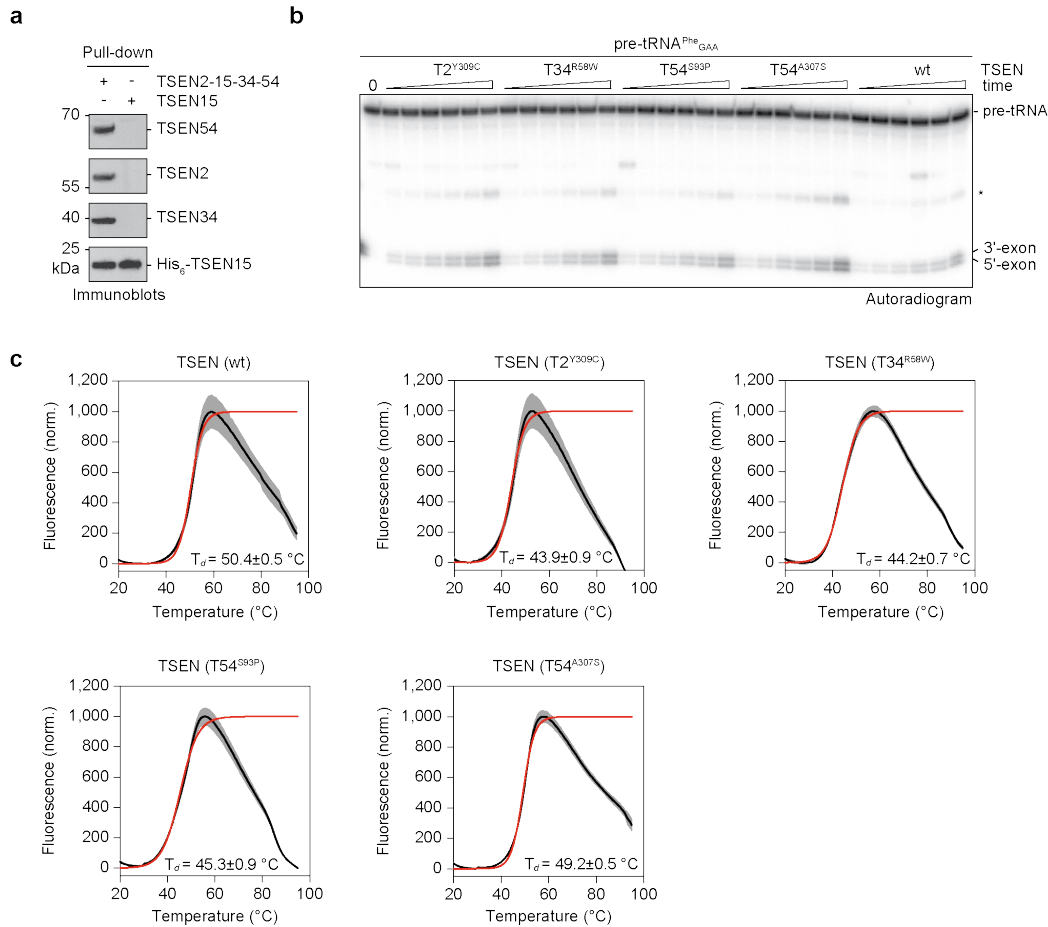
1185 mutant TSEN complexes assessed by DSF. Shown is the denaturation temperature (T_d) of each

1186 complex with fit errors derived from Boltzmann sigmoids. Fit errors were derived from means of

1187 technical triplicates and are representative of biological duplicates. Unprocessed gels for **a**, **b**, and **c**

1188 are shown in [Source Data 7 and 8](#).

1189



1190

1191 **Extended Data Fig. 4 | PCH mutations affect thermal stability but not activity of recombinant**

1192 **TSEN *in vitro*.** **a**, Pull-down assay from HEK293T cells overexpressing TSEN subunits TSEN2, His₆-

1193 TSEN15, TSEN34, and TSEN54, or His₆-TSEN15 alone. Co-precipitated proteins were visualized by

1194 immunoblotting. **b**, Pre-tRNA cleavage assay (time course) of radioactively labelled S.c. pre-

1195 tRNA^{Phe}_{GAA} with wt or mutant TSEN complexes revealed by phosphorimaging. The asterisk indicates

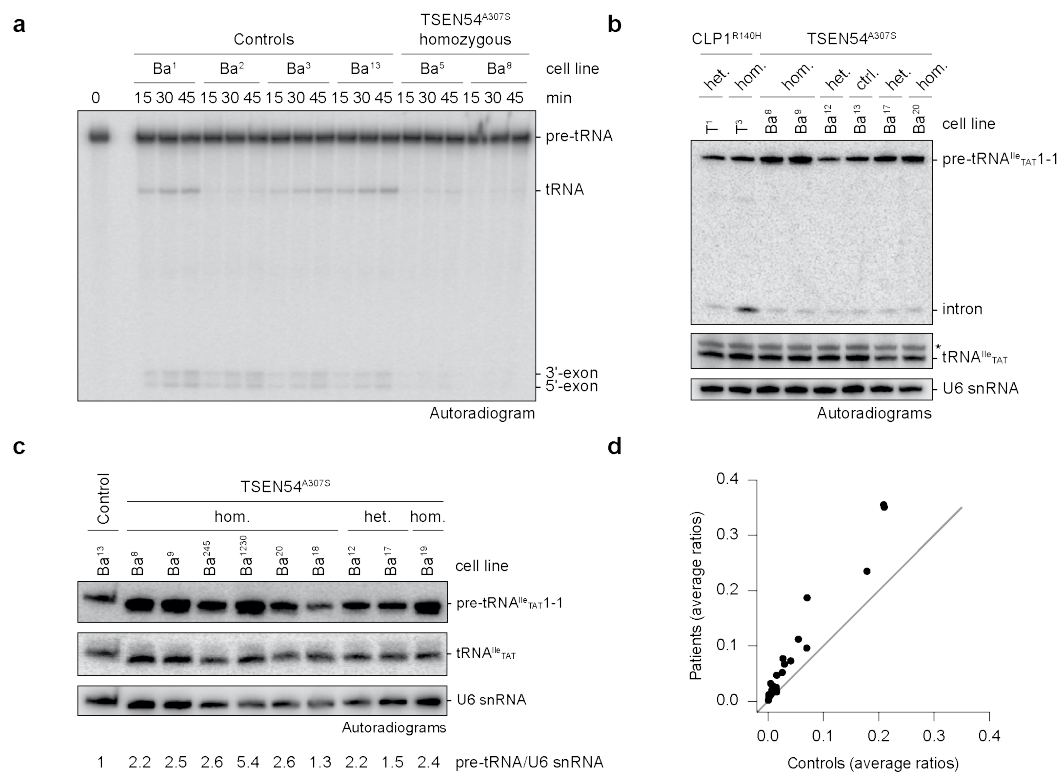
1196 an intermediate cleavage product. **c**, Representative thermal denaturation curves as shown in [Fig. 4d](#)

1197 of recombinant wt and mutant TSEN complexes derived from DSF with sigmoidal Boltzmann fits as

1198 red lines. Grey zones show SDs from technical triplicates. Denaturation temperature (T_d) is presented

1199 with error of fit. Unprocessed gels for **a** and **b** are shown in [Source Data 9](#).

1200



1201

1202 **Fig. 5 | TSEN54 c.919G>T fibroblasts exhibit reduced splicing activity *in vitro* and accumulation**

1203 **of intron-containing pre-tRNAs. a**, Pre-tRNA splicing assay (time course) with radioactively labelled

1204 S.c. pre-tRNA^{Phe}_{GAA} and cell extracts derived from control and PCH patient fibroblasts. Splice products

1205 were separated by Urea-PAGE and monitored by phosphorimaging. **b**, Comparison of pre-tRNA^{lle}-TAT1-

1206 1 intron abundance between control cells and fibroblasts carrying the heterozygous or homozygous

1207 TSEN54 c.919G>T (TSEN54^{A307S}) or the CLP1 c.419G>A (CLP1^{R140H}) mutation by northern blotting. **c**,

1208 Northern blot analysis comparing pre-tRNA^{lle}-TAT1-1 and mature tRNA^{lle}-TAT1-1 levels to levels of U6

1209 snRNA in control fibroblasts and fibroblast carrying the heterozygous (het.) or homozygous (hom.)

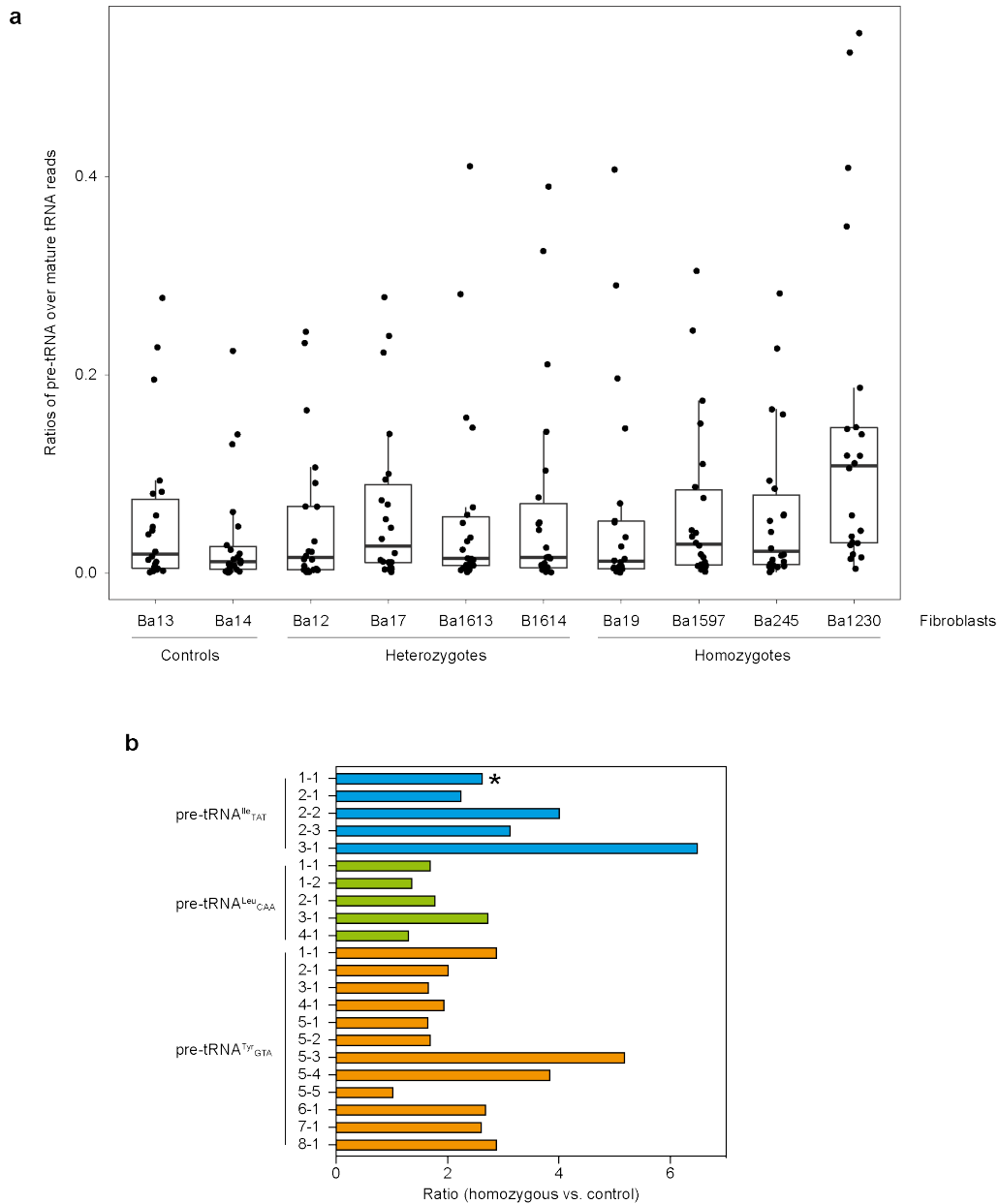
1210 TSEN54 c.919G>T mutation with quantification. **d**, Average ratios of pre-tRNAs to mature tRNAs

1211 derived from Hydro-tRNAseq for all intron-containing tRNAs comparing PCH patients to control

1212 samples. The black line indicates a slope of 1. Panels in **a**, **b**, and **c**, are representative of at least two

1213 independent experiments. Unprocessed gels for **a**, **b**, and **c**, are shown in [Source Data 10](#).

1214



1215

1216 **Extended Data Fig. 5 | Hydro-tRNAseq reveals accumulation of intron-containing pre-tRNAs in**

1217 **TSEN54 c.919G>T fibroblasts. a,** Boxplots showing ratios of pre-tRNA over mature tRNA reads for

1218 all intron-containing tRNAs from hydro-tRNAseq of PCH patient-derived and control fibroblasts. Pre-

1219 tRNA reads for all samples and tRNAs are less abundant than mature tRNA reads (ratios < 1). The

1220 lowest median corresponds to a homozygous control, and the highest median to a homozygous

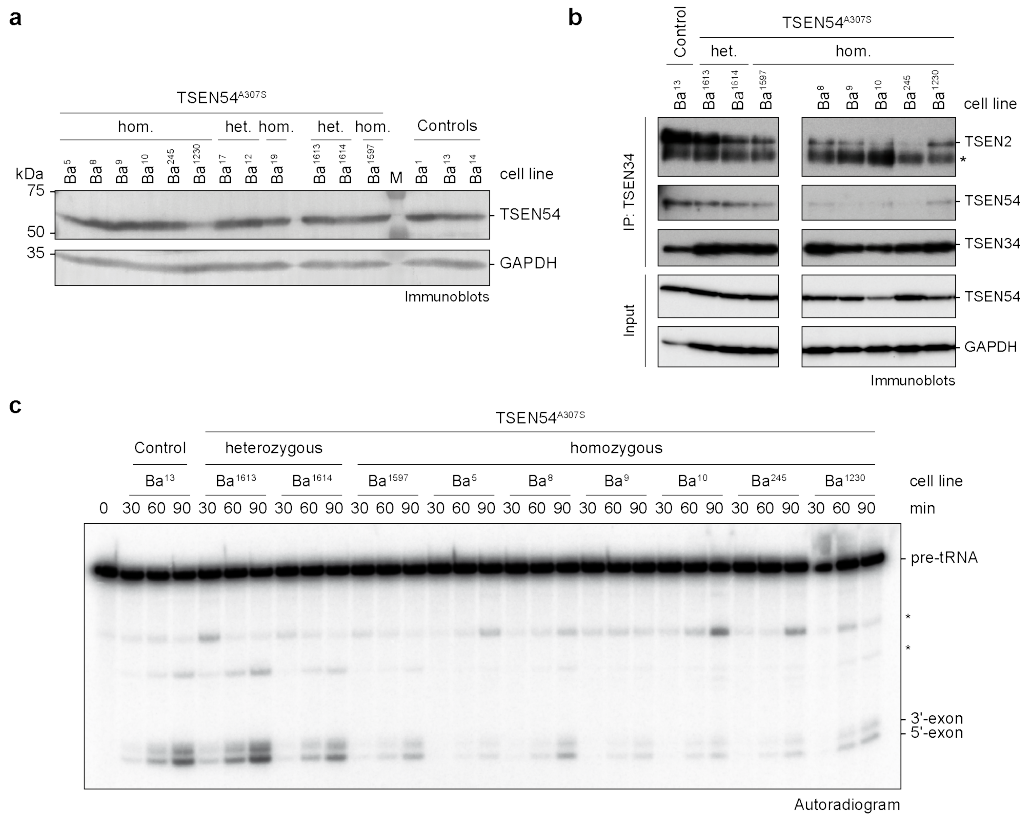
1221 TSEN54^{A307S} patient. **b,** For every intron-containing tRNA, the ratio of hydro-tRNAseq reads mapped

1222 to pre-tRNAs over mature tRNAs was calculated. The average ratio of all patients over the average

1223 ratio of all homozygous controls is shown. TSEN54 c.919G>T fibroblasts exhibit an increase of pre-

1224 tRNA/mature tRNA ratios for all intron-containing tRNAs. tRNA^{Ile}_{TAT} isodecoders targeted by a 5' exon

1225 probe shown in Fig. 5b are highlighted in blue. tRNA^{Ile}_{TAT}1-1 targeted by an intron probe in Fig. 5b,c is
1226 marked with an asterisk.
1227



1228

1229 **Fig. 6 | Reduced pre-tRNA cleavage activity in PCH patient-derived cell extracts is associated**

1230 **with altered composition of TSEN. a**, Comparison of TSEN54 protein levels between control and

1231 heterozygous or homozygous PCH patient fibroblasts analyzed by immunoblotting. GAPDH served as

1232 a loading control. **b**, Co-immunoprecipitation (IP) assay using an α -TSEN34 antibody with cell lysates

1233 derived from control and heterozygous or homozygous *TSEN54* c.919G>T fibroblasts analyzed by

1234 immunoblotting. The asterisk indicates the heavy chain of the α -TSEN34 antibody. **c**, On-bead pre-

1235 tRNA cleavage assay (time course) with radioactively labelled S.c. pre-tRNA^{Phe}_{GAA} and

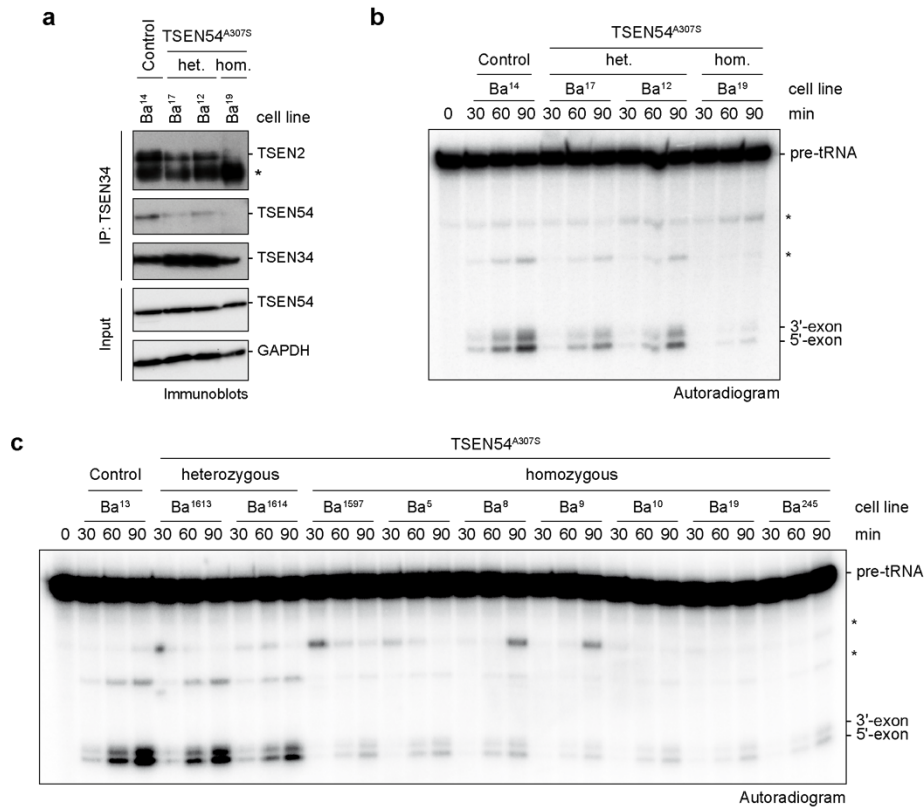
1236 immunoprecipitated TSEN complexes (α -TSEN34 antibody-coupled resin) derived from control

1237 fibroblasts and from fibroblasts carrying heterozygous or homozygous *TSEN54* c.919G>T mutation

1238 shown in (b). Unspecific bands are indicated by asterisks. Data are representative of at least two

1239 independent experiments. Unprocessed gels for a, b, and c are shown in [Source Data 11-13](#).

1240



1241

1242 **Extended Data Fig. 6 | Reduced pre-tRNA cleavage activity in PCH patient-derived cell extracts**

1243 **is associated with altered composition of TSEN. a**, Co-immunoprecipitation (IP) assay using an α -

1244 TSEN34 antibody with cell lysates derived from a control cell line and fibroblast derived from a PCH

1245 patient (Ba¹⁹) and the parents (Ba¹⁷ and Ba¹²) analyzed by immunoblotting. The asterisk indicates the

1246 heavy chain of the α -TSEN34 antibody. GAPDH served as a loading control. **b**, On-bead pre-tRNA

1247 cleavage assay (time course) with radioactively labelled S.c. pre-tRNA^{Phe}_{GAA} and immunoprecipitated

1248 TSEN complexes (α -TSEN34 antibody-coupled resin) shown in (a). Unspecific bands are indicated by

1249 asterisks. **c**, On-bead pre-tRNA cleavage assay (time course) with radioactively labelled S.c. pre-

1250 tRNA^{Phe}_{GAA} and immunoprecipitated TSEN complexes (α -TSEN2 antibody-coupled resin) derived from

1251 control fibroblasts and from fibroblasts carrying heterozygous or homozygous *TSEN54* c.919G>T

1252 mutation. Unspecific bands are indicated by asterisks. Data are representative of at least two

1253 independent experiments. Unprocessed gels for **a**, **b**, and **c** are shown in [Source Data 14 and 15](#).

1254