New insights into RNA processing by the eukaryotic tRNA splicing endonuclease

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Through its role in intron cleavage, tRNA splicing endonuclease (TSEN) plays a critical function in the maturation of intron-containing pre-tRNAs. The catalytic mechanism and core requirement for this process is conserved between archaea and eukaryotes, but for decades, it has been known that eukaryotic TSENs have evolved additional modes of RNA recognition, which have remained poorly understood. Recent research identified new roles for eukaryotic TSEN, including processing or degradation of additional RNA substrates, and determined the first structures of pre-tRNA–bound human TSEN complexes. These recent discoveries have changed our understanding of how the eukaryotic TSEN targets and recognizes substrates. Here, we review these recent discoveries, their implications, and the new questions raised by these findings.

tRNA introns can be found in all three domains of life and need to be removed to make mature, functional tRNAs. tRNA introns in archaea and eukaryotes are distinct from most other introns because they are spliced by an all-protein machinery. The removal of almost all other introns, including those in bacterial tRNAs, is catalyzed by RNA (although often as part of a ribonucleoprotein complex) in the form of group I and group II introns or by the concerted action of the small nuclear ribonucleoprotein particles of the spliceosome. In archaeal and eukaryotic tRNA splicing, a tRNA splicing endonuclease (TSEN) separates the exons from the intron, and a tRNA ligase subsequently joins the exons (Fig. 1*A*). The TSEN enzymes of archaea and eukaryotes are all related to each other, but the ligation step seems to be carried out by two very different ligase families. TSEN activity was discovered in the 1970s with the realization that tRNA cleavage in yeast is a two-step process with different enzymes performing the cleavage and ligation steps (1–3). Partially purified endonuclease was shown to cleave at two sites within the pre-tRNA to produce 5′ and 3′ exon halves containing 2′-3′ cyclic phosphate and 5′ hydroxyl ends, respectively. Subsequently, all four subunits of yeast TSEN were identified, with TSEN2 and TSEN34 recognized as the catalytically active subunits that cleave at the 5′ and 3′ ends of the intron, respectively (4) (see Box1). Four orthologous genes encoding human TSEN were subsequently identified, leading to the assumption that eukaryotes share these four subunits. However, the human enzyme complex copurified with a fifth protein, the RNA kinase CLP1, whose function in tRNA splicing remains unresolved (5). Recent cryo-EM structures of human TSEN highlighted its molecular architecture and delineated principles of substrate recognition (6–8).

The human TSEN enzyme shares many characteristics with archaeal TSENs, including a common catalytic triad of Tyr, His, and Lys residues which cleave the phosphodiester backbone using a mechanism similar to RNase A (Fig. 1*B*). Binding of the substrate in the active site of one catalytic subunit is further enhanced by cation-π stacking of residues from the other catalytic subunit with a base of the substrate (Fig. 1*B*). The human TSEN structures reveal many commonalities with the archaeal enzymes, but they also show novel aspects of TSEN biology, including a distinct mechanism to recognize substrates. An important distinction is that the archaeal enzymes are all symmetrical (homodimers or homotetramers) and rely on recognition of a pseudosymmetric local secondary RNA structure, the bulge-helix-bulge (BHB) motif. This pseudosymmetric motif presumably can bind the symmetric enzyme in two different orientations. In contrast, the human enzyme is an asymmetric heterotetramer and relies more on recognition of the asymmetric tertiary structure of the tRNA. This feature is likely conserved in fungi and other animals, which also have a heterotetrameric TSEN.

Although the TSEN enzymes were first identified for their role in tRNA splicing, there is growing evidence that archaeal TSEN also plays a key role in pre-rRNA processing, separating the 16S and 23S rRNA from a common precursor (9–12) and a less well conserved role in mRNA splicing (9, 13, 14), while the yeast TSEN also cleaves mRNAs to initiate their degradation (15). Some of these functions have only recently been discovered and others may remain to be discovered.

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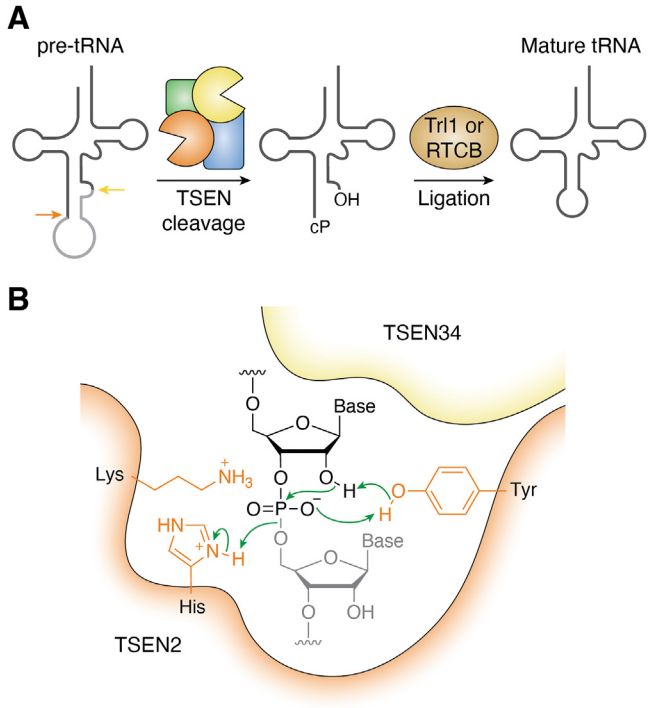

Figure 1. TSEN initiates the splicing of intron-containing pre-tRNAs. *A*, the TSEN2 and TSEN34 subunits of human (or yeast) TSEN cleave the 5′ and 3′ end of the pre-tRNA intron, respectively. Depicted is a typical human tRNA with the intron in the canonical position between nucleotides 37 and 38 and folded in a bulge helix loop motif. Archaeal pre-tRNAs can have introns in other positions, can have more than one intron, and are typically folded into a bulge-helix-bulge motif. tRNA splicing is then completed by an RNA ligase. Two different families of ligase (Trl1 and RTCB) perform this step in different species. *B*, proposed catalytic mechanism of TSEN. The proposed roles of the catalytic triad of TSEN2 (Tyr, Lys, His) are depicted as is the contribution of TSEN34 to the composite active site. For the other catalytic site, the roles of TSEN2 and TSEN34 are reversed. Equivalent active sites are formed by archaeal TSEN homologs even though they do not have distinct TSEN2 and TSEN34 subunits. TSEN, tRNA splicing endonuclease.

The presence of pre-tRNA introns and TSEN in archaea and eukaryotes suggests that the introns have an important function. Although the presence of pre-tRNA introns is conserved, they are short (generally 15–30 nucleotides) and poorly conserved in sequence (http://gtrnadb.ucsc.edu/). Identification of pre-tRNA intron function is further complicated by the extensive duplication of tRNA genes (http://gtrnadb.ucsc.edu/). For example, the yeast genome contains 61 introns in the genes for ten different tRNAs (16). In a heroic effort, Hayashi *et al.* (16) created ten different yeast strains lacking all the introns in a particular set of tRNA isodecoders. For example, one strain had the introns deleted from all ten genes for tRNA-Phe, while another strain lacked the intron in the sole serine tRNA with a CGA anticodon. Surprisingly, none of the introns across the ten different yeast tRNAs proved essential. The same conclusion was reached in other studies that showed the tRNA ligase in yeast, trypanosomes, and nematodes is not essential if a full complement of intron-less tRNA genes is artificially expressed (17–19). While tRNA introns are not essential, some of the yeast intron deletion strains showed phenotypes, such as cold sensitivity, slow growth, respiratory defects, or hypersensitivity to translational inhibitors, suggesting there is a physiological role for tRNA introns (16, 17). The growth defects were relatively mild under standard lab conditions; however, the benefits of maintaining introns could be sufficient to select for them over evolutionary timescales in the natural environment.

One well defined role of some tRNA introns in a variety of eukaryotes is that they are required for tRNA modification. A subset of tRNA modifying enzymes rely on recognition of intron-containing pre-tRNA. For example, pre-tRNA-Tyr-GUA, pre-tRNA-Ile-UAU, pre-tRNA-Phe-GAA, and pre-tRNA-Leu-CAA are modified only when the intron is present (20–24). Each of these enzymes recognizes and modifies the intron-containing precursor, but not the mature tRNA. These modifications, and thus the introns as well, affect translational fidelity. Why these enzymes only recognize pre-tRNAs and whether this may explain the conservation of introns across eukaryotes remains unclear. The recent structures of the human TSEN complex also reveal the structure of an intron-containing pre-tRNA and how it is recognized by the TSEN complex (6–8). Whether tRNA-intron–modifying enzymes use similar strategies to recognize the intron-containing pre-tRNAs remains to be determined.

Box 1. TSEN subunit nomenclature.

TSEN variants with a similar overall structure and function, but with five different subunit compositions, have now been described [α_4, $(\alpha\beta)_2$, α'_2, ε_2, and $\alpha\beta\gamma\delta$; see main text] leading to a confusing nomenclature. The conflicting rules for official names of proteins in the different organisms further complicates arriving at a common nomenclature. The official protein names for the human subunits are TSEN2, TSEN15, TSEN34, and TSEN54; for clarity, in this review, we use the same nomenclature for the orthologs in other animals and fungi. However, we note that in *Saccharomyces cerevisiae*, the official names are Sen2, Sen15, Sen34, and Sen54. Commonly used names for archaeal proteins and genes are also confusing. The gene name for the catalytic subunit of archaeal TSEN enzymes is generally called EndA, irrespective of whether it is from an α_4, $(\alpha\beta)_2$ α'_2 or ε_2 enzyme. The structural subunit of the $(\alpha\beta)_2$ enzyme is called EndB (111). Confusingly, in the α_4 enzymes, all four subunits are called EndA, but two of them function like the EndA subunit of the $(\alpha\beta)_2$ enzymes, and the other two function like the EndB subunit. Thus, nomenclature does not correspond to function across species. Additionally, the single subunit of α'_2 and ε_2 are also called EndA, but α and ε are two different ways to fuse a catalytic domain to a structural domain. Finally, EndA is also the official gene name in *Escherichia coli* that encodes the completely unrelated DNA-specific endonuclease I.

Variation and conservation of the archaeal TSEN complexes

Although archaea and eukaryotes use a related TSEN, the subunit composition has diverged (Fig. 2, *A–F*). The simplest archaeal subunit composition (*e.g.*, *Methanocaldococcus jannaschii*) is a homotetramer (α_4; Fig. 2*A*). However, the structure reveals that even in the α_4 TSEN, two subunits adopt a catalytically active conformation and two perform non-catalytic, structural roles (25). In some archaea, including *Aeropyrum pernix*, the gene for TSEN is duplicated, with one gene encoding a catalytic subunit (α) and the other gene encoding a structural subunit (β), an example of sub-functionalization (Fig. 2*B*). The overall structure of these TSEN enzymes is conserved, but they have a distinct $(\alpha\beta)_2$ subunit composition. In yet other archaea, a single gene

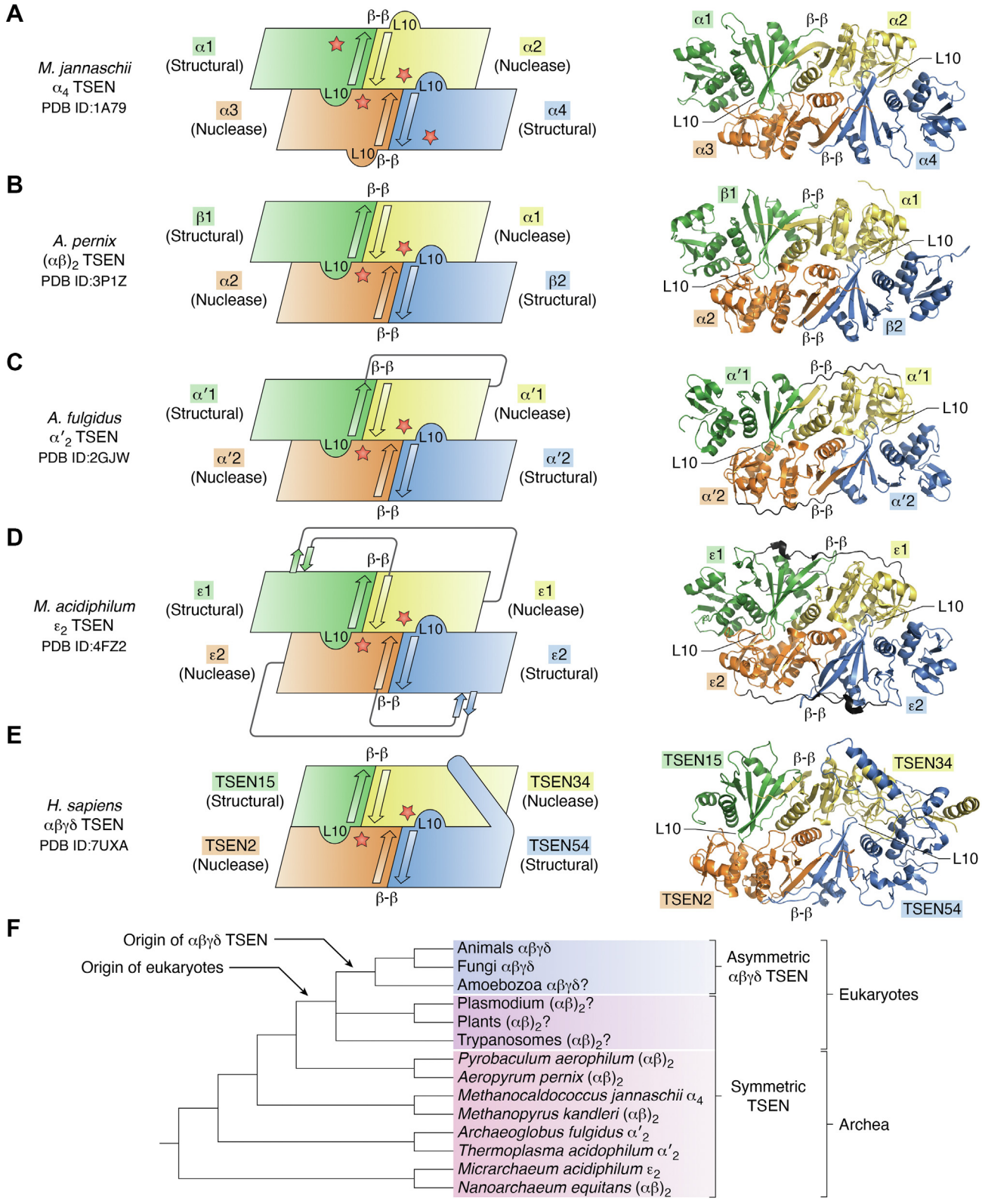

Figure 2. TSEN occurs in five distinct subunit compositions that form a conserved overall structure. *A*, box and cartoon structure of *Methanocaldococcus jannaschii* α_4 TSEN (PDB ID 1A79). Each alpha subunit is colored individually to indicate whether it is a structural (*green* and *blue*) or nuclease (*yellow* and *orange*) subunit. *B*, box and cartoon structure of the *Aeropyrum pernix* $(\alpha\beta)_2$ TSEN (PDB ID 3P1Z) with the structural beta subunits colored in *green* and *blue* and the alpha nuclease subunits colored in *yellow* and *orange*. *C*, box and cartoon structure of the *Archaeoglobus fulgidus* α'_2 TSEN (PDB ID 2GJW). TSEN is composed of only two subunits, and the structural and nuclease domains of this protein are colored as in (*A*). *D*, box and cartoon structure of the *Candidatus Micrarchaeum acidiphilum* ε_2 TSEN (PDB ID 4FZ2) colored as in (*C*). *E*, box and cartoon structure of the human TSEN complex (PDB ID 7UXA). *F*, phylogenetic tree of select archaea and eukaryotes. Included are nine species for which a TSEN structure has been determined experimentally. The TSEN subunit composition is indicated in *parentheses* on the *right*. Also included are three groups of eukaryotes for which we predict an $(\alpha\beta)_2$ TSEN, which is indicated with a *question mark*. This architecture is similar to the archaea most closely related to eukaryotes. This phylogeny suggests that a hetero-tetrameric TSEN arose later in evolution than previously appreciated. TSEN, tRNA splicing endonuclease.

encodes what is essentially a fusion protein of a catalytic subunit to a structural subunit (26, 27). This fusion has occurred in at least two different ways, resulting in α'_2 and ε_2 TSEN complexes found in *Archaeoglobus fulgidus* and *Micrarchaeum acidiphilum*, respectively (Fig. 2, *C* and *D*).

The structures of the archaeal TSEN variations [α_4, $(\alpha\beta)_2$, α'_2 and ε_2] have remarkable similarity (111, 26–33) (Fig. 2). The crystal structure of the α_4 TSEN from *M. jannaschii* (26) revealed that the archaeal subunit has a bi-lobed architecture. The N-terminal subdomain is composed of three small α helices and a β-sheet, and the C-terminal subdomain is composed of two α helices surrounding a central five-stranded β-sheet (Fig. 2*A*) (13). Oligomerization of the archaeal subunits is facilitated by two key interactions (25, 34). The first interaction is promoted by the final β strand (β9) from the C-terminal subdomains, thus generating a β–β interface between two subunits (Fig. 2*A*). The second interaction is driven by the negatively charged L10 loop from the C subdomain, which inserts into a positively charged pocket on the opposite side of another C subdomain. Both these β–β and L10 loop interfaces are conserved in the α_4, $(\alpha\beta)_2$, α'_2, and ε_2 TSENs (Fig. 2, *A–D*).

Architecture of the human TSEN complex

The architecture of the human TSEN complex was recently revealed by cryo-EM from three independent groups (6–8). Six structures of the human TSEN complex bound to intron-containing pre-tRNAs were resolved at resolutions ranging from 2.9 to 3.9 Å. A 3.9 Å-resolution structure of full-length WT TSEN complex bound to pre-tRNA-Arg-UCU, in which the 2′ OH of the riboses at the 3′ and 5′ splice sites were replaced with fluorine to prevent cleavage, revealed the overall architecture of the complex, the arrangement of the four subunits, and the tRNA-binding sites (6). Similar observations were made from a structure at 3.8 Å resolution of the full-length complex, with point mutations in both active sites to prevent cleavage of the bound pre-tRNA-Tyr-GUA (8). Another structure of the TSEN complex containing TSEN2 and TSEN34 active site mutants, bound to pre-tRNA-Arg-UCU, was determined at 3.3 Å, which provided sufficient resolution for model building (6). Based on the observation that TSEN2, TSEN34, and TSEN54 contain large, disordered insertions not visible in the other structures (Fig. 2*E*), a truncated version of the complex, in which disordered regions were deleted, was created. The resulting 3.1 Å-structure of the complex bound to pre-tRNA-Arg-UCU enabled unambiguous model building of most of the heterotetramer and pre-tRNA (8). Finally, two additional structures of the complex bound to pre-tRNA-Arg-UCU were determined in the precleavage and postcleavage state at 2.9 Å-resolution (7). However, the atomic models for these two final structures include several regions of the protein and tRNA modeled into weak or missing density. The postcleavage state is similar in overall structure to the precleavage structures (6–8) with only minor changes associated with cleavage of the 3′ splice site, suggesting that the TSEN complex does not undergo large-scale conformational changes following cleavage. The rigid architecture of the core of the TSEN complex is likely an important feature of TSEN as it must be able to recognize and process all intron-containing pre-tRNAs. Furthermore, all of the structures of the TSEN complex trapped in the precleavage state display nearly identical architecture, revealing that mutation of active site residues, truncation of flexible regions from multiple subunits, the identity of the bound pre-tRNA, and the sequence/size of the intron do not cause large-scale conformational changes within the complex (6–8).

The CLP1 protein was also present in most of these recombinant complexes, but it was not visible in any of the high-resolution cryo-EM reconstructions, suggesting that CLP1 associates with the complex through a flexible region. One of the studies attributed weak density from low-pass filtered maps to CLP1 in their deposited structures (7). However, it is unclear whether this weak density indeed reflects the position of CLP1 within the TSEN complex, in part because the sample was chemically cross-linked prior to structure determination.

The four subunits of the TSEN complex assemble into a highly interconnected quaternary structure that resembles the arrangement of the subunits from the four archaeal TSEN types (Fig. 2, *A–D*). The archaeal subunit interfaces including the β–β interfaces and L10 loops are conserved within human TSEN and these interfaces help support the rigid core of the complex. Within the human TSEN complex, the β–β interfaces are formed between TSEN15-TSEN34 and TSEN2-TSEN54. The L10 loops from TSEN15 and TSEN54 support dimer interfaces with TSEN2 and TSEN34, respectively (Fig. 2*E*). While the general structure and arrangement of the four subunits are well conserved across TSEN complexes from archaea to humans, we describe several notable differences below.

TSEN15 is the smallest subunit of the human TSEN complex and the only member of the complex where no direct interactions were observed with the pre-tRNA (Fig. 3, *A–E*). TSEN15 forms an interface with TSEN34 through the β-β subunit interface. In some of the structures, TSEN15 also forms an interface with TSEN2 through TSEN15's L10 loop and additional residues from the TSEN15 C subdomain. Generally, the position of TSEN15 in each of the structures supports its proposed role as a structural subunit and the β–β and L10 interfaces with the two catalytic subunits. TSEN15 has a flexible N-terminal tail not observed in the cryo-EM reconstructions, and it lacks the N subdomain found in all the other subunits. The missing N subdomain is reminiscent of the crenarchaeon *Pyrobaculum aerophilum* $(\alpha\beta)_2$ TSEN, whose structural subunit also lacks this region (31). Another perplexing difference between TSEN15 and the other three subunits is its overall negative electrostatic surface potential. The negative surface charge of TSEN15 could play an important role in preventing tRNA from binding to the complex in the wrong orientation and/or it may facilitate product release following cleavage.

TSEN34 is the endonuclease that is responsible for catalyzing cleavage at the 3′ splice site, and it is positioned surrounding the 3′ splice site in all the pre-tRNA–bound cryo-EM structures (Fig. 3, *A–E*) (4, 6–8, 35). TSEN34 interacts with all the other subunits within the complex. Beyond the L10 loop

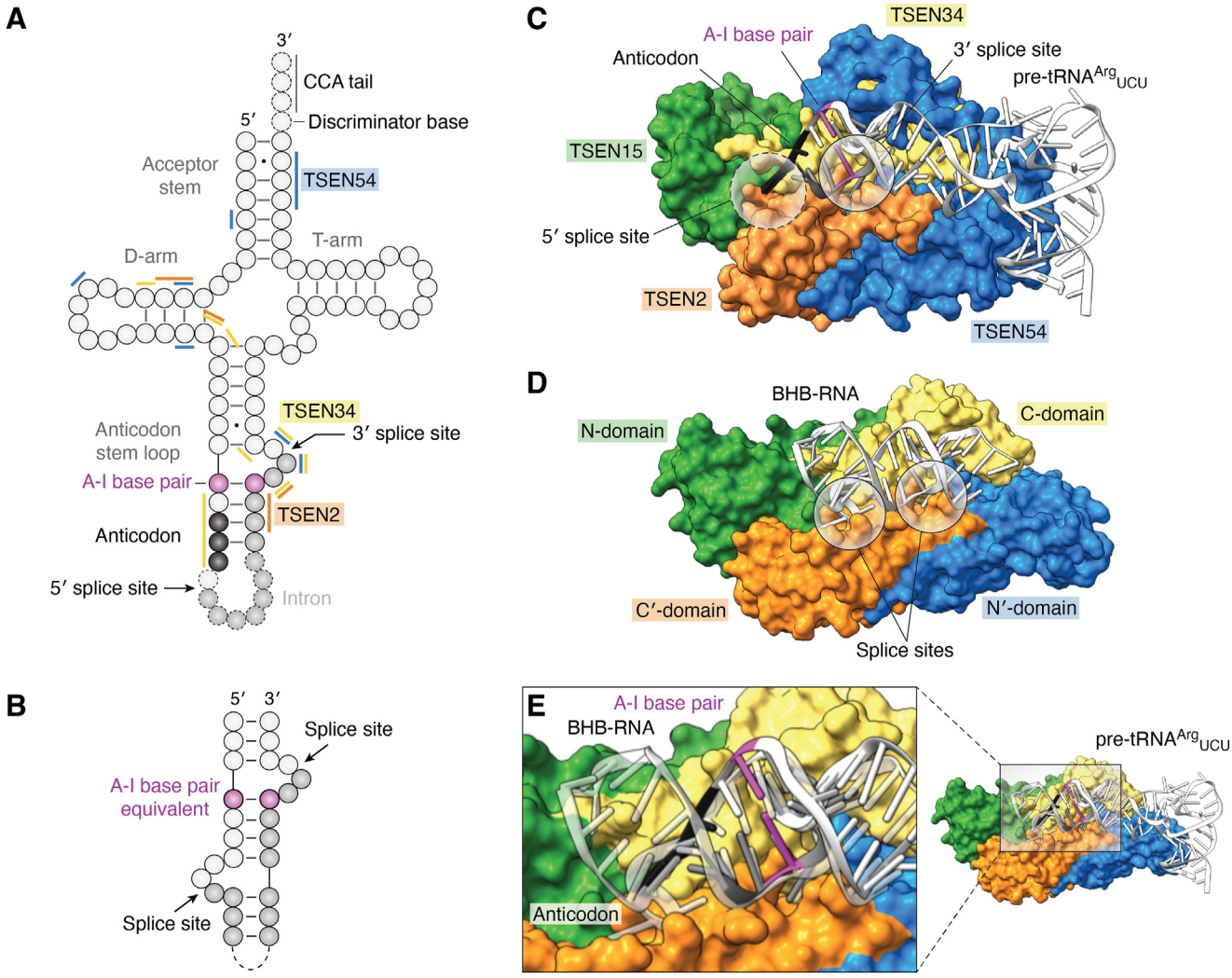

Figure 3. Pre-tRNA recognition by human and archaeal TSEN. *A*, cloverleaf representation of an intron-containing eukaryotic pre-tRNA. Sites of pre-tRNA recognition by subunits of TSEN are indicated by colored bars (TSEN2 – *orange*, TSEN34 – *yellow*, TSEN54 – *blue*). *Highlighted* are a critical base pair, the A-I base pair, between the anticodon loop and intron (*magenta*), nucleotides of the intronic region (*gray*), the anticodon (*black*), and the remainder of the mature tRNA (*white*). 5′ exon and intron nucleotides indicated as *dashed lines* are not well-defined in any of the available structures. *B*, schematic representation of an archaeal BHB motif with highlighted splice sites and A-I base pair equivalent (*magenta*). *C*, structural model of human TSEN bound to pre-tRNA-Arg-UCU color-coded as described in (*A*) (TSEN15 – *green*). Note that the 5′ splice site is not adequately resolved in any of the cryo-EM reconstructions. *D*, X-ray crystal structure of α'_2 TSEN from *Archaeoglobus fulgidus* with a BHB RNA (PDB ID 2GJW). The color coding matches the domain arrangement of the human complex as shown in (*C*). N- and C-terminal domains of the two *A. fulgidus* endonuclease polypeptides are indicated. *E*, superposition of the pre-tRNA-Arg-UCU structure from panel *C* with the structure of *A. fulgidus* TSEN bound to BHB RNA (transparent) from (*D*). Human TSEN is not shown for clarity. BHB, bulge-helix-bulge; TSEN, tRNA splicing endonuclease.

interface, TSEN34 and TSEN54 form an extensive interface with one another with a buried surface area of ∼2230 Å^2. TSEN34 also contains a large insertion between the N and C subdomains. AlphaFold structure predictions for TSEN34 orthologs including those from yeast, human, and *Drosophila* predict that this insertion can be divided into a long α helix (34H) and a disordered region (36, 37). Weak density for 34H was observed in the human cryo-EM structures, positioning it near the elbow of the tRNA, hinting that it may play a role in facilitating tRNA recruitment or sensing its correct tertiary fold. Truncation of the disordered region (residues 121–179) does not impair complex formation or activity *in vitro* (8). TSEN34 also contacts the D-arm of the tRNA, and modeling of the intron around the 5′ splice site suggests that residues from TSEN34 are critical for supporting 5′ cleavage as well (Fig. 3*A*). Thus, TSEN34 is a multifunctional subunit of the complex, playing roles in tRNA binding, supporting cleavage at the 5′ splice site, and catalyzing cleavage at the 3′ splice site.

TSEN2 is the endonuclease that is responsible for catalyzing cleavage at the 5′ splice site (6–8, 35). Well-ordered density for the intron surrounding the 5′ splice site is missing in all of the cryo-EM reconstructions, suggesting that the 5′ splice site is dynamic, which could be important for allowing the complex to accommodate different substrates. Analogous to TSEN34, TSEN2 interacts with all the other subunits of the complex (Figs. 2*E* and 3*C*). TSEN2 contains a large insertion within its N subdomain. AlphaFold predicts that part of this insertion contains an additional ordered region (36, 37). However, no high-resolution density was observed for this region in any of the cryo-EM structures. Moreover, truncation of the insertion

(residues 82–291) does not impair complex formation or activity *in vitro* (8). The local resolution for the N subdomain of TSEN2 was substantially lower than other regions of the TSEN structure indicating that this region is dynamic. Similar to TSEN34, TSEN2 makes contact with the D-arm of the tRNA and with residues surrounding the 3′ splice site suggesting that, beyond catalyzing cleavage at the 5′ splice site, it is also important for tRNA binding and supporting cleavage at the 3′ splice site.

TSEN54 is the largest subunit of the complex and was originally predicted to function as a molecular ruler to help position the pre-tRNA for cleavage (4, 25, 38). Beyond its N- and C-terminal lobes, TSEN54 contains an N-terminal extension (NTE) of approximately 75 residues that forms part of the extensive interface with TSEN34 and the pre-tRNA. The TSEN54-NTE clamps around the TSEN34 N subdomain, adding an additional subunit interface to the human TSEN complex that is not observed in the archaeal TSEN families (Fig. 2, *A–E*). The C subdomain also contains a very large insertion not observed in any of the cryo-EM structures. Truncation of this insertion (residues 179–424) impairs binding of CLP1 but not TSEN complex formation or pre-tRNA cleavage *in vitro* (8). Moreover, crosslinking analysis supports the idea that CLP1 associates with this region of TSEN54 (6). The combined structural and functional analyses confirm that TSEN54 provides structural support to the TSEN2 and TSEN34 subunits, plays a pivotal role in pre-tRNA binding, and facilitates the recruitment of CLP1 through a dynamic insertion.

Origin of heterotetrameric TSEN and occurrence of $(\alpha\beta)_2$ TSEN in some eukaryotes

The first cryo-EM reconstructions of the human TSEN complex reveal that the αβγδ TSEN family shares structural similarity with the four archaeal types but also contains unique features, such as the TSEN54-NTE and TSEN34-34H that support complex assembly and tRNA binding (6–8). Advances in structure prediction combined with the human TSEN structures provide a template for deriving structural models of other eukaryotic TSEN complexes. The heterotetramer has been experimentally identified in yeast and humans, and predicted structures for the four yeast subunits closely match the cryo-EM structures of human TSEN. Thus, the features discovered by the cryo-EM structures are very likely conserved in yeast. Computational searches of sequences (with BLAST and PSI-BLAST) or predicted AlphaFold structures (with Dali) readily identify TSEN2, TSEN15, TSEN34, and TSEN54 orthologs in many other animals and fungi, as well as the slime mold *Dictyostelium* (all belonging to the taxonomic supergroup Amorphea, previously known as unikonta) (39–41) (Fig. 2*F*). However, in other eukaryotic taxa, only one catalytic subunit and one structural subunit is generally recognizable with these same tools (Fig. 2*F*). These groups include *Leishmania*, *Plasmodium*, and some plants, including maize. Thus, TSENs in these eukaryotes are likely of the $(\alpha\beta)_2$ type. Although the model plant *Arabidopsis thaliana* has two catalytic subunits, these seem to be recent paralogs that are much more closely related to each other than would be expected for TSEN2 and TSEN34 (42). Importantly, *Arabidopsis* mutants lacking one of the catalytic subunits (AT3G45590 or AT5G60230) are viable, while double mutant plants are very early zygotic lethal (43). This finding suggests that *Arabidopsis* has an $(\alpha\beta)_2$ TSEN with alternative α subunits being encoded by the two paralogs. Because the origin of the αβγδ architecture seems to be more recent than the last common ancestor of all eukaryotes, the human and yeast enzymes are unlikely to reflect the diversity of eukaryotic TSENs and future studies of more diverse eukaryotic TSENs are likely to reveal new biology (Fig. 2*F*).

The difference between an αβγδ TSEN in humans and yeast—but likely an $(\alpha\beta)_2$ TSEN in plants—may explain some differences in substrate recognition. A wheat germ extract can correctly process pre-tRNAs from other plants, including tobacco and *Arabidopsis*, but is completely inactive on human, *Xenopus*, or yeast tRNAs (44). The converse, however, is not true, as a HeLa extract readily splices pre-tRNAs from tobacco (45). One possible explanation that should be explored is that the plant $(\alpha\beta)_2$ TSEN enzyme resembles the archaeal enzymes in substrate recognition. If the $(\alpha\beta)_2$ TSEN enzymes from plants and other early-diverging eukaryotes are indeed more like the archaeal enzymes, they may also be able to splice introns from noncanonical positions. The αβγδ TSEN recognizes the overall tRNA structure and can only remove introns that are inserted between nucleotides 37 and 38, named the canonical position. However, archaeal TSENs recognize only local secondary structure and can therefore remove introns from other positions. Interestingly, some red algae are known to have introns in noncanonical positions but have an uncharacterized TSEN (46). These noncanonical introns were discovered because essential tRNAs appeared to be missing from the genome. In most eukaryotic genomes, tRNAs are annotated with the use of the tRNAscan algorithm, but the eukaryotic settings of this program only consider canonical introns (47) and thus might be more reliable for animal, fungal, and amoebozoan genomes than for earlier-diverging eukaryotes. These potential eukaryotic $(\alpha\beta)_2$ TSEN enzymes and their ability to recognize introns in noncanonical positions will be an interesting area for future research.

Structure of intron-containing tRNAs

Early chemical and enzymatic structure probing experiments revealed that the main body of eukaryotic intron-containing pre-tRNAs shares a fold similar to the canonical L-shaped structure of mature tRNAs, albeit with a significantly altered structure of the anticodon arm (48, 49). Intermolecular interactions, mainly between the D- and T-loops, fold tRNAs into their typical L-shape, which is a key requirement for the fidelity of several processing steps, nuclear export, and aminoacylation (50, 51). The long arm of the L-shape is formed by the D-stem stacking onto the anticodon stem (Fig. 3, *A* and *C*). In mature tRNA, the three residues of the anticodon (nucleotides 34–36) are centered in the seven nucleotide-long anticodon loop.

In eukaryotes with an αβγδ TSEN, tRNA introns are invariably positioned one nucleotide downstream of the anticodon between residues 37 and 38 of matured tRNAs (38, 52). Nucleotides within these introns were predicted to base pair with nucleotides from the anticodon loop. The structures of pre-tRNA bound to TSEN are consistent with this base pairing and with stacking of this helix onto the long arm of the L-shape, further extending it. Although this anticodon-intron helix is visible in the TSEN structures (6–8), the remainder of the intron, including the 5′ splice site, is not defined in any of the cryo-EM reconstructions by high-resolution density maps.

The extended L-shape of intron-containing pre-tRNAs is in contrast to archaeal precursor tRNAs, where introns are found at almost all positions in the molecule with the exception of the acceptor stem region (53). Special to archaea (and the red algae mentioned above) is the presence of multiple introns in some tRNA genes and the occurrence of *trans*-splicing, a process by which tRNA exons encoded by separate genes are joined to make one tRNA (54–56). Consistent with the presence of introns in diverse positions within the pre-tRNA, archaeal pre-tRNA introns are only defined by the BHB motif (12, 57, 58), that consists of a central helical element of four base pairs flanked on each side by a three-nucleotide bulge (27), and are independent of the global tRNA structure (53).

Pre-tRNA recognition and cleavage by human TSEN

Splice site selection in eukaryotes does not solely rely on local secondary structure but additionally relies on a defined distance from the short arm of the L-shape to the splice sites, referred to as the molecular ruler mechanism of splice site selection (38, 59, 60). Indeed, the recent high-resolution cryo-EM models of TSEN bound to a pre-tRNA substrate revealed recognition of the acceptor stem structure by TSEN54 (Fig. 3*A*). In contrast to archaeal tRNA splicing endonucleases, human TSEN forms an extensive interface sensing several regions of pre-tRNAs (6, 8). TSEN nestles up to the pre-tRNA at the intersection of the acceptor stem, the D-arm, and the anticodon arm. Phosphate backbone interactions between the acceptor stem and D-arm with TSEN54 anchor the pre-tRNA to TSEN and represent the largest protein–RNA interface within the complex. This arrangement positions the extended helical anticodon arm towards the active sites of TSEN2 and TSEN34 (Fig. 3, *A*–*E*) and explains why insertions in the anticodon arm can impact splice site selection (27, 38, 59). Remarkably, the N-terminal regions of TSEN34 and TSEN54, which do not have sequence homology to archaeal TSENs, mediate pre-tRNA binding, which may explain why mature tRNAs bind to TSEN with similar dissociation constants as pre-tRNAs (61).

Near the active site, there is more similarity between archaeal and human TSEN. Only one of the available archaeal structures includes a bound RNA, a short 21-oligomer that includes a BHB motif. The crystal structure of the BHB RNA-bound state revealed two Arg residues that act in *trans* at both active sites and two catalytic triads that are each made up of Tyr, His, and Lys residues (25, 27, 35). The Arg residues form cation-π sandwiches with pinched out bases of the bulges and were proposed to position the splice sites for cleavage (27, 35) (reviewed in (36, 62, 63)). The requirement for such a cation-π sandwich was also described for 5′ cleavage in the yeast tRNA splicing endonuclease (35). Presence of the cation–π sandwiches provides cooperativity between the active sites by cross-subunit stabilization, whereas positioning of the BHB motif is primarily driven by interactions with the RNA backbone in a sequence-independent manner (27, 35).

In human TSEN, the active sites of TSEN2 and TSEN34 each harbor the catalytic triad, but only TSEN2 provides a conserved arginine tweezer at the 3′ splice site (6, 8, 61, 64). In both the yeast and human enzyme, one Arg in the tweezer motif of TSEN34 at the 5′ splice site is substituted by a Trp (6, 8, 35). TSEN2 may be involved in sensing anticodon-intron helix stability as it contacts a wobble base pair distal of the anticodon-intron helix (6, 8). Whereas the molecular environment of 3′ splice site is well resolved in all cryo-EM reconstructions and is reminiscent of the archaeal splice site conformation, the orientation of residues that form a productive 5′ splice site remains obscure. In the cryo-EM reconstructions of the precatalytic and postcatalytic states of TSEN (7), the active site Lys and Tyr of TSEN2 are more than 8 Å away from the scissile phosphate. Taken together, all structures suggest substantial local remodeling to allow cleavage of the pre-RNA at the 5′ splice site. Thus, structures that unambiguously show the conformation and RNA sequence of a productive 5′ splice site will be needed to fully resolve the cleavage mechanism by eukaryotic TSEN.

Relaxed sequence requirements around the splice sites and in the central anticodon-intron helix were postulated for eukaryotic pre-tRNAs, suggesting the additional need for recognition of the mature body by TSEN (4, 38, 59, 60). Only the proximal base pair of the anticodon-intron helix (magenta in Fig. 3) seems conserved among eukaryotic pre-tRNAs with a critical role for intron excision (38, 60, 61, 65, 66). These features imply that there are notable differences in substrate recognition between archaeal and eukaryotic tRNA splicing endonucleases, whereas the underlying intron excision mechanism from pre-tRNAs is conserved.

Beyond tRNA splicing: TSEN cleaves pre-mRNAs, pre-rRNA, mRNAs, and possibly other RNAs

Though TSEN has been extensively studied for its namesake role in tRNA splicing, other functions of the complex have been discovered. Some archaeal species use TSEN for mRNA splicing (Fig. 4*A*). The first noted example was the *cbf5* pre-mRNA in *Sulfolobus* (13). TSEN was found to cleave at the exon-intron junctions of *cbf5*, which is followed by ligation of the two exons to produce the Cbf5 protein. The *cbf5* pre-mRNA has a secondary structure that is similar to the BHB motif of pre-tRNAs, but it is more relaxed. A trend the authors noted is that most of these species have a $(\alpha\beta)_2$ TSEN and hypothesized that this form of TSEN may differ in requirements for substrate recognition (13). Only a relatively small group of related archaea seem to have a *cbf5* intron (14). However, in other archaea such as *Haloferax volcanii*, the α_2 TSEN cleaves another mRNA (systematic name *HVO_1309*)

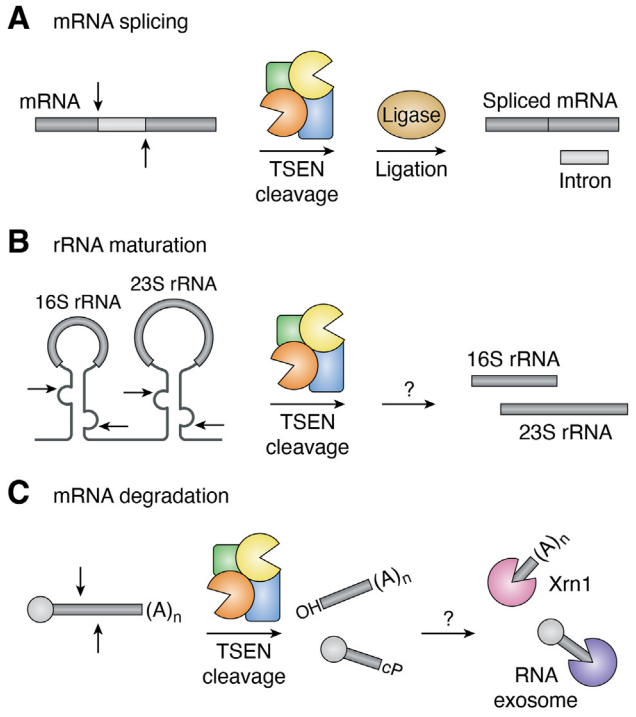

Figure 4. TSEN cleaves pre-mRNAs, pre-rRNAs, and mRNAs. *A*, in some archaea, TSEN cleaves mRNAs (*black*) such as *cbf5* and *HVO_1309*, which is the first step in splicing out an intron (*gray*). *B*, in archaea, TSEN participates in rRNA maturation. The pre-rRNA forms two bulge-helix-bulge motifs that are recognized by archaeal TSEN. Two of the cleavage products are further matured by unknown enzymes (indicated with a *question mark*) to the RNA of the large and small ribosomal subunit, respectively (16S and 23S rRNA). This rRNA maturation pathway has been described for diverse archaea. *C*, yeast TSEN cleaves mRNAs in a pathway referred to as tRNA endonuclease-initiated decay (TED) to facilitate degradation of the transcripts. The 3′ product is degraded by Xrn1 (*pink*), and the 5′ product is degraded by the RNA exosome (*purple*), possibly after their ends are modified (*question mark*). The extent of conservation of this pathway remains to be determined. TSEN, tRNA splicing endonuclease.

(9). Although the original report (9) did not show that the flanking regions are ligated together, RNAseq reads from other studies (*e.g.*, (67)) clearly show that the spliced RNA is the major product of this gene. Thus, TSEN functions in splicing of at least two archaeal genes and it appears likely that more remain to be discovered.

In many archaeal species, the pre-rRNA also has a pair of BHB motifs flanking both the 16S and 23S rRNAs (Fig. 4*B*). TSEN from *H. volcanii* can cleave these BHB motifs *in vitro* and, upon TSEN depletion, rRNA precursors accumulate, indicating that TSEN is necessary for correct pre-rRNA processing (9). This pre-rRNA maturation function of TSEN is likely to be conserved in diverse archaea (10–12). Together, these results demonstrate that archaeal TSEN can cleave pre-rRNAs and pre-mRNAs in addition to its canonical pre-tRNA substrates and that it uses similar rules for recognition of local structure in each case.

Like archaeal TSENs, eukaryotic TSENs can cleave additional RNA targets. Introducing a BHB structure into an mRNA was sufficient for *Xenopus* TSEN to cleave this non-tRNA substrate *in vitro*, suggesting that eukaryotic TSENs could have targets besides pre-tRNAs (68). This implication was validated as yeast TSEN was found to cleave the *CBP1* mRNA (69). Both 5′ and 3′ cleavage products of *CBP1* could be detected, but when TSEN was impaired, the mRNA fragments were no longer observed. If TSEN cleaves one mRNA, could it have even more targets? A specialized type of RNAseq, Parallel Analysis of RNA Ends (PARE), identified additional targets of yeast TSEN (15). PARE specifically identifies endonuclease cleavage products by ligating an adapter onto the resulting 5′ phosphate (70, 71). This adapter is then used for amplification during RNAseq. Using this high-throughput method, several novel targets of TSEN were identified (15). While *Sulfolobus* TSEN mediates splicing of the *cbf5* mRNA, cleavage of *CBP1* or other mRNAs by yeast TSEN does not produce spliced products. Instead, the cleavage products are stabilized when *XRN1* is deleted or the cytosolic RNA exosome is inactivated (Fig. 4*C*) (13, 15, 69). Because Xrn1 and the cytosolic RNA exosome are the major cytosolic exoribonucleases, these data suggest that cleavage of eukaryotic mRNAs by TSEN results in mRNA decay. This novel decay mechanism was termed TED or tRNA Endonuclease-initiated mRNA Decay (15).

If TSEN specifically cleaves a small number of mRNAs, these mRNAs must share some feature(s) that would allow substrate recognition. These features appear to be a combination of location, structure, and sequence. Importantly, all of the endogenous yeast mRNAs that are cleaved by TSEN encode proteins localized to the mitochondria (15, 69). Such proteins are often imported cotranslationally, suggesting that the mRNA is localized near the mitochondria surface (72, 73). For some mRNAs, including *CBP1*, this localization occurs because the mitochondrial protein import machinery (the TOM complex) recognizes a “transit peptide” on the nascent protein (73). Importantly, *CBP1* cleavage is dependent on the transit peptide of the protein (69). In addition, mislocalization of TSEN by a small deletion in the Sen54 subunit prevented cleavage of *CBP1* while artificially restoring TSEN localization rescued cleavage (69). Overall, these results indicate that part of the mRNA substrate specificity of TSEN is explained by colocalizing the mRNA and enzyme. In addition to the transit peptide, a second region within *CBP1* is essential for TSEN cleavage and was predicted to form a stem-loop structure (69). This proposed structure has no obvious resemblance to a pre-tRNA, and for the other yeast mRNAs, no specific structure was predicted (15). These observations suggest yeast TSEN differs in substrate requirements from the archaeal TSEN, as the *cbf5* mRNAs cleaved by archaeal TSEN form a BHB motif similar to that of archaeal pre-tRNA introns. Notably, sequence specificity was assessed by comparing TSEN cleavage sites in yeast mRNAs. This comparison showed that most of these mRNAs shared an adenosine before the TSEN cleavage site (15). This mRNA specificity is distinct from tRNA specificity, as A, G, or U often precede the yeast pre-tRNA splice sites (http://gtrnadb.ucsc.edu/). Mutating this adenosine residue in *CBP1* decreased cleavage by TSEN both *in vitro* and *in vivo*. Therefore, yeast TSEN appears to recognize mRNA substrates based on mitochondrial colocalization, some sequence specificity, and possibly structure. However, these factors are likely only part of how TSEN identifies its mRNA targets, as only a subset of mitochondrial-localized mRNAs are TSEN targets.

The expanding list of TSEN substrates has generated new questions about the mechanism of recognition of all these targets. TSEN has two catalytic sites, and *CBP1* is cut at two sites (74). Do the two catalytic sites each cut at one site in the mRNA? It appears that one cut should be sufficient to initiate mRNA degradation. TSEN cleavage of mRNAs produces 5′ hydroxyl and 2′,3′ cyclic phosphate ends (75). The 3′ product is degraded by Xrn1, but Xrn1 strongly prefers 5′ monophosphate substrates over 5′ hydroxyl substrates (69, 76). This preference suggests that some enzyme may need to convert the TSEN product into a 5′ monophosphate RNA before Xrn1 can act. The 5′ product is known to be degraded by the RNA exosome (15, 69), but whether it can directly degrade from the 2′,3′ cyclic phosphate end or needs another enzyme to convert this is also unknown. Finally, the PARE technology requires that the cleavage product is converted to a 5′ monophosphate; therefore, any RNAs that persist with a 5′ hydroxyl would require an alternative strategy such as 5′-hydroxyl RNA-seq (77).

Yeast genetic experiments revealed that TSEN must have another essential function independent of tRNA processing, but we do not yet know what that function is. Yeast pre-tRNA splicing can be relocalized to the nucleus, and this nuclear TSEN can produce mature tRNAs that are correctly exported to the cytosol to be used for translation (78). Despite tRNA processing appearing normal by all metrics, these yeast cells were still inviable when the nuclear-localized TSEN was the sole TSEN enzyme, suggesting TSEN has an essential role in the cytosol. In addition, a cytosolic catalytic TSEN mutant was not able to rescue the nuclear-localized TSEN (78). Independent confirmation of an additional essential function was derived from expressing artificial intron-less tRNA genes. As mentioned above, this experiment bypasses the essentiality of tRNA ligase in yeast, trypanosomes, and *Caenorhabditis elegans* (17–19). However, the same yeast study showed that these intron-less tRNA genes do not bypass the need for TSEN (17). The fact that tRNA ligation is no longer essential with the intron-less tRNA plasmid suggests that the tRNA splicing pathway is successfully bypassed by the plasmid. Despite this bypass, all four subunits of TSEN were still essential. Although these results indicate that TSEN has another essential function independent of tRNA processing, the nature of this function remains unknown.

Perplexing localization of TSEN

The observation that yeast TSEN cleaves mRNAs that are localized near the mitochondria and that encode mitochondrial proteins provides a rationale for the long-puzzling observation that yeast TSEN is localized to the outside of the mitochondria. This localization is firmly established and was thought to be a peculiar feature of budding yeast TSEN. Interestingly, the same localization has been observed for fission yeast (79), which is a distant relative, suggesting that this localization pattern has been conserved for at least half a billion years. Evidence for localization to mitochondria includes GFP-tagging of the endogenous genes, immunolocalization with antibodies against the endogenous proteins, and immunolocalization with antibodies against an epitope-tagged version expressed from the endogenous locus (80). All three strategies revealed clear mitochondrial localization. In addition, TSEN subunits and activity cofractionated with markers for the outer mitochondrial membrane during glycerol, sucrose, or Nycodenz gradient centrifugation (80). This finding rigorously confirms the observation that yeast TSEN is enriched in a membrane fraction from which it can be solubilized with nonionic detergents (3) and raises the question of whether cytosolic tRNA splicing is widespread.

When the subunits of human TSEN were initially identified, GFP-tagged TSEN2 and myc-tagged TSEN34 were found localized to the nucleus (5). This study has been widely cited as showing that human TSEN is a nuclear enzyme. However, the localization of a single overexpressed subunit does not always faithfully reflect the endogenous complex. Nuclear localization of vertebrate TSEN is supported by *Xenopus* experiments, where injection of intron-containing pre-tRNAs into the nucleus results in their splicing, but injection into the cytosol does not (81). Furthermore, incubation of intron-containing pre-tRNAs with *Xenopus* oocyte nuclei or nuclear extract results in splicing, but the cytosolic extract is inactive (82). One caveat in these experiments is that oocytes are minimally active in transcribing and processing tRNAs. One possibility is that in rapidly dividing cells, a steady supply of spliced tRNAs is needed, but in nondividing cells such as oocytes, TSEN may perform a different role. On the other hand, intron-containing pre-tRNAs have been reported to be enriched in the human cytoplasm, both by cell fractionation and *in situ* hybridization (83). Localization of human TSEN and pre-tRNA was recently revisited using several methods to localize the endogenous complex (84). First, the authors fractionated cells to enrich for nuclear and cytosolic RNAs and proteins. The cytosolic fraction was also enriched for intron-containing tRNAs and TSEN. They also localized the intron-containing pre-tRNAs by FISH and again found them enriched in the cytosol. Finally, immunofluorescence with anti-TSEN antibodies showed specific cytoplasmic fluorescence that was reduced upon siRNA-mediated depletion of TSEN. In contrast, the nuclear signal was not reduced upon TSEN depletion, suggesting that it may result from cross-reaction of the antibody and may not reflect nuclear TSEN. Overall, some data suggest that human TSEN is cytosolic, while other data suggest it is nuclear, and thus localization of human TSEN activity remains controversial. With CRISPR/Cas technology, it should be possible to tag the endogenous TSEN genes with an epitope or fluorescent protein, an approach that would provide further confirmation of its localization. Noteworthy is that all of the studies on human TSEN localization have been done with cultured cells (HeLa, U2OS, 293T, and RPE-1). It is possible that TSEN localizes to different compartments in different cell types. Importantly, TSEN localization has not been characterized in the developing mammalian brain, which is an important issue because mutations in TSEN cause pontocerebellar hypoplasia (PCH), a disease characterized by underdevelopment of the fetal brain, further discussed below. It will be important to determine what cell types are primarily affected (*e.g.*, neurons, glia) and to localize TSEN in those cells most relevant to the disease.

A few studies have also addressed TSEN localization outside the animal/fungi lineage. Specifically, in trypanosomes (*Trypanosoma brucei*), intron-containing pre-tRNAs and splicing intermediates are enriched in the cytosol upon cell fractionation (18). In addition, when the catalytic subunit of TSEN was epitope-tagged, ectopically expressed, and immunolocalized, it was found diffused in the cytosol and not associated with either the nuclei or mitochondria (18). This cytosolic localization was confirmed in a high-throughput study (http://www.tryptag.org/), where both subunits were tagged at either the N or C terminus with mNeonGreen (85). All four fusion proteins localized to the cytoplasm (85). Importantly, this high-throughput study tagged the endogenous loci and avoids the caveats associated with overexpression. The same question has also been addressed in *Arabidopsis*. As discussed above, many plants only have one catalytic subunit, but *Arabidopsis* has two paralogs. Both paralogs were separately and transiently overexpressed in either bean or onion cells, which resulted in both nuclear and cytoplasmic localization (86). Notably, the cytoplasmic localization did not overlap with mitochondria. Since this experiment involves overexpression of a single subunit in a heterologous host, it is difficult to draw definitive conclusions, but the results are consistent with both cytosolic and nuclear localization of plant TSEN.

Because TSEN from *Saccharomyces* and *Schizosaccharomyces* are associated with the outer membrane of mitochondria and these two species are diverse members of the ascomycete fungi, it is reasonable to expect similar distribution in other ascomycetes. Furthermore, one might expect a localization sequence that is conserved in ascomycetes. Deletion of 32 amino acids of TSEN54 results in its failure to be localized to mitochondria, but TSEN2 still localizes to the mitochondria suggesting that yeast TSEN may contain multiple localization signals (80). These 32 amino acids are also unexpectedly poorly conserved among the ascomycetes. The corresponding region of human TSEN54 is much larger and was not captured in the structures of the TSEN complex as it is part of a large unstructured region (Fig. 2*E*). Thus, the exact sequence features that localize yeast TSEN remain unclear. The observation that TSEN54 and TSEN2 contain distinct localization signals is consistent with the hypothesis that TSEN subunits are localized to the mitochondrial membrane to increase local concentration and thereby facilitate complex assembly (87).

Similarly, the *trans*-acting factors responsible for TSEN localization are largely unknown. As mentioned above, yeast TSEN purifies with membrane-containing organelles. Treatment with 1% Triton X-100 or 0.2 M Na_2CO_3 releases the enzyme from the membrane (3, 80), consistent with a peripheral membrane association. A genetic screen in yeast indicated the translocase of the outer mitochondrial membrane (TOM) and sorting and assembly machinery (SAM) complexes are required for mitochondrial localization of TSEN (79). The requirement for the SAM and TOM complexes might reflect that TSEN is associated with one of these complexes. However, these complexes are required for targeting many other proteins into the outer membrane (including the SAM and TOM complexes themselves) and therefore TSEN could associate with any of these other TOM clients. Full understanding requires further biochemical studies and structural details of the ascomycete TSEN complex.

Are tRNA splicing and TSEN activity regulated?

Although there is limited evidence for regulation of either TSEN activity or tRNA splicing, there is clear evidence for the regulation of TSEN's pre-tRNA substrates. In particular, the human genome contains five genes for tRNA-Arg-UCU. Four of these contain an intron and are ubiquitously expressed. The fifth tRNA-Arg-UCU gene is a brain-specific tRNA that lacks an intron (88) (http://gtrnadb.ucsc.edu/). This intron distribution is conserved in mouse, where there are four intron-containing tRNA-Arg-UCU genes plus a brain-specific intron-less gene. A mutation in this brain-specific intron-less tRNA-Arg-UCU gene in mice leads to ribosomal pausing at AGA codons (88). If GTPBP2, a GTPase that releases stalled ribosomes, is also absent, this pausing is exacerbated, resulting in neurodegeneration (88). One possible explanation for the need of a brain-specific intron-less tRNA-Arg-UCU gene is that TSEN activity may be lower than in other cells. A relatively low TSEN activity level in the brain could also explain why modest defects in TSEN preferentially affect the brain.

A puzzling observation is that the fifth protein that associates with metazoan TSEN, CLP1, is a polynucleotide kinase that can phosphorylate the tRNA 3′ exon and intron in addition to other RNAs *in vitro* (89). This CLP1-TSEN association is conserved between *Drosophila* and human (90). CLP1 can phosphorylate the 5′ end of RNAs that contain a 5′ hydroxyl, but the context in which it performs this activity is not clear. In addition to being part of the TSEN complex, CLP1 is also part of the cleavage and polyadenylation machinery (hence its name, cleavage factor polynucleotide kinase subunit 1), and it has been implicated in phosphorylating siRNAs (89). Although it is unclear if CLP1 affects tRNA splicing *in vivo*, it does have the biochemical capacity to inhibit the ligation step of tRNA splicing in Metazoa, which requires a 5′ hydroxyl. This requirement is in contrast to the yeast ligase, which requires a 5′ monophosphate. Yeast Clp1 is catalytically inactive and does not associate with yeast TSEN. One suggestion is that the 5′ end on the intron might be more accessible to CLP1 than the 5′ end on the 3′ exon, which would allow CLP1 to stimulate intron degradation by 5′ monophosphate-specific exonucleases without preventing exon ligation (7). Similarly, converting the 2′-3′ cyclic phosphate to 2′ and 3′ hydroxyls would inhibit ligation of the tRNA exons. The mammalian ANGEL2 protein has the required biological activity (91), but whether it inhibits tRNA splicing *in vivo* remains unknown. While CLP1- and ANGEL2-modified tRNA exons cannot be ligated by the well-characterized tRNA ligase RTCB, a recently discovered alternative ligase, C12orf29, could ligate these modified RNAs (92).

TSEN and PCH

With advances in whole genome sequencing, a study in 2008 identified the first mutations in TSEN linked to the

human disease PCH (93). PCH is a condition where the cerebellum and sometimes other parts of the brain atrophy during development, resulting in a variety of symptoms including but not limited to seizures, microcephaly, developmental failure, respiratory conditions, loss of motor development, intellectual disabilities, and an overall failure to thrive very early in childhood (94–96). PCH is usually lethal before the patient reaches adolescence, though milder forms of the disease have recently been identified. Mutations in all four subunits of the TSEN complex have been linked to PCH (Fig. 5). Many of the causative mutations result in single amino acid changes, although some patients have one allele with a single amino acid change and a second more damaging allele (*e.g.*, a nonsense or frame shift allele). Interestingly, one particular single amino acid change in CLP1 (R140H) has also been shown to cause PCH (97).

Animal models of TSEN (98, 99) and CLP1 (97, 99–103) mutations recapitulated related phenotypes and showed a marked loss of motor neurons. It is unclear what happens at the cellular level that causes these phenotypes to manifest and whether CLP1 and TSEN mutations lead to PCH through a common mechanism. As pointed out above, it is possible that the developing human brain is more sensitive to altered tRNA levels or that some other TSEN function is critical for brain development. Both recent and older experiments show that archaeal and yeast TSEN have functions beyond tRNA splicing and these roles could also be relevant to PCH. Some studies suggest that TSEN dysregulation causes apoptosis (97, 99, 104), specifically with a role for p53 (97, 100, 105), while a recent report using human organoids did not find apoptosis present in the organoid model (106). Regardless, these studies suggest that mutated TSEN and CLP1 relate to dysregulated RNA processing (97, 100–102). Two major themes for this mechanism are a tRNA- and an mRNA-targeted route, though neither is mutually exclusive. In the case of the former, studies have shown that mutations in CLP1 lead to dysregulated tRNA processing and an accumulation of potentially toxic tRNA fragments, with impacts that vary across pre-tRNAs (97, 101, 102, 105). At this time, there is more evidence for a role of fragments in altered cellular homeostasis instead of altered mature tRNA pools or an accumulation of pre-tRNAs. Furthermore, a recent study of a mouse model of the PCH mutation for CLP1 found that while relative abundance of tRNA fragments did change, there was also an important change in the landscape of mRNA cleavage due to altered polyadenylation site selection (101), further supporting an mRNA-driven model of PCH. While no mRNA targets of the TSEN complex in humans have yet been identified, identification of novel substrates may elucidate if there are parallel roles for TED in humans as has been identified in yeast (15) or if there are other important roles for TSEN in human mRNA processing in the brain.

From the disease perspective, it would be valuable to understand what cell type(s) are primarily affected in PCH, which would likely require animal models that reflect the single amino acid changes in TSEN seen in patients. How does TSEN change the state of these cells? We do not yet understand why mutations in TSEN and CLP1 cause similar diseases, but this picture becomes even more complicated if one considers all 17 types of PCH (omim.org). While all the PCH types share some symptoms, other aspects of the disease differ. Furthermore, the genes mutated in PCH types 2, 4, 5, 7, and 10 are linked to tRNA function in the cytosol (TSEN, CLP1, and SEPSECS), but mutations for PCH type 6 are linked to mitochondrial tRNA dysfunction (RARS2). PCH types 1 and 7 are more broadly linked to RNA function (RNA exosome and TOE1, both 3′ exonucleases), and the mutations for yet other PCH types have no apparent link to RNA biology at all (omim.org). Does this diverse set of genes that are mutated in the PCH types have a common function/effect or should these even be classified as the same disease?

Not only is the underlying cause of PCH poorly understood, the individual contributions of PCH-causing mutations to cellular disruptions also remains an open question.

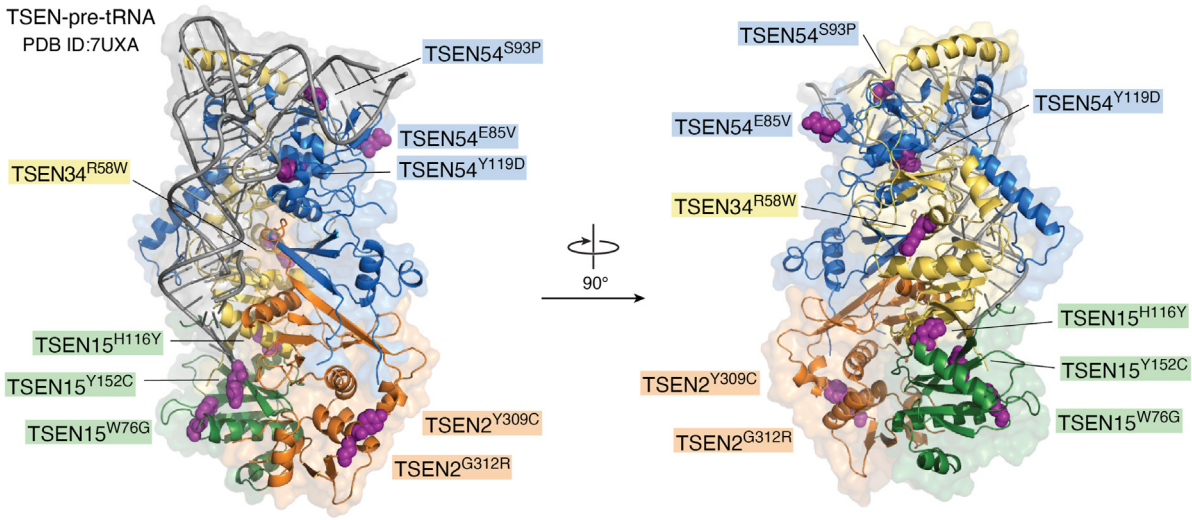

Figure 5. Pontocerebellar hypoplasia causing mutations mapped on the TSEN complex. Amino acids that are well documented to be mutated in PCH are shown mapped on a TSEN-pre-tRNA structure (PDB ID: 7UXA) and displayed as *purple atomic spheres* for contrast. TSEN, tRNA splicing endonuclease.

Reconstitution of the full complex is necessary to study the TSEN complex *in vitro* (64). In such a reconstitution system, there was no difference in the catalytic function of several purified PCH mutation–containing complexes. Instead, PCH-causing TSEN mutations affected the thermal stability of purified TSEN and lysates from patient-derived cells had reduced tRNA cleavage activity (61). These findings suggest both protein/complex stability and/or intracellular regulation may play a role in the altered complex's function. Contextualizing these mutations remained a challenge because, until recently, there was no structure for any full eukaryotic TSEN complex. The recent structures of human TSEN in complex with a pre-tRNA (6, 8) reveal the molecular environment in which PCH mutations occur and allow for more accurate predictions of how each mutation might impact the structure, function, or regulation of the TSEN complex. Analysis of these structures suggests that mutations affect the inter- and intra-molecular interactions of the complex and that the endonuclease active sites are not affected in known PCH-causing mutations. It is not surprising that the mutations do not occur in the active sites of the endonuclease subunits since these proteins are essential for life (4, 107).

Potential impacts of many PCH mutations can now be summarized based on the recently solved structures (Fig. 5). For TSEN34, a known PCH-causing mutation TSEN34-R58W eliminates a salt bridge between TSEN34-R58 and TSEN34-E218 (6, 8), potentially causing a reduction in TSEN34's stability, as demonstrated previously (61). Similarly, PCH mutations in TSEN15 may affect complex/subunit stability. TSEN15-H116 forms an important hydrogen bond network at the interface with TSEN34, and mutating this residue reduced thermal stability *in vitro* (61). Similarly, TSEN15-W76 and TSEN15-Y152 also appear to form important stability-linked bonding networks within TSEN15. In contrast, PCH mutations at residues TSEN2-Y309 and TSEN2-G312 are in surface-accessible regions of TSEN2 that could be important for either complex stability or other protein–protein interactions. These residues are close but not connected, which suggests this shared surface interface may be important for binding to another factor. Furthermore, TSEN2-Y309 likely participates in a complex hydrogen bonding network, which potentially explains why its mutation leads to a major decrease in its *in vitro* thermal stability (61). Finally, PCH mutations in TSEN54 reside in regions that have varying roles. TSEN54-S93P is in a region that seems to be important for conformational rigidity (8), while TSEN54-E85V is in a surface-exposed region that is also near the tRNA-binding interface, with no observed direct binding to tRNA (6). The mutation TSEN54-Y119D would disrupt an important hydrogen bonding network that presumably coordinates both subunit stability and, indirectly, coordination to tRNA binding through a hydrogen bond between TSEN54-Y119 and TSEN34-R41 (6, 8). The most prevalent mutation, which contributes to the most severe forms of PCH, TSEN54-A307S (95, 108, 109), was not visualized in any cryo-EM structure, suggesting this region may be disordered. Two studies found that TSEN54 mediates CLP1 binding (6, 8), with one study determining that residues 179 to 424 of TSEN54 are required for CLP1 binding (8). These findings suggest that the TSEN54-A307S mutation may interfere with CLP1's association with the TSEN complex.

The recent cryo-EM structures provide valuable insights into how PCH mutations in TSEN disrupt TSEN biology, but no unifying conclusion has yet emerged. Some mutations likely disrupt folding and thermal stability, but others occur in disordered regions. Much remains to be understood about how these mutations impact interactions of the complex with RNA, CLP1, and potentially other proteins.

Answering some questions raises many new ones

We have learned a great deal about TSEN function over the last 50 years, but much remains to be discovered. TSEN clearly plays a critical role in tRNA splicing, but its function extends beyond tRNAs. It will be important to determine all of the substrates of TSEN as well as the noncanonical roles of the complex. Yeast has proven to be a powerful model organism to probe these questions, but studies need to be extended to human TSEN and other metazoan TSENs. By leveraging the new human TSEN structures, it should be possible to uncover principles of RNA recognition by TSEN and the potential role TSEN mutations play in disease.

A better understanding of yeast and human TSEN also leads us to rethink whether these enzymes reflect biology shared with all eukaryotes. The early branches of the eukaryotic tree have not been entirely resolved, but it is clear that fungi, animals, and Amoebozoa are on one branch of the tree (the Amorphea) and that they likely share a heterotetrameric TSEN that is asymmetric and recognizes the asymmetric L-shape of the pre-tRNA. However, other eukaryotes have a symmetric TSEN that is a homodimer of heterodimers $(\alpha\beta)_2$ that may recognize pre-tRNAs by local secondary structure. These other eukaryotes include plants, as well as important human parasites (*Plasmodium*, trypanosomes). Perhaps this difference in biology can be exploited to develop badly needed antiparasitic drugs. In addition, a better appreciation of which different TSENs architectures restrict introns to the "canonical" position should help improve algorithms to detect tRNA introns in noncanonical positions and annotate eukaryotic genomes with $(\alpha\beta)_2$ TSENs. It was proposed almost 20 years ago that different archaeal TSEN architectures correlated with the capacity to cleave noncanonical tRNA introns (110), but it would be interesting to revisit the correlation between TSEN architecture and tRNA intron position, mRNA splicing function, rRNA processing functions, or substrate secondary structure, now that thousands of archaeal genomes have been sequenced.

With an increased knowledge of the diversity of TSENs and TSEN substrates, we also need more structural insights. For example, it remains unclear how yeast TSEN recognizes and cleaves mRNA substrates. Moreover, we lack structural insight of plant and parasite TSENs. Continued advances in tRNA sequencing, protein and RNA structure prediction, *in situ* structure determination by cryo-EM, and other emerging methods will no doubt add new dimensions to our

understanding of TSEN biology in the years to come. It is an exciting time to study TSEN.

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Abbreviations—The abbreviations used are: BHB, bulge-helix-bulge; NTE, N-terminal extension; PARE, Parallel Analysis of RNA Ends; PCH, pontocerebellar hypoplasia; SAM, sorting and assembly machinery; TOM, translocase of the outer mitochondrial membrane; TSEN, tRNA splicing endonuclease.

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