Crystal structures and insights into precursor tRNA 5′-end processing by prokaryotic minimal protein-only RNase P

Yangyang Li¹, Shichen Su², Yanqing Gao¹, Guoliang Lu², Hehua Liu¹, Xi Chen¹, Zhiwei Shao¹, Yixi Zhang¹, Qiyuan Shao¹, Xin Zhao¹, Jie Yang¹, Chulei Cao¹, Jinzhong Lin², Jinhiao Ma² & Jianhua Gan¹

Besides the canonical RNA-based RNase P, pre-tRNA 5′-end processing can also be catalyzed by protein-only RNase P (PRORP). To date, various PRORPs have been discovered, but the basis underlying substrate binding and cleavage by HARPs (homolog of Aquifex RNase P) remains elusive. Here, we report structural and biochemical studies of HARPs. Comparison of the apo- and pre-tRNA-complexed structures showed that HARP is able to undergo large conformational changes that facilitate pre-tRNA binding and catalytic site formation. Planctomycetes bacterium HARP exists as dimer in vitro, but gel filtration and electron microscopy analysis confirmed that HARPs from Thermococcus celer, Thermocrinis minervae and Thermocrinis ruber can assemble into larger oligomers. Structural analysis, mutagenesis and in vitro biochemical studies all supported one cooperative pre-tRNA processing mode, in which one HARP dimer binds pre-tRNA at the elbow region whereas 5′-end removal is catalyzed by the partner dimer. Our studies significantly advance our understanding on pre-tRNA processing by PRORPs.
Transfer RNA (tRNA) decodes mRNA and transfers the genetic information from mRNA to protein. The precursor of tRNA (pre-tRNA) undergoes a series of post-transcriptional processes, including nucleobase modification\(^1\)-\(^3\), splicing\(^4\)-\(^7\), 5’-end cleavage\(^8\)-\(^10\) and 3’-end cleavage and conserved CCA addition\(^1\)-\(^3\);\(^11\)-\(^14\). Correcting processing is essential for the function of tRNA. In addition to pre-tRNA coding genes, mutations in many pre-tRNA processing enzymes have also been linked with serious diseases\(^15\)-\(^21\). RNase P catalyzes the 5’-end cleavage of pre-tRNA. The canonical RNase P was first discovered in Bacteria\(^22\)-\(^23\), it was later found in Archaea and Eukarya\(^24\). The canonical RNase P occurs as ribonucleoprotein (RNP) composed of one RNA and various number of proteins\(^24\)-\(^25\), but pre-tRNA 5’-end is all cleaved by the RNA molecule.

Interestingly, recent studies showed that pre-tRNA 5’-end can also be cleaved by protein-only RNase Ps (PRORP). To date, PRORP has been found in the mitochondria of human, Caenorhabditis elegans (C. elegans), Drosophila and many other animals\(^26\)-\(^27\). These mitochondrial PRORPs are composed of three proteins, MRPP1-3. Pre-tRNA 5’-end cleavage is catalyzed by MRRP3 (Fig. 1A), but it strictly requires the assistance of MRPP1 and MRPP2. PRORP is also present in plants and protists\(^28\), such as Arabidopsis thaliana (A. thaliana) and Trypanosoma brucei (T. brucei)\(^29\). Although AtPRORPs and TbPRORPs are homologous to human MRPP3 (Fig. 1A), they are active on their own.

Bacteria and Archaea do not express eukaryotic-like PRORP proteins, but in vitro pre-tRNA 5’-end cleavage activity has been confirmed for protein Aq_880 (Fig. 1A) encoded by the hyperthermophilic bacterium Aquifex aeolicus (A. aeolicus)\(^30\). Besides A. aeolicus, bioinformatic analysis showed that homologs of Aquifex RNase P (termed HARP hereafter) are also present in some other Bacteria and are even more wide-spread in Archaea\(^30\)-\(^31\). Like the eukaryotic PRORPs, HARPs are members of the PIN domain superfamily, but belong to a different subgroup (PIN_5 cluster)\(^32\). Eukaryotic PRORP contains three domains: the pentatricopeptide repeat (PPR) domain, the central domain and the catalytic metallonuclease domain. Compared to eukaryotic PRORP, HARP is much shorter and it lacks both PPR and the central domains. The catalytic metallonuclease domain is conserved, but it only shares very limited sequence similarity (<25%) between HARP and eukaryotic PRORP.

In addition to Aq_880, the in vitro pre-tRNA 5’-end cleavage activity has also been confirmed for HARPs from Haloflexax volcanii (H. volcanii) and Matheosarcina maezi (M. maezi), which are members of the euryarchaeotes\(^33\). Aq_880 shares conserved residues with HvHARP, MmHARP and HARPs from many other species (Supplementary Fig. 1A). Knockout of the HARP gene showed no growth defects in H. volcanii and M. maezi, indicating that the RNA-based RNase P is the main and essential RNase P in these archaea\(^33\). Different from euryarchaeotes, HARP is the only RNase P expressed in A. aeolicus and many related Aquificaceae, such as Hydrogenobacter thermophiles and Thermococcus albus DSM. Aq_880 is able to replace the RNase P complex in budding yeast Saccharomyces cerevisiae and rescue the growth of E. coli strain BW, in which the chromosomal expression of RNase P RNA is switched off by replacing arabinose with glucose in the medium\(^30\). To improve our understanding on pre-tRNA binding by HARP, we performed extensive structural and biochemical studies. Here, we report the crystal structures of Planctomyces bacterium HARP (PbHARP) and Thermococcus celer HARP (TcHARP). Comparison of the apo- and pre-tRNA-complexed PbHARP structures showed that HARP is able to undergo large conformational changes that facilitate pre-tRNA binding and catalytic site formation. Electron microscopy analysis of Thermococcus ruber HARP (TrHARP) showed that the dimer of HARP can assemble into oligomers. Structural analysis, mutagenesis, in vitro cleavage and cross-linking assays all suggested a HARP pre-tRNA binding and cleavage mode involving the cooperation of two dimers.

**Results**

**Pre-tRNA 5’-end cleavage activity is conserved in HARPs.** To investigate whether the pre-tRNA 5’-end cleavage activity is conserved in HARPs, we constructed and purified four HARPs, PbHARP, TcHARP, TmHARP and TrHARP, which are coded by Planctomyces bacterium GWF2_40_8 (P. bacterium), Thermococcus celer (T. celer), Thermococcus minerva (T. minerva) and Thermococcus ruber (T. ruber), respectively. T. celer is a hyperthermophilic archaeon, T. minerva and T. ruber are all members of Aquificaceae. PbHARP, TcHARP, TmHARP and TrHARP share 60-80% sequence similarities with Aq_880, and the sequence identity is about 30% among the five proteins (Supplementary Fig. 1B).

All proteins were purified to homogeneity. During purification, we noticed that the oligomerization state of HARP varies (Supplementary Fig. 2). PbHARP exists as dimer in buffer containing 300–500 mM salt; at lower concentration of salt, it precipitates out. The oligomerization state of TcHARP is changeable. At higher concentration of salt (200–500 mM), it exists as dimer; however, it assembles into larger oligomers in buffer containing 100 mM salt. Using 1.0 μM Thermus thermophiles pre-tRNA\(^{Gly}\) (Supplementary Fig. 3A) purified in the laboratory, we performed in vitro cleavage assay (Fig. 1B) for PbHARP, TcHARP, TmHARP, TrHARP and Aq_880, which served as a positive control. The HARP concentrations are all controlled to be equal. About 6% pre-tRNAGly at a reaction time of 20 min.

**Overall folding and dimerization of apo- PbHARP and TcHARP.** Puzzled by their different oligomerization states and catalytic efficiencies, we performed crystallographic studies for all the HARPs. No crystal grew for Aq_880, TmHARP or TrHARP, but we successfully solved the apo-form structures of PbHARP and TcHARP at atomic resolution (Supplementary Table 1). PbHARP crystal belongs to P3\(_1\) space group, per asymmetric unit contains two PbHARP dimers. TcHARP crystal belongs to P2\(_1\)2\(_1\)2\(_1\) space group and there is only one TcHARP dimer in the asymmetric unit. Both PbHARP and TcHARP are of α/β fold in nature (Fig. 1C-F, Supplementary Fig. 4); they share six β-strands (β1-β6) and ten α-helices (α1-α10). Compared to TcHARP, PbHARP is 14 amino acid longer at its N-terminus, which forms one short extra helix, α0. β1-β3 and β5-β6 are parallel to each other, forming one flat β-sheet at the center of the metallonuclease domain. Helices α1-α6 and α10 reside on one side of the β-sheet, whereas α9 and the C-terminus of α8 locate on the opposite site.

β4 and α7 are not involved in the metallonuclease domain formation. β4 is linked to the metallonuclease domain through one short linker that is perpendicular to the C-terminus of α8 (Fig. 1C). α7 is separated from α8 by a short linker. Indicated by the weak electron density and different conformations (Supplementary Fig. 4C), the α7-α8 connecting linker is flexible. Both α7 (amino acids 97-122) and α8 (amino acids 131-165) are very long, forming a four-helix bundle (HB) with α7 and α8 from the partner molecule. Due to the presence of Lys or Arg residues, the surface of HB is highly positive in charge (Fig. 1D). The
The metallonuclease domain is also positive on the surface distal from HB, whereas is highly negative at the region facing HB. 

β4 plays an important role in PbHARP dimerization. As depicted in Fig. 1G, the β4 strands of the two monomers form four hydrogen (H) binding interactions: two between the backbone carbonyl and amino functions of the two V95 residues and the other two mediated by M93 and A97 residues. These H-bond interactions are very stable, supported by their short distances (2.7–2.85 Å). The dimerization manner of TcHARP is similar to that of PbHARP. However, structural analysis revealed some conformational differences between the HB regions (Fig. 1F). Further analysis showed that the overall folds of the two TcHARP monomers are similar to that of PbHARP monomer A (or C) (Supplementary Fig. 4D), the conformational differences are mainly caused by the bending of α8 of PbHARP monomer B (or D), hinged at residue E147 (Fig. 1H). The root-mean-square deviation (RMSD) value between TcHARP and PbHARP A or C monomers is 1.0 Å, whereas is 1.4 Å between TcHARP and PbHARP B or D monomers.

### Pre-tRNA recognition by PbHARP

Besides apo-proteins, we also performed co-crystallization trials using pre-tRNA\(^{\text{Gly}}\), but no crystal was obtained. We then transcribed and purified another three pre-tRNAs, including E. coli pre-tRNA\(^{\text{His}}\), mitochondrial pre-tRNA\(^{\text{Cys}}\) and chloroplastic pre-tRNA\(^{\text{Phe}}\) (Supplementary Fig. 3A). After extensive trials, we solved the complex structure of PbHARP/pre-tRNA\(^{\text{His}}\) at 2.8-Å resolution (Supplementary Table 1). The crystal belongs to P41212 space group and it contains two PbHARP dimers (AB and CD) and one pre-tRNA\(^{\text{His}}\) molecule in the asymmetric unit (Fig. 2A). As indicated by the low RMSD value (0.6 Å), the overall folds of PbHARP AB and CD dimers are very similar (Supplementary Fig. 5A). Out of the 85 nucleotides of pre-tRNA\(^{\text{His}}\), G-10 to U-2, U32 to A36 and C73/
A74 located at the 5'-end, anticodon loop and the 3'-end are disordered, whereas other nucleotides are all well-defined (Supplementary Fig. 5B).

Pre-tRNA\textsuperscript{His} is recognized by the AB dimer of PbHARP. Although the overall folds of the metallonuclease domains and the HB domains are conserved, their relative orientations are different within the two monomers (Supplementary Fig. 5C). The asymmetric dimerization orients the side chains of four HB domain Arg residues, including R116 and R123 of monomer B and R138 and R142 of monomer A, toward the pre-tRNA\textsuperscript{His} T\textsubscript{Ψ}C-loop where they form extensive H-bond interactions with the phosphate backbones of G52-G56 region (Fig. 2B). In details, R116 forms two H-bonds: one with the OP1 atom of G52 and the other with the OP2 atom of U53 (Fig. 2C). Both R123 and R138 interact with G56. Unlike G52-C55 region, the phosphate backbone of G56 is severely bent and packs against the nucleobase of U54 (Fig. 2C). R123 interacts with the OP1 atom of G56, whereas R138 interacts with both OP1 and OP2 atoms. Besides G56, R138 also interacts with the OP2 atom of C55 (Fig. 2D). R142 forms two H-bonds: one with the OP1 atom of U53 and the other with the OP2 atom of U54 (Fig. 2E).

In the complex structure, the acceptor stem and the predicted 5'-end cleavage site of pre-tRNA\textsuperscript{His} are far away from PbHARP (Fig. 2A), which prompted us to ask whether PbHARP has pre-tRNA\textsuperscript{His} cleavage activity. To this end, we carried out in vitro cleavage assay using 0.05 \textmu M PbHARP and 1.0 \textmu M pre-tRNA\textsuperscript{His} (Supplementary Fig. 3B). Although it is weak, PbHARP can remove the 5'-end of pre-tRNA\textsuperscript{His}. Besides pre-tRNA\textsuperscript{His}, we showed that PbHARP is also able to process pre-tRNA\textsuperscript{Cys} and pre-tRNA\textsuperscript{Phy} under identical reaction conditions (Supplementary Fig. 3B). We then asked whether the pre-tRNA cleavage by PbHARP is specific. To address this question, we performed pre-tRNA\textsuperscript{Phe} (5.0 \textmu M) cleavage assay using 0.25 \textmu M PbHARP, Aq\textsubscript{880} or \textit{E. coli} RNase P. The reaction mixtures were separated on 20% denaturing UREA-PAGE gels (Supplementary Fig. 3C). No cleavage product was observed in the absence of RNase P. Two 5'-leader cleavage products were observed, one being identical in size to that generated by Aq\textsubscript{880} and \textit{E. coli} RNase.
P (Supplementary Fig. 3C, outer right lane). This shows that PbHARP cleaves pre-tRNA at the canonical cleavage site in addition to some apparent miscleavage at the neighboring upstream site.

To verify whether the interactions observed in the complex are functionally relevant, we constructed five PbHARP mutants, including R116A, R123A, T135A, R138A and R142A. T135 interacts with pre-tRNAHis in the complex (Fig. 2E), but it is not conserved (Supplementary Fig. 1). Surprisingly, in vitro binding assays showed that Ala substitution of the five pre-tRNA recognizing residue has no obvious impacts on pre-tRNA binding by PbHARP (Supplementary Fig. 6A). Unlike many other homologs proteins, PbHARP is longer at its N-terminus. The extra PbHARP terminus is highly positive in charge; out of the total 13 residues, 7 are Arg or Lys (Supplementary Fig. 1B).

To test whether the extra terminus contributes to the unexpected pre-tRNA binding results, we constructed one truncated protein, PbHARP_ΔN (aa 14-203). Compared to the full-length protein, the pre-tRNA binding ability of PbHARP_ΔN is weaker (Supplementary Fig. 6B). Started from PbHARP_ΔN, we then constructed several PbHARP mutants with pre-tRNA recognizing residue mutation. Although it has no strong impact on the overall pre-tRNA binding ability, Ala substitution of either R116 (for the PbHARP_ΔN _R116A mutant) or R123 (for the PbHARP_ΔN _R123A mutant) altered the pre-tRNA binding mode of PbHARP, indicated by the smeared bands on the gel. The pre-tRNA binding ability of PbHARP_ΔN _R138A is weaker than that of PbHARP_ΔN. No obvious difference was observed for the PbHARP_ΔN _R142A mutant, but the PbHARP_ΔN _R138A/R142A mutant showed weaker pre-tRNA binding ability, compared to the PbHARP_ΔN _R138A mutant.

The above mutation and binding assay results indicated that R116, R123, R138 and R142 affect pre-tRNA binding by PbHARP. To further confirm the functional importance of these residues, we performed in vitro cleavage assay using equimolar (1.0 μM) pre-tRNAHis and proteins (Fig. 2F). Substitution of T135 by Ala has no obvious impacts on pre-tRNAHis cleavage, compared to the wild-type (WT) PbHARP. In contrast to T135, R116, R123, R138 and R142 are all highly conserved (Supplementary Fig. 1). Ala substitution of either R116, R123 or R142 caused a dramatic reduction of the protein's pre-tRNAHis cleavage activity. No detectable pre-tRNAHis cleavage activity at all was observed for the R138A mutant. Altogether, these observations suggested that R116, R123, R138 and R142 are critical for assembly of the catalytically active PbHARP/pre-tRNA complex.

In the apo-form PbHARP structure, both D151 and D155 reside at the central region of a8 and D173 locate at the N-terminus of a9 (Fig. 3A, Supplementary Fig. 8A). The side chains of D155 and D173 are close to each other, forming water-mediated H-bond interactions. In contrast, the side chain of D151 points away from D155, the averaged distance between their carboxylate groups exceeding 7 Å. R110 locates at the middle of a7; its side chain points toward D155, forming two direct H-bond interactions between their guanidine and carboxylate groups. Such conformations are incompatible with Mg2+ coordination with D151, D155 and D173, suggesting that the apo-form PbHARP structure is actually in an inactive state.

As demonstrated for many nucleases, such as RNase III35,36, RNase D27 and RNase H28, Ca2+ is insufficient in supporting the cleavage activity but can mimic Mg2+ in coordination. The crystal of PbHARP/pre-tRNAHis was grown in the presence of 10 mM CaCl2. In the structure, several well-defined Ca2+ ions were captured (Supplementary Fig. 8B). As demonstrated by the A monomer of PbHARP (Fig. 3B), Ca2+ directly coordinates with the side chain OD1 atom of D151. In addition, it also coordinates with the main chain O atoms of R146 and I149. The side chains of D151, D155 and D173 are close to each other. Although not observed in the structure, this arrangement may allow PbHARP to coordinate a second divalent cation, thereby utilizing the common two metal ion-assisted mechanism of catalysis35,37,38.

Proper dimerization is essential for the function of PbHARP. PbHARP exists as dimer in both the apo- and pre-tRNAHis-complexed PbHARP structures. As depicted in Fig. 3C, the relative orientations of the metallonuclease domains are preserved, but the orientations of the HB domains are significantly different in the two structures. Compared to the apo-structure, all the HB domain helices are anticlockwise rotated in the complex. Further structural analysis (Supplementary Fig. 8C-E) revealed that the inactive and active state switching of PbHARP is mainly mediated by the rearrangement of the HB domain, including rotation of a7 and shifting of the N-terminus of a8. Due to the structural rearrangement, a8 is split into two helices (termed a8a and a8b) in the complex. a8a and a8b are linked by one short loop, starting at the hinge residue E147 and ending at the catalytic residue D151 (Fig. 3D).

Proper dimerization is essential for the function of PbHARP. PbHARP exists as dimer in both the apo- and pre-tRNAHis-complexed structures. The dimerization interactions mediated by β4 (Fig. 1F, G) are maintained in both structures; however, due to the rearrangement of the HB domain, the dimerization interactions mediated by a7 and a8 varied. In the apo-form structure, L103, I104, V107 and I111 of a7 and L145 of a8 all locate around the central axis of HB, forming extensive hydrophobic interactions (Fig. 4A). V107 of the two partner molecules directly interact with each other, whereas L103 and I111 interact with I104 and L145 of the partner molecule, respectively. In the pre-tRNA-complexed structure (Fig. 4B), L103 of the two PbHARP monomers interact with each other. Stable hydrophobic interactions are also observed between the two V107 residues. Compared to the apo-form structure, the averaged distance between I111 of the two monomers is increased by 2 Å, altering the interactions between I111 and L145 in the complex.

In the apo-form structure, the N-termini of a8 helices are close to each other (Fig. 1C). However, the C-termini of a7 helices gathered together in the complex structure, allowing cooperative pre-tRNA binding by R116, R123, R138 and R142 of the two monomers (Fig. 2B-E, Supplementary Fig. 5A, C). Like the four
Arg residues, A118 is highly conserved (Supplementary Fig. 1). In the complex structure, the A118 residues center around the HB domain central axis, the distance between their side chains is only 3.6 Å (Fig. 4C). Except A118, many other hydrophobic residues, including A121 and Y141, are also arranged around the central axis (Fig. 4C).

To investigate the functional importance of HARP dimerization, we constructed and purified one PbHARP mutant, L103E/V107E, in which the hydrophobic L103 and V107 are simultaneously substituted by negatively charged Glu residues. Different from WT PbHARP, the size exclusion chromatographic study indicated that the L103E/V107E mutant mainly exists as monomer with an elution volume of 15.6 mL (Fig. 4D). Compared to WT PbHARP, the L103E/V107E mutant is less stable, indicated by its lower melting temperature (51.1 °C) measured by the Nano-DSF method (Supplementary Fig. 9A, B). In addition, we also performed CD spectra analysis for the two PbHARP proteins (Supplementary Fig. 10). In consistent with the crystal structure (Supplementary Fig. 4A, B), the CD results (Supplementary Fig. 10A, B) showed that WT PbHARP possesses ~50% α-helices and 10% β-strands (Parallel + Antiparallel) at 20 °C; the calculated Tm value is ~70.0 °C. Different from the WT protein, the L103E/V107E mutant (Supplementary Fig. 10C, D) possesses ~30% α-helices and 20% β-strands (Parallel + Antiparallel) at 20 °C; the Tm value of the mutant is about 53.0 °C. These results suggested that the L103E/V107E mutation affected the proper folding of PbHARP, which may play important role in the lower stability of the L103E/V107E mutant.

In addition to L103E/V107E, we also constructed the A118E/A121E mutant of PbHARP, in which A118 and A121 are substituted by Glu. The elution volume (14.4 mL) of A118E/A121E is similar to that of WT PbHARP, suggesting that it also exists as dimer (Fig. 4D). However, different from WT PbHARP, the pre-tRNA binding affinity of A118E/A121E is weak; no pre-tRNA binding at all was observed for the L103E/V107E mutant (Supplementary Fig. 11A). WT PbHARP can cleave pre-tRNA, but detectable pre-tRNA cleavage activity was neither observed for the L103E/V107E nor for the A118E/A121E mutant under identical reaction conditions (Fig. 4E), probably due to their low stability and/or difficulty to form a functional dimer.

Length of the acceptor stem affects pre-tRNA cleavage by HARP. Compared to pre-tRNA Gly, pre-tRNA Cys and pre-tRNA Phe, the pre-tRNA cleavage activity of PbHARP is significantly weaker (Fig. 1B, Supplementary Fig. 3B). In the structure of the complex, PbHARP mainly recognizes the shape and backbone of the TwC-loop (Fig. 2B–E), a structural element conserved in all tRNAs. However, compared to other pre-tRNAs (Supplementary Fig. 3A), the acceptor stem of pre-tRNA His is one base pair longer, due to the pairing of G-1 and C72 which is not visible in Supplementary Fig. 5B. During pre-tRNA 5′-end maturation, the phosphodiester bond between nucleotides -1 and +1 is usually cleaved. To investigate whether the extra base pair contributes to the weaker pre-tRNA cleavage activity of PbHARP, we transcribed three pre-tRNA variants (Fig. 5A), His-7bp, His-6bp and His-9bp, which have seven, six and nine base pairs in the acceptor stem region, respectively.

Using 0.05 μM PbHARP and 1 μM native or mutated pre-tRNA, we performed in vitro cleavage assays. As depicted in Fig. 5B, no His-9bp cleavage activity was observed for PbHARP. Although it can be cleaved, the cleavage efficiency of native pre-tRNA was much weaker than those of His-7bp and His-6bp. In fact, the cleavage efficiency of His-7bp was comparable to those of pre-tRNA Cys and pre-tRNA Phe under identical reaction conditions (Supplementary Fig. 3B). Besides PbHARP, we also performed in vitro cleavage assay using 0.05 μM TrHARP.
Similar to PbHARP, TrHARP failed to cleave His-9bp and also showed a clear preference for His-7bp.

Oligomerization and assembly of TrHARP. Both TrHARP and PbHARP preferably cleaved the His-7bp variant (Fig. 5B, C), but their oligomerization states are different. TrHARP eluted from the size exclusion column at ~11.4 mL in all buffers containing 100, 200 or 500 mM salt (Fig. 6A). A previous study suggested the possibility that Aq_{880} may form either six trimers or three hexamers (inferred from size exclusion chromatography profiles), but both PbHARP and TrHARP formed dimers in the crystal structures (Fig. 1C–F). To gain more insights into the assembly of HARPs, TrHARP collected from the peak of the size-exclusion columns was subjected to electron microscopy. As depicted in Fig. 6B and S12A, TrHARP produced many well-defined particles on the negative stain CCD images, which can be divided into 20 different classes (Fig. 6C).

The 2D class average showed an open ring-like conformation of TrHARP. The shape and size of the ring matched well with an assembly of six PbHARP dimers that arrange in a left-handed orientation (Fig. 6D). Oligomerization of TrHARP is mainly mediated by the metallonuclease domains, which form the main body of the ring; the HB domains of TrHARP all point toward the outside and are roughly vertical to the central rotation axis of the ring. Dimer_1 and Dimer_6 reside near the cleft of the ring, the rotation angle between them is ~80° (Supplementary Fig. 12B). The rotation angles are ~60°–70° between Dimer_4 and the two neighboring dimers (Dimer_3 and Dimer_5). The rotation angles are ~50° between Dimer_1 and Dimer_2, Dimer_2 and Dimer_3, and Dimer_5 and Dimer_6. On the average, the rotation angle between all the interacting dimers is ~55°.

HARP binds and cleaves pre-tRNA in a cooperative mode. In addition to the AB dimer that interacts with pre-tRNA_{His}, there is one more PbHARP dimer (the CD dimer) within the asymmetric unit of the PbHARP/pre-tRNA_{His} complex. Possibly due to crystal packing, no pre-tRNA_{His} is found near the CD dimer. Similar to TrHARP, the AB and CD dimers of PbHARP interact with each other via their metallonuclease domains; the rotation angle between the two dimers is ~55° (Supplementary Fig. 12C), which well matched with the averaged angle observed in the TrHARP model (Supplementary Fig. 12B). PbHARP appeared as dimer during the size-exclusion chromatographic analysis, but the complex structure and its similarity with TrHARP suggested that PbHARP has the potential to form larger oligomers under proper conditions. The incorporation of additional PbHARP dimers can be easily modeled by rotating the complex structure along the 2-fold axis of either the AB dimer (Fig. 7A) or the CD dimer.

Based on pre-tRNA_{His}, the original AB dimer and the predicted EF dimer, one plausible pre-tRNA binding and cleavage model can be produced (Fig. 7B). In the model, the AB dimer is responsible for pre-tRNA binding, whereas the processing is performed by the EF dimer. The model can well explain the substrate preference of PbHARP (Fig. 5B). For His-7bp, the acceptor stem is composed of 7 base pairs. When the elbow region of His-7bp is recognized by PbHARP AB dimer, the first base pair of the acceptor stem, G1:C71, reaches the catalytic site of molecule E of the EF dimer. The phosphate backbone of G1 can readily coordinate with the catalytic cations, allowing the cleavage reaction to occur. G-1 pairs with C72 in native pre-tRNA_{His}, and such pairing may interfere with the conformational change and metal ion coordination at the scissile phosphodiester bond between G-1 and G1. The acceptor stem of His-9bp is 2 bp longer than that of His-7bp; the longer acceptor stem likely

**Fig. 4** Verification of the functional importance of PbHARP dimerization. A Interactions involved in PbHARP dimerization in the apo-form structure. B, C Interactions involved in PbHARP dimerization in the pre-tRNA-complexed structure. D Size-exclusion chromatographic analysis of wild-type, L103E/V107E and A118E/A121E mutants of PbHARP. E In vitro pre-tRNA_{His} cleavage assays catalyzed by WT PbHARP, L103E/V107E and A118E/A121E mutants. The substrate cleavage percentage (%) is shown at the bottom of the gels. Experiments were repeated independently three times with similar results. Source data are provided in Source Data file.
prevents His-9bp from simultaneously interacting with the AB and EF dimers.

Encouraged by the consistence between the in vitro cleavage assay results (Fig. 5B, C) and the pre-tRNA binding and cleavage model (Fig. 7B), we did further sequence and structural analysis. Besides the four Arg residues involved in pre-tRNA elbow recognition (Fig. 2B), two other Arg residues are also highly conserved in HARPs, corresponding to R108 and R146 in PbHARP (Supplementary Fig. 1B). In the proposed pre-tRNA binding and cleavage model, the side chains of R146 of molecule E and R108 of molecule F are very close to the phosphate backbones of G-1 and G1 (Fig. 7C). To investigate the potential function of these Arg residues, we constructed two PbHARP mutants, R108A and R146A. Although Ala substitution of R108 and R146 had no strong impact on pre-tRNA binding by PbHARP (Supplementary Fig. 11B), no detectable pre-tRNAHis cleavage activity was observed for the R108A or the R146A mutant (Fig. 7D).

Pre-tRNAHis is recognized by the PbHARP AB dimer in the complex, but neither R108 nor R146 of the AB dimer is involved in the interaction. These structural observations combined with the inactivity of these mutants further suggested that PbHARP binds and cleaves pre-tRNA in a cooperative mode. To better demonstrate the cooperation between PbHARP dimers, we performed in vitro crosslinking assays using WT PbHARP and His-7bp (Supplementary Fig. 13). In addition to the monomer, PbHARP dimers were also observed on the SDS-PAGE gel upon the addition of suberic acid bis (3-sulfo-N-hydroxysuccinimide ester) sodium salt (BS3), a chemical reagent that can crosslink the side chains of two lysine residues. Although not clearly detectable in the absence of His-7bp, bands tentatively assigned to the PbHARP tetramer (complex of two PbHARP dimers) were clearly enhanced in the presence of His-7bp.

Discussion

Here we present results of structural and biochemical analysis of HARPs from four organisms, *P. bacterium*, *T. celer*, *T. minervae* and *T. ruber*. Like Aq_880 from *A. aeolicus*30, in vitro cleavage assays showed that PbHARP, TcHARP, TmHARP and TrHARP can all remove the 5'-end of pre-tRNA, suggesting that pre-tRNA 5'-end processing is one conserved activity of HARP. HARP and the eukaryotic PRORP are all members of the PIN domain superfamily32. However, unlike PRORP enzymes constituting the PRORP subfamily, HARPs belong to the PIN_5 cluster subgroup. The metallonuclease domain is shared by all PIN superfamily members. In the reported *At*PRORP134 and *Hs*MRPP339 structures, the metallonuclease domains of HARP are of α/β fold in nature, but only two central β strands (β1 and β4) and one α helix (α8b) can be superimposed with the corresponding regions in the eukaryotic PRORP structure (Supplementary Fig. 14C, D).

The activities of HARP and eukaryotic PRORP proteins are all cation-dependent. The apo-form *Hs*MRPP3 structure is in an inactive form, the active center is blocked by one of its own Arg residues39. Activation of *Hs*MRPP3 requires the help of the two
partner proteins, HsMRPP1 and HsMRPP2. The apo-form structures of PbHARP and TcHARP are also in an inactive form. However, in vitro cleavage assays confirmed that all HARPs are active on their own (Fig. 1B). Structural comparison of the apo- and the pre-tRNA-complexed PbHARP structure suggested that HARP undergo self-activation via a large conformational change to the catalytically active form (Fig. 3B–D). When the catalytic PbHARP and AtPRORP1 structures are superimposed based on their central β strands and α helix, D155 and D173 of PbHARP can align well with D475 and D493 of AtPRORP1, respectively. However, the third catalytic Asp residue (D151) of PbHARP is positioned in mirror image to D399 of AtPRORP1 (Supplementary Fig. 14E). This mirror-image position of D151/D399 and the self-activation ability distinguish HARP from eukaryotic PRORPs.

As observed in their crystal structures, both AtPRORP1 and HsMRPP3 contain an N-terminal PPR domain. AtPRORP1 PPR is composed of five intandem PPR motifs; it is one of the typical proteins with multiple PPR motifs. In the complex structure of PbHARP, the pre-tRNA^{His} is recognized by the HB domain. Similar to the typical PPR motif, the HB domain is mainly composed of α–helices. Dimerization of HARP brings two HB domains together, structurally mimicking two PPR motifs arranged in tandem. Comparison with the recently reported AtPRORP1 PPR/tRNAPhe complex showed that the HB domain of HARP and AtPRORP1 PPR both recognize the TψC-loop of pre-tRNA (Supplementary Fig. 15A, B), but the orientations of the HB domain and PPR domain are roughly vertical to each other (Supplementary Fig. 15C). Sequence and structure-based alignment indicated that the helices of the HARP HB and the AtPRORP1 PPR domain are arranged in a reversed order (Supplementary Fig. 15D, E). However, one Arg residue is positioned at identical position in the two protein/tRNA complex structures (Supplementary Fig. 15F).
The apo-form AtPRORP1 structure adopts a V-shaped conformation. The two arms are formed by the metallonuclease domain and the PPR domain, linked by the central domain at the bottom. Although it has not been experimentally verified, it is likely that the V-shaped conformation is important for the function of AtPRORP1. With such arrangement, once pre-tRNA is recognized by the PPR domain, the acceptor stem and 5’-end of pre-tRNA will be placed near the active site of the metallonuclease domain. Neither a single monomer nor dimer of HARP is sufficient for binding and cleavage of pre-tRNA substrate, but the structure, mutagenesis and crosslinking assay results suggest that two dimers should be sufficient for the function of HARP. Interestingly, although not identical to AtPRORP1, the two HARP dimers also seem to adopt a V-shaped conformation (Fig. 7B).

The total size of HARP is only about 1/3 of AtPRORP1 and HsMRPP3, the latter even requiring two additional protein cofactors (HsMRPP1 and HsMRPP2) for in vivo activity. However, HARP performs the same reaction as AtPRORP1 and the HsMRPP complex. A similar phenomenon has also been observed for some other RNA processing enzymes, such as RNase III and Trax. Bacterial RNase III is around 200 amino acids in length and functions as a homodimer. The human RNase III homology protein, Dicer, is about 2000 amino acids in length. Although different in size and domain architecture, bacterial RNase III and human Dicer catalyze the identical dsRNA cleavage reaction. In the RNAi pathway in human and Drosophila, the nicked RNA passenger strand is degraded by C3PO, the complex of Trax and Translin. Very recently, the cryo-EM structures of Aq_880 and HARP from Halorhodospira halophila SL1 (Hh) were reported. Similar to TrHARP, Aq_880 and HhHARP assembled into dodecameric structures. Structural comparison revealed that the overall folding of the individual HhHARP dimer is similar to that of PbHARP in the apo-form structure (Supplementary Fig. 16C), whereas the folding of Aq_880 dimer is more close to PbHARP in the complex structure (Supplementary Fig. 16D). Superposition of Aq_880 and the PbHARP/pre-tRNAHis complex structures further confirmed the cooperative pre-tRNA binding and cleavage mode of HARP (Supplementary Fig. 16E). Both our and the reported structures of HARP support the idea that oligomerization and division of function between mono- or dimers may be a more common way (than previously thought) to provide small enzymes with substrate binding and catalytic abilities.

RNase P exists in two distinct forms (PRORP and the RNA-based RNase P) and has been considered the most excellent example for the evolutional transition theory. Very recently, the cryo-EM structures of Aq_880 and HARP from Halorhodospira halophila SL1 (Hh) were reported. Similar to TrHARP, Aq_880 and HhHARP assembled into dodecameric structures. Structural comparison revealed that the overall folding of the individual HhHARP dimer is similar to that of PbHARP in the apo-form structure (Supplementary Fig. 16C), whereas the folding of Aq_880 dimer is more close to PbHARP in the complex structure (Supplementary Fig. 16D). Superposition of Aq_880 and the PbHARP/pre-tRNAHis complex structures further confirmed the cooperative pre-tRNA binding and cleavage mode of HARP (Supplementary Fig. 16E). Both our and the reported structures of HARP support the idea that oligomerization and division of function between mono- or dimers may be a more common way (than previously thought) to provide small enzymes with substrate binding and catalytic abilities.
chioroplast, and trypanosomal mitochondria, very little is known about HARP from Archaeal and Bacteria. The PbHARP and TcHARP structures we report here are the only available crystal structures of HARP. Since no metallonucleic domain is present in the AtPRORP1 PPR/rRNAPh complex, the PbHARP/pre-rRNAPh structure represents the only rRNA-complexed structure of a full-length protein-only RNase P. Instead of being dodecamers, PbHARP and TcHARP exist as dimer in the structures (Fig. 1C, E). In vitro size-exclusion chromatography showed that the oligomerization state of TcHARP is changeable (Supplementary Fig. 2B). We believe that a tetramer is the minimal catalytic unit of HARP in vivo, whereas dodecamerization may allow HARP to bind and cut multiple pre-rRNAs simultaneously, as proposed recently5,44,45. Our study reveals the molecular basis for substrate binding and cleavage by HARP proteins.

Methods

Construction of recombinant plasmids. All genes that contain codon-optimized DNA sequences of Aq_880, RnpA (Supplementary Table 2) were purchased from Shanghai Generay Biotech (Supplementary Table 2) was inserted into a pUC18 vector at the HindIII and Smo tag at their N-termini. The recombinant plasmids of were cloned into pET28a-Sumo vectors. All recombinant proteins contain one 6×His-tag at their C-termini for substrate binding and cleavage by HARP proteins.

Expression and purification of recombinant proteins. All HARP proteins were expressed and purified using similar procedures. The recombinant plasmids were transformed into Escherichia coli BL21(DE3) competent cells and cultured in Lysogeny broth (LB) medium supplemented with 50 μg/mL kanamycin at 37 °C. When the OD₆₀₀ reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration of 0.2 mM, Sangon Biotech, Shanghai, China) was added to induce target protein expression. For accumulation of recombinant proteins, the cultures were incubated at 18 °C for an additional 18 h. For expression of Selonemethionine (Se-Met) substituted TcHARP protein, cells were cultured in M9 medium supplemented with 30 mg/L L-selenomethionine (HRK Scientific, Shanghai). Cells were collected by centrifugation, resuspended in Buffer A (20 mM Tris-HCl pH 8.0, 500 μM NaCl, 25 μM Imidazole), and lysed by ultrasonic-pressure homogenizer. Cellular debris was removed by centrifugation and the supernatant was loaded onto a HiTrap HP column (GE Healthcare). After washing with high salt buffer (20 mM Tris-HCl pH 8.0, 2 M NaCl), the bound protein was eluted with a linear gradient of buffer B (20 mM Tris-HCl pH 8.0, 500 μM NaCl, 500 μM imidazole). The proteins were treated with U1p1 and dialyzed against buffer C (20 mM Tris-HCl pH 8.0, 500 μM NaCl). The samples were loaded onto the HiTrap HP column again, the flow-through was collected, concentrated, and applied to a HiLoad Superdex 200 column (GE Healthcare). For purification of PbHARP or Aq_880, the nphl gene with T7 promoter and hammerhead ribozyme (Supplementary Table 2) was inserted into a pUC18 vector at the HindIII and EcoRI restriction sites. Sequences of all plasmids were confirmed by DNA sequencing.

In vitro RNA transcription. All pre-tRNAs and E. coli RNase P RNA were synthesized by in vitro transcription using the same method. The template that contains a T7 promoter sequence upstream of the pre-tRNA or RNase P RNA coding region was obtained by overlap PCR using the primers listed in Supplementary Table 4. In vitro transcription reaction was carried out by mixing T7 RNA polymerase (prepared in laboratory), NTPs and template together and incubated at 37 °C overnight. Next day, the DNA template was digested by RNase-free DNAse I (Takara, 2276A) at 37 °C. To remove the hammerhead ribozyme and increase the stability of the pre-tRNA, 25 μM of 5′-endo with a 1:1 molar ratio in processing buffer. The mixture was incubated at 4 °C for 5 min. The reactions were initiated through addition of 4 μl diluted protein. The final concentrations of HARP, pre-tRNA, MgCl₂ were 1 μM (or 0.05 μM), 1 μM, respectively. At specific time points, 5 μl aliquots of the reaction were quenched with 5 μl 2 x Formamide loading buffer (90% (v/v) formamide denaturated, 20 mM EDTA, 1% SDS, 0.2% bromophenol blue, 0.02% xylene cyanol) and heated at 95 °C for 5 min. Samples were centrifuged and separated by 20% denaturing UREA-PAGE gels (10 W for 2 h) in 1 x TBE buffer. The bands were stained by Gel Red and visualized by Typhoon FLA 9000 Imaging Scanner.

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In vitro chemical crosslinking assays. The buffer of PbHARP was exchanged with 20 mM HEPES pH 7.9, 500 μM NaCl through protein concentration in an ultracentrifugation tube. For crosslinking of complex, PbHARP (the final concentration is 20 μM) and His-7bp (10 μM) were incubated at 4 °C for 2 h in gel filtration buffer. The mixture was incubated at 4 °C for 40 min and divided into 10 μl per tube. Chemical crosslinking experiments were started by adding 200 μl of solution of BS3 (ThermoFisher Scientific, 21580) at a final concentration of 0.5 μM. Samples were incubated at 25 °C for 1 h. In the crosslinking system, the final concentration of PbHARP and RNA is 37.6 μM and 45.1 μM, respectively. To terminate the crosslinking reactions, 20 mM Glycine was added and then incubated at 25 °C for 15 min. Finally, 3 μl of 5×SDS PAGE loading buffer [250 mM Tris-Cl, pH 6.8, 50% (v/v) glycerol, 10% (w/v) SDS, 0.05% bromophenol blue, 5% (v/v) β-mercaptoethanol] was added into each tube and 5 μl samples were loaded to a 12% SDS-PAGE (300 V for 30 min) for analysis.

Size-exclusion chromatography and electron micrographic analysis. To analyze the oligomerization state of HARP’s, 500 μL HARP proteins (300 μg) were applied to Superdex 200 Increase 10/300 GL column equilibrated with 20 mM Tris-Cl pH 8.0 and 100–500 μM NaCl. The flow rate was set at 0.4 mL/min. Besides HARP proteins, we also performed size-exclusion chromatography analysis for 100 μL standard marker proteins using the same column equilibrated with 20 mM Tris-Cl pH 7.9 and 150 μM NaCl buffer. To further determine the oligomerization state of TcHARP, 400 μg protein was applied to Superdex 200 Increase 10/300 GL column equilibrated with 20 mM Tris-HCl pH 8.0 and 200 μM NaCl. The peak fraction was diluted to 40 μg/mL using the same buffer. 5 μl sample was placed onto the glow-discharged carbon-coated copper grids (200 mesh) purchased from Beijing Zhongxingkeying Technology Co., Ltd, China and incubated for 1 min, the excess solution was blotted off. After washing the grid with pre-TRNAln sample on the grid, the grid was stained with uranyl acetate for 3.5 min and then air-dried. Finally, the grid was viewed with the Talos L120C transmission electron microscope (ThermoFisher Scientific) equipped with a electron. The images were recorded using the Morada software and analyzed using the Bruker Xelis software (Bruker, Germany) for micrograph classification.
4 K × 4 K CETA CCD camera (FEI) at an accelerating voltage of 120 kV. Data were collected and images were recorded at a nominal magnification of 92 000×, corresponding to a pixel size of 1.55 Å. The contrast-transfer function (CTF) parameters of each micrograph were determined using CTF FIND4. Single particles were picked and processed using RELION3.0. Reference-free 2D classification was generated using RELION. 2D class-averages were used to generate de novo 3D model and 3D classification by RELION.

**Nano-differential scanning fluorimetry (nano-DSF) analysis.** Nano-DSF experiments were performed using a Prometheus NT.48 instrument from NanoTemper Technologies (Munich, Germany). 20 μl of 1.0 mg/ml WT PHARP and the L103E/V107E mutant samples were prepared. Each sample was dissolved in 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% (v/v) glycerol buffer. For each analysis, 10 μl samples were loaded into NR NT.48 standard capillaries from NanoTemper Technologies. Samples were subjected to a temperature gradient of 1°C/min from 20°C to 90°C and the fluorescence was constantly monitored. Protein unfolding was detected by following the change in tryptophan fluorescence at emission wavelengths of 330 and 330 nm. The ratio between the emission intensities at 350 nm and 330 nm was used to track the structural changes with increasing temperature. Tm value was determined from the first derivative nano-DSF curve.

**Circular dichroism (CD) spectra analysis.** Both WT PHARP and the L103E/V107E mutant were dissolved in 5 mM Tris pH 8.0, 500 mM NaCl buffer with a final concentration 0.2 mg/ml. 300 μl sample or buffer was loaded into a quartz cuvette of 1 mm path length (Hellma, Germany). The CD spectra was recorded on a nitrogen-flushed Chirascan CD spectrometer (Applied Photophysics, UK). The wavelength range of 190–260 nm was recorded. The step and band width were set at 1 nm. The scanning time per point was 0.5 s. For temperature control, the heating rate was set at 1°C/min in a range of 20°C–80°C (with a temperature interval measurement of 2°C). Secondary structure content (%) was calculated by CDNN software. Tm value was determined using nonlinear regression (curve fit) in GraphPad Prism software.

**Crystallization and data collection.** For crystallization of apo-form HAR, 10 mg/ml protein samples were used. For crystallization of HAR/pre-tRNA complex, HAR was mixed with pre-tRNA and 10 mM CaCl2 in gel filtration buffer, with a molar ratio between HAR and pre-tRNA of 1:1.2. The mixture was incubated at 4°C for 1 h. The initial crystallization conditions were identified at 16°C using the Gryphon crystallization robot system and commercial crystallization kits. The sitting-drop vapor diffusion method with the 3-drop intelligent plate was utilized during both initial screening and optimization process. The crystallization condition for TcHARP and Se-TcHARP was 30% (v/v) PEG 3000, 100 mM Tris-HCl pH 7.0, 200 mM NaCl. The crystals of apo-form PHARP grew in the buffer composed of 2000 mM Ammonium sulfate, 100 mM Sodium citrate/ Citric acid pH 5.5, whereas the crystallization conditions are composed of 8% (v/v) Tcascinate pH 8.0, 20% (v/v) PEG 3350 for the PHARP/pre-tRNA complex crystals. All the samples were cryoprotected in reservoir solution supplemented with 25% (v/v) glycerol and snap-frozen in liquid nitrogen. The X-ray diffraction data were collected on beamlines BL17U and BL19U at the Shanghai Synchrotron Radiation Facility (SSRF). HKL3000 program was used to process the data. The data collection and processing statistics are summarized in Supplementary Table 1.

**Structure determination and refinement.** The apo-form Se-Met substituted TcHARP structure was solved by the single-wavelength anomalous diffraction (SAD) method with the Autosol program embedded in the Phenix suite56. The initial model was built using the Autobuild program and then refined against the diffraction data using the ReFmac5 program of the CCP4 suite77. The Fo - Fc and Fo - Fc electron density maps were regularly calculated and used as guide for the building of the missing amino acids using COOT55. The apo-form PHARP structure was solved by molecular replacement using the apo-TcHARP structure as the search model with the phaser program of the CCP4 suite. The pre-tRNAHiocomplexed PHARP structures were solved by molecular replacement using the apo-form PHARP structure as the search model. Nucleic acids, ions, water, and other molecules were all built manually using the COOT program. The complex structures were refined using the ReFmac5 program of the CCP4 suite or the phenix.refine program of Phenix suite. The structural refinement statistics were summarized in Supplementary Table 1.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Structural factors and coordinates have been deposited in the Protein Data Bank under accession codes 7E8J, 7E8K, and 7E8O for the apo-form TcHARP, apo-form PHARP, and PHARP/pre-tRNAHiocomplex, respectively. Source data are provided with this paper.

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Author contributions

Y.Y.L. performed the crystallization and biochemical studies with the help of H.H.L., X.C., Z.W.S., Y.X.Z., Q.Y.S., X.Z., J. Y. and C.L.C. Y.Y.L. and Y.Q.G. collected and processed the crystal diffraction data. S.C., Y.Y.L and G.L.L. performed the electron microscopy study. Y.Y.L., J.Z.L., J.B.M, and J.H.G. analyzed the data. Y.Y.L. and J.H.G. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Jianhua Gan.

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