Recent advances in epitranscriptomics research have demonstrated the chemical diversity and biological importance of RNA modifications1–4. Thus far, about 150 types of RNA modification have been reported in various RNA molecules from all domains of life2. In particular, tRNAs contain the widest variety and largest number of modified nucleosides, with 80% of RNA modifications identified in tRNA molecules. Diverse RNA modifications are clustered in the anticodon loop, especially at positions 34 and 37 (refs. 1,8). These modifications have critical roles in stabilizing and modulating codon–anticodon interactions on the ribosome, ensuring accurate and efficient protein synthesis. Many RNA modifications are also found in the tRNA body composed of the D-loop, TΨC loop (T-loop) and variable loop (V-loop)9,10 (Fig. 1a). These modifications are required for correct folding and stability of the tRNA core structure. In particular, 2′-O-methyl modifications (Nm) confer conformational rigidity to the tRNA core region by fixing C3′-endo ribose puckering9,11.

In thermophilic bacteria and archaea, unique RNA modifications contribute to the thermal adaptation of tRNAs11. 5-Methyl-2-thiouridine (m5s2U) or s2T) is found at position 54 in the T-loop of tRNAs from thermophiles12. m5s2U54 adopts a rigid conformation with C3′-endo ribose puckering, thereby stabilizing the tRNA body in high-temperature environments13. The 2-thiolation level of m5s2U54 increases as the growth temperature rises14,15. m5s2U54 contributes to the thermotolerance of Thermus thermophilus15. In Pyrococcus furiosus, the relative abundance of N-acetylcystidine (acC) and its 2′-O-methyl derivative (acCm) were markedly increased with rising growth temperature16. acC is a prevalent modification that is present in tRNAs, rRNAs and other RNAs in hyperthermophilic archaea16,17. acC favours the C3′-endo form and stabilizes tRNAs16,18. Loss of acC results in a growth defect in Thermococcus kodakarensis at high temperature, contributing to cellular thermotolerance19. In Bacillus steathermophilus, 2′-O-methylation in tRNAs increases when the growth temperature rises20. Archaeosine (G+) is a unique 7-deazaguanosine derivative found at position 15 in the D-loop of archaeal tRNAs21. On the basis of quantum mechanics calculations, G+ stabilizes the Levit base pair with C48 (ref. 22). In line with this, biochemical and genetic studies have shown that G+ confers thermal stability to tRNAs and contributes to thermotolerance21,23.

Here we identified 2′-phosphouridine (Up) at position 47 of tRNAs from thermophilic archaea. Up47 confers thermal stability and nuclease resistance to tRNAs. Atomic structures of native archaeal tRNA showed a unique metastable core structure stabilized by Up47. The 2′-phosphate of Up47 protrudes from the tRNA core and prevents backbone rotation during thermal denaturation. In addition, we identified the arkI gene, which encodes an archaeal RNA kinase responsible for Up47 formation. Structural studies showed that ArkI has a non-canonical kinase motif surrounded by a positively charged patch that efficiently dephosphorylates Up47 in vitro and in vivo. Taken together, our findings show that Up47 is a reversible RNA modification mediated by ArkI and KptA that fine-tunes the structural rigidity of tRNAs under extreme environmental conditions.

**Discovery of Up in tRNA**

To explore tRNA modifications in hyperthermophilic archaea, we isolated 12 tRNA species from the thermoacidophilic crenarchaeon Sulfurisphaera tokodaiii using our original method for RNA isolation by reciprocal circulating chromatography (RCC) (Extended Data Fig. 1)24.
First, we comprehensively analysed all post-transcriptional modifications of tRNA by mass spectrometry (RNA-MS) and mapped 13 types of RNA modification at 18 positions in this tRNA molecule (Fig. 1a, Supplementary Table 1). Among the modifications, we detected an unknown uridine derivative with molecular mass of 324 (tentatively named N324) at position 47 of the RNA fragments digested with RNases (Fig. 1b, c). The relative intensity of the mass chromatograms showed that N324 occurred at a frequency of 96.8% (Fig. 1d), indicating that N324 is an abundant modification. We also detected N324 in the seven other tRNA species (Extended Data Fig. 3a, b, Supplementary Note 1, Supplementary Table 2), indicating that N324 is a prevalent and abundant (82–100%) modification in class I tRNAs bearing U47 in the V-loop, but not in class II tRNAs with a long V-loop (Extended Data Fig. 3a). We also detected N324 in tRNA precursors (Extended Data Fig. 3c, Supplementary Note 1).

High-resolution mass analysis of the N324-containing fragment showed that the additional mass of N324 attached to the uridine residue was 79.9706 Da, equivalent to one phosphate group (theoretical mass, 79.96632 Da), with a low error value of 4.4 millimass unit, indicating that N324 is a phosphorylated uridine residue. This prediction explains why the N324 nucleoside was not detected in our nucleoside analysis (Extended Data Fig. 2a), owing to N324 being dephosphorylated during nucleoside preparation. To determine the phosphorylation site of N324, we prepared the N324-containing nucleotide and analysed its chemical structure through collision-induced dissociation (CID) and biochemical approaches (Supplementary Note 2, Extended Data Fig. 4a–h). We found that phosphorylation occurs on the 2′-OH group of the ribose moiety and concluded that N324 is 2′-phosphouridine (Up), where ‘p’ is superscript to discriminate it from 3′-phosphate (Fig. 1e).

**U47 stabilizes tRNA structure**

Given that U47 is a thermophile-specific modification found in the tRNA core region, we investigated whether U47 stabilizes the tertiary structure of tRNA. To this end, we treated *S. tokodaii* tRNA with yeast Tpt1p (2′-phosphotransferase) to remove the 2′-phosphate of Up47. We measured the melting temperature (Tm) of *S. tokodaii* tRNA with and without U47 (Fig. 1f). In the melting curves, the tRNA without U47 gradually melted at around 65 °C whereas the tRNA with U47 remained stable even at 70 °C. The Tm values of the tRNA with and without U47 were 85.8 ± 0.5 °C and 79.2 ± 0.5 °C, respectively. These observations clearly demonstrate that a single U47 modification increases the thermal stability of tRNA by 6.6 °C.

We next performed an RNase probing experiment to examine the nuclease resistance of tRNA with and without U47. *S. tokodaii* tRNA and its Tpt1p-treated form were labelled with 32P at their 3′ termini and were probed with RNase I at 65 °C (Fig. 1g). Over time, the intact tRNAs were gradually degraded into RNA fragments. Compared with...
the intact tRNA with Up47, the Tpt1p-treated tRNA was degraded more rapidly, within 5 min, indicating that the tRNA without Up47 was highly sensitive to RNase I. This observation demonstrates that Up47 stabilizes and protects tRNAs from nucleolytic degradation.

**Structural study of Up47 in native tRNA**

To determine the molecular basis for thermal stabilization of tRNA by Up47, we crystallized *S. tokodaii* tRNA<sup>Val</sup> and determined its atomic structure at a resolution of up to 1.9 Å by X-ray crystallography (Fig. 2a, Extended Data Fig. 3, Supplementary Note 3, Supplementary Fig. 2). One unit cell contains two tRNA molecules, denoted as molecule A and molecule B. Molecule A formed a canonical tRNA core structure (Fig. 2b, c, Extended Data Fig. 5a), whereas molecule B had an altered core structure with a non-canonical base triple (Fig. 2b, c, Extended Data Fig. 5b). We clearly observed electron densities for tRNA modifications, including Up47 (Fig. 2a, Extended Data Fig. 6a, b). When Up47 was virtually introduced to yeast tRNAPhe (Fig. 2e, f, Extended Data Fig. 6a, b), G46 changed its ribose pucker from C2′-endo to C3′-endo with altered torsion angles (δ, ε and ζ were changed by –58°, –28° and 68°, respectively) (Extended Data Fig. 6b, Supplementary Table 3). In addition, the Up47 backbone was substantially rotated with the α and γ angles changing by –113° and 167°, respectively (Extended Data Fig. 6b, Supplementary Table 3). The m<sup>1</sup>C48 backbone was also rotated, with the α and γ angles changing by –36° and –86°, respectively (Extended Data Fig. 6b, Supplementary Table 3).

Although molecule A had a canonical tRNA core structure stabilized by multiple tertiary interactions between the D-arm and V-loop, including the base triples s<sup>U</sup>8–A14–A21, Ψ13–G22–G46, C12–G23–C9 and m<sup>5</sup>G10–C25–G45 (Fig. 2b, c), molecule B unexpectedly had a non-canonical core structure (Fig. 2b, c). In molecule B, G46 was dissociated from the base triple Ψ13–G22–G46 and stacked with Up47.
The ΔV-amino group of G46 formed hydrogen bonds with A21 by inserting itself between the base triple and U^47 (Fig. 2d). This interaction pushes A21 towards A14 to make additional hydrogen bonds that stabilize the s^U8–A14–A21 triple (Fig. 2d). Because U^47 does not substantially change its backbone conformation (Extended Data Fig. 6b, Supplementary Table 3, Supplementary Video 1), the G46 base was stably trapped by U^47 in molecule B (Fig. 2d, f). To compensate for this conformational change, C9 comes up from the lower layer (C12–G23–C9) (Fig. 2g, Supplementary Video 1) to form the backbone bulge outwards, flipping the G46 base out with the χ angle altered by ~70° (Extended Data Fig. 6b, Supplementary Table 3). C9 changes its backbone, altering the α, β, γ and angles by 171°, ~37°, ~180° and 26°, respectively (Supplementary Table 3).

To further investigate the structural role of U^47, we also solved a crystal structure for Tpt1p-treated S. tokodaiii tRNA^Val (Extended Data Fig. 7a). Both molecules A and B of the Tpt1p-treated RNA showed the canonical structure with the standard core (Extended Data Fig. 7b–f). In both molecules, U^47 was dissociated from the s^U8–A14–A21 base triple (Extended Data Fig. 7b–e) with backbone angles α, β and altered by 153°, ~109° and ~37°, respectively (molecule A) (Extended Data Fig. 6b, Supplementary Table 3), thereby placing the uracil base of U^47 outwards (Fig. 2h, Extended Data Fig. 7b–d). In another aspect of the Tpt1p-treated tRNA, C9 was detached from the C12–G23–C9 base triple in both molecules (Extended Data Fig. 7f). These findings imply that U^47 stabilizes the metastable tRNA core structure with a non-canonical base triple during thermal denaturation.

Identification of an RNA kinase for U^47

To identify a gene responsible for U^47 formation, we narrowed down the candidate genes in the S. tokodaiii genome by performing a comparative genomics analysis of sequenced genomes using RECOG (http://mbgd.genome.ad.jp/RECOG/). According to our analysis of U^47 distribution in archaeal species (Supplementary Note 4, Extended Data Fig. 7a–d), U^47 is present in seven archaeal species, including S. tokodaiii, but is absent in two species (Fig. 3a). Among the 2,826 genes encoded in the S. tokodaiii genome, only nine genes (Supplementary Table 4) were commonly found in all seven archaeal species with U^47 (Fig. 3b). Among them, five genes (Supplementary Table 4) were of uncharacterized function (Fig. 3b). We chose one gene encoding a putative protein kinase, STK_09530 (hypothetical serine/threonine kinase, COG2112), as a strong candidate (Fig. 3b). STK_09530 resides in an operon containing a gene for a tRNA nucleotidyltransferase (STK_09530) with a hinge (positions 96–109) (Extended Data Fig. 9). Compared with the canonical ePK, the characteristic sequences in the conserved motifs were altered in TkArkI. The HRD triplet in the catalytic core of TkArkI had subdomains I–V in the N-terminal lobe and subdomains Vlβ, VI and IX in the C-terminal lobe, but lacked subdomains Vlβ and VII, respectively (Fig. 4b, Extended Data Fig. 9). The conserved motifs of the P-loop (positions 31–38), catalytic loop (positions 128–140) and metal-binding loop (positions 145–153) were present in subdomains I, VI and VII, respectively (Fig. 4b, Extended Data Fig. 9).

Crystal structure of TkArkI

To find the structural basis of U^47 formation, we crystallized TkArkI and determined its atomic structure at a resolution of 1.8 Å using X-ray crystallography (Fig. 4a, Extended Data Table 1). On the basis of its amino acid sequence, TkArkI belongs to a superfAMILY of euKaryotic protein kinases (ePKs)25. As observed for ePKs, TkArkI also consisted of two lobes, termed the N-terminal and C-terminal lobes, which were connected by a hinge (positions 96–109) (Fig. 4a, Extended Data Fig. 9). ePKs consist of 12 conserved subdomains that fold into the catalytic core. TkArkI had subdomains I–V in the N-terminal lobe and subdomains Vlβ, VI and IX in the C-terminal lobe, but lacked subdomains VIII and X (Fig. 4b, Extended Data Fig. 9). The conserved motifs of the P-loop (positions 31–38), catalytic loop (positions 128–140) and metal-binding loop (positions 145–153) were present in subdomains I, VI and VII, respectively (Fig. 4b, Extended Data Fig. 9).

Although we demonstrated that TkArkI is an ATP-dependent RNA kinase involved in the formation of U^47 (Fig. 3e, f), we observed a clear electron density for guanosine in the cleft of the two lobes (Fig. 4a, c, d), which corresponds to the ATP-binding site of ePKs surrounded by the hinge and metal-binding, catalytic and P-loops (Supplementary Fig. 6a, b). We confirmed guanosine (and deoxyguanosine) as a ligand that tightly binds to TkArkI (Supplementary Note 6, Supplementary Fig. 7a–c). These observations indicate that TkArkI has binding affinity for guanosine and deoxyguanosine but uses ATP as a major phosphate donor. In the ATP-binding site of mouse PRKACA (Supplementary Fig. 6a, b), the triphosphate of ATP coordinates two

(double-knockout strain, in which queE is responsible for archaeosine (G15) formation, because G15 thermally stabilizes tRNAs and contributes to cellular thermotolerance26. We confirmed the absence of U^47 and G15 in tRNAs from the double-knockout strain (Supplementary Fig. 3). The ΔqueE strain grew well at 83 °C, slowly at 87 °C and not at all at 91 °C (Fig. 3d), as reported26. The ΔarkIΔqueE strain grew slower than the wild-type, ΔarkI and ΔqueE strains at 83 °C (Fig. 3d). The strain exhibited a severe growth phenotype at 87 °C (Fig. 3d) and was unable to survive at 91 °C (Fig. 3d). This finding indicates that U^47 and G15 cooperatively stabilize the tRNA core structure at high temperatures, thereby contributing to cellular thermotolerance.
Mn\(^{2+}\) ions and interacts tightly with the conserved motifs, especially the metal-binding loop and P-loop. However, in the guanosine-bound TkArkI structure, the P-loop was dislocated from the ligand-binding site (Fig. 4c, d). Thus, ATP does not bind the ligand-binding site of the observed structure. In homology modelling to ePKs (Supplementary Fig. 6c), ATP virtually bound to the active site of the ligand-binding site of TkArkI. It is likely that the P-loop and other motifs form the active pocket for ATP binding following tRNA binding to TkArkI. Although the biological relevance of guanosine binding to TkArkI is not known, guanosine may compete with ATP to regulate tRNA phosphorylation, similar to the mechanism by which nucleoside derivatives inhibit protein kinases\(^{30,32}\). Judging by its high \(K_m\) value for ATP (1.2 mM) (Fig. 3f), TkArkI might sense the cellular energy status and guanosine binding to TkArkI might have a regulatory role in Up47 formation. Given that TkArkI was a recombinant protein expressed in \textit{Escherichia coli}, we cannot rule out the possibility that guanosine was an artificial ligand bound to the inactive form of TkArkI. It is unclear whether guanosine actually binds to TkArkI within archaeal cells at high growth temperatures.

The electrostatic surface potential showed a large positive area on one side of the TkArkI structure (Fig. 4e). The positively charged surface covered the ATP-binding site in the N-terminal loop and extended to the ArkI-specific elongated \(\alpha_7\) helix in the C-terminal loop (Extended Data Fig. 9). Instead of the missing subdomain VIII involved in recognition of substrate peptide in ePKs (mouse PRKACA), the basic surface in the C-terminal lobe might bind substrate tRNA through electrostatic interaction.

To characterize the conserved residues in TkArkI, we constructed 14 TkArkI mutants in which targeted residues were replaced by alanine (Fig. 4b, f). All mutants were expressed in soluble form and purified. The tRNA phosphorylation activity of each mutant was measured (Fig. 4g). In the ATP-binding site, K32A, G33A, K31A and E65A substitutions markedly reduced activity, whereas the R95A substitution caused a mild reduction in activity. In addition, a severe reduction in activity was observed in the H130A, Q132A and K137A mutants with substitutions in the catalytic loop. No activity was detected for the D149A mutant, in which the mutated residue is in subdomain VII involved in metal binding. These results clearly confirm the critical role of catalytic residues in kinase activity. The N160A and T162A substitutions in subdomain IX led to decreased activity. We mutated the YKR motif in the \(\alpha_7\) helix, finding a severe reduction in activity with the K201A substitution and a mild reduction with the Y200A and R202A substitutions. These observations indicate the importance of the conserved residues and positively charged surface in the C-terminal lobe.

\textbf{KptA acts as an eraser for Up47}

TpTlp removes the 2'-phosphate from tRNA precursors during maturation\(^{31}\). TpTlp/KptA homologues are distributed across all domains of life\(^{34,35}\) (Supplementary Fig. 4). Although TpTlp/KptA homologues are also present in thermophilic archaea and bacteria (Supplementary Fig. 4), natural RNA substrates with 2'-phosphate have not been identified.

Efficient removal of Up47 by yeast TpTlp prompted us to speculate that archaean KpTAl is capable of removing the 2'-phosphate of Up47 from tRNAs in the cell (Fig. 5a). To explore this possibility, we conducted in vitro dephosphorylation of Up47 with \textit{T. kodakarensis} KptA (TkKptA) in the presence of NAD\(^+\), with the results indicating that the 2'-phosphate of Up47 was efficiently removed (Fig. 5b). In the same reaction conditions used for Up47 formation by TkArkI, we measured the kinetic parameters of Up47 dephosphorylation catalysed by TkKptA: the \(K_m\) and \(V_{\text{max}}\) values for tRNA were 180 nM and 27 nM s\(^{-1}\), respectively (Fig. 5c). The \(K_m\) value for dephosphorylation by TkKptA is comparable to that of phosphorylation by TkArkI, implying that TkKptA acts as an eraser for Up47 in the cell.
We then examined the in vivo function of Tpt1/KptA homologues in Up47 dephosphorylation, using E. coli as a model organism. Because E. coli tRNAs have m"G46 and acp"U47 modifications, which inhibit Up47 formation in the V-loop, we used the E. coli ΔtrmBΔtapT strain as a host cell in which both of these tRNA modifications are absent and then expressed Nitrososphaera viennensis ArkI (NvArkI), because N. viennensis is a mesophilic archaeon and its ArkI homologue was predicted to have efficient activity in E. coli. The class I tRNA fraction prepared from this strain was subjected to shotgun analysis to detect the Up47 modification. We clearly detected four Up47-containing fragments derived from various E. coli tRNA species (Fig. 5d, Supplementary Table 5). Each fragment was sequenced by higher-energy collision dissociation analysis, confirming the presence of Up at position 47 (Supplementary Fig. 8). Next, we introduced TkKptA under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter and quantified the peak intensity of each Up47-containing fragment when TkKptA expression was induced by addition of 10 or 100 μM IPTG (Fig. 5d, e). All four Up47-containing fragments had decreased abundance as a function of IPTG concentration, demonstrating that TkKptA erases Up47 in E. coli. We obtained similar results with E. coli KptA (Extended Data Fig. 10a, b) and S. cerevisiae Tpt1p (Extended Data Fig. 10c, d). Together, these data demonstrate that Tpt1/KptA homologues dephosphorylate Up47 of tRNAs in vivo.

Discussion

Up47 is, to our knowledge, the first known instance of internal phosphorylation as a stable RNA modification (Supplementary Note 7). 2'-Phosphate at an internal residue appears transiently during tRNA splicing in fungi and plants. However, this moiety is not formed by phosphorylation but rather through hydrolysis of 2',3'-cyclic phosphodiester via the healing and sealing pathway. Because the 2'-phosphate is removed by Tpt1p, it is not present in mature tRNAs.

In S. tokodaii tRNAs isolated in this study, Up47 was detected in nine class I tRNA species with high frequency (85–100%) (Fig. 1d, Extended Data Fig. 3a) but was absent in two class I tRNAs (tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Val}) and two class II tRNAs (tRNA\textsuperscript{AUA} and tRNA\textsuperscript{SER}) (Extended Data Fig. 3a). Judging from the primary sequences of these species (Supplementary Fig. 9), it is likely that ArkI introduces Up47 in tRNAs bearing a V-loop with five bases, as tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Val} have four and six bases in the V-loop, respectively. Supporting this finding, only the class I tRNA fraction was phosphorylated in total RNA by in vitro reaction (Supplementary Fig. 5b, c).

RNA hydrolysis is mediated by the 2'-OH group in the presence of divalent metal ions such as Mg\textsuperscript{2+}. Especially at high temperatures, RNA is rapidly degraded. Similarly to 2'-O-methylation, the 2'-phosphorylation of Up47 also serves to prevent tRNA degradation. This property partly explains the RNase resistance of tRNA conferred by Up47 (Fig. 1g). It is known that Up adopts C2'-endo ribose puckering, which confers flexibility to the RNA strand by extending the backbone structure\textsuperscript{36,38}. Hence, Up47 presumably acts as a defining mark for single-stranded RNA.

In the process of tRNA folding, Up47 might have a role in preventing the V-loop from being accidentally incorporated into stem structures, ensuring correct folding of the tRNA L-shape structure. Especially in thermophiles, tRNA might frequently misfold owing to its high G+C content. Thus, Up47 deposition in the tRNA precursor might be required to loop out the V-loop region to ensure correct folding of the tRNA. We clearly detected four Up47-containing fragments derived from various E. coli tRNA species (Fig. 5d, Supplementary Table 5). Each fragment was sequenced by higher-energy collision dissociation analysis, confirming the presence of Up at position 47 (Supplementary Fig. 8). Next, we introduced TkKptA under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter and quantified the peak intensity of each Up47-containing fragment when TkKptA expression was induced by addition of 10 or 100 μM IPTG (Fig. 5d, e). All four Up47-containing fragments had decreased abundance as a function of IPTG concentration, demonstrating that TkKptA erases Up47 in E. coli. We obtained similar results with E. coli KptA (Extended Data Fig. 10a, b) and S. cerevisiae Tpt1p (Extended Data Fig. 10c, d). Together, these data demonstrate that Tpt1/KptA homologues dephosphorylate Up47 of tRNAs in vivo.

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Up47 to U47 by transferring the phosphate group of Up47 to NAD+, producing ADP as a by-product. KptA converts the indicated tRNA concentrations. Data represent the average values of technical triplicates ± s.d. The relative abundance of the Up47-containing fragments was measured in Tpt1p-treated tRNA has the canonical structure with the standard core (Extended Data Fig. 7a–f). It is likely that the structural alteration is caused by Up47. During thermal denaturation of tRNAs, the core region and D-arm are unwound first. In molecule B, G46 is released from the base triple Ψ13–G22–G46 and stacks with the uracil base of Up47 (Fig. 2d, f). Presumably, this unique conformation is a metastable structure of tRNA during heat denaturation. Curiously, in the structural transition from molecule A to molecule B (Supplementary Video 1), the torsion angle of G46 changes substantially, whereas that of Up47 does not (Extended Data Fig. 6b). Up47 catches the G46 base that is dissociated from the base triple to restrict further rotation of the V-loop, thereby stabilizing the metastable core structure of the tRNA to prevent its heat denaturation. In addition, C9 comes up from the lower layer (C12–G23–C9) to fill in for the missing G46, forming the non-canonical base triple Ψ13–G22–C9 (Fig. 2f). Up47 does not fix the tRNA rigidly but rather maintains a metastable structure when the tRNA core thermally fluctuates, thereby preventing further collapse of the core structure, as well as increasing the chance of return to the canonical structure.

ArkI homologues are mainly distributed in thermophilic archaea but are also present in some bacteria (Supplementary Fig. 4). We confirmed the activity of tRNA phosphorylation for bacterial ArkI homologues (Supplementary Fig. 5a, c). In silico analysis of protein kinases suggested that ArkI-family proteins were originally classified as members of the AQ578 family found in bacterial and archaeal genomes44; the AQ578 family was proposed to have emerged by gene duplication in the early archaean lineage. The bacterial AQ578 family might have been acquired by horizontal gene transfer of the archaean homologue, suggesting that the strategy of stabilizing tRNA by internal phosphorylation might have spread across the domains of life.

The ΔarkI strain of T. kodakarensis exhibited weak temperature sensitivity (Fig. 3d), demonstrating that Up47 by itself contributes to cellular thermostolerance. Because multipletRNA modifications cooperatively stabilize the tRNA structure, we chose to analyse the G15 modification, showing a synthetic phenotype with Up47 loss. We found that the ΔarkIΔqueF double-knockout strain was extremely susceptible to high temperature (Fig. 3d), suggesting that Up47 and G15 cooperatively stabilize the tRNA core structure and contribute to cellular thermostolerance. Up47 flexibly deals with the structural change due to thermal denaturation of the core structure, like a padlock, whereas G15 tightly fixes the core structure, like a screw bolt (Supplementary Note 3). On the basis of these findings, we propose a new mechanism of tRNA stabilization mediated by two distinct but concerted actions of tRNA modification.

In eukaryotic miRNAs and non-coding RNAs, N6-methyladenosine (m6A) has a critical role in RNA metabolism and function as a reversible RNA modification45. If Up47 is a reversible modification, it is expected that RNA function and stability are dynamically regulated by a writer and eraser, raising the possibility of epitranscriptomic regulation of tRNAs in translation. The mechanism closely resembles post-translational modification of proteins. Phosphorylation and dephosphorylation rapidly and dynamically control protein function46–48. Because tRNAA is a stable molecule with a low turnover rate and long lifetime in the cell, it would be reasonable for tRNA function to be regulated by Up47 modification. We found efficient dephosphorylation of Up47 by TkKptA in vitro (Fig. 5b) and confirmed the in vivo activity of Tpt1p/TkKptA homologues in E. coli cells (Fig. 5d, e and Extended Data Fig. 10a–d). In fact, tRNA stability is regulated by thermophile-specific tRNA modifications including m5s2U and ac4C, which become much more abundant as the growth temperature increases49–51 but are not reversible. Reversible Up47 modification would be beneficial for hyperthermophilic organisms in extremely harsh environments. Future studies will be necessary to investigate Up47 frequency and the expression levels of ArkI and KptA under various growth conditions, including during rapid changes in growth temperature and introduction of environmental stresses.
Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-022-04677-2.
Preparation of tRNA fractions

For small-scale preparation (-100-ml culture), archaeal cells were resuspended in 3 ml solution D (25 mM guanidine thiocyanate, 25 mM citrate-NaOH (pH 7.0), 0.5% (wt/vol) N-lauroylsarcosine sodium salt and 1 mM 2-mercaptoethanol) and mixed with an equal volume of water-saturated phenol and 1/10 volume of 3 M sodium acetate (pH 5.3). The mixture was shaken for 1 h on ice and mixed with 1/5 volume of chloroform, followed by centrifugation at 8,000 g for 10 min at 4 °C. The supernatant was collected and mixed with an equal volume of chloroform, followed by centrifugation at 8,000 g for 10 min at 4 °C. Total RNA was obtained from the resultant supernatant by isopropanol precipitation. The total RNA prepared in this manner was separated by 10% denaturing PAGE, followed by staining with SYBR Gold or toluidine blue. The visualized tRNA fraction including class I and class II tRNAs was cut out and eluted from the gel slice with elution buffer (0.3 M sodium acetate (pH 5.3) and 0.1% (wt/vol) SDS), followed by filtration to remove the gel pieces and ethanol precipitation for RNA-MS analysis of the tRNA fraction.

For large-scale preparation of tRNA fractions from *S. tokodaii*, cell pellets (3 g) were resuspended in 530 ml solution D and then mixed with 53 ml of 3 M sodium acetate (pH 5.3) and 425 ml neutralized phenol. The mixture was shaken for 1 h on ice to which 106 ml chloroform/isoamyl alcohol (24:1) was added. The aqueous phase was collected and precipitated with isopropanol. The prepared total RNA (608 mg) was dissolved in 250 ml of buffer consisting of 20 mM HEPES-KOH (pH 7.6), 200 mM NaCl and 1 mM DTT and then loaded on a DEAE Sepharose Fast Flow column (320 ml beads) and fractionated with a gradient of NaCl from 200 to 500 mM. Fractions containing tRNA were collected by isopropanol precipitation.

Isolation of individual tRNAs

Isolation of individual tRNAs from thermophilic organisms is extremely difficult owing to their high melting temperatures, which are the consequence of their high G+C content and complex modifications. We thus optimized our original method for RNA isolation by RCC (28) or capillary column chromatography (CCC) (29). Approximately 200 absorbance at 260 nm (A260) units of the *S. tokodaii* tRNA fraction was subjected to RCC. The isolation procedure was carried out as follows: hybridization and elution at 66 °C in 6× NHE buffer (30 mM HEPES-KOH (pH 7.5), 15 mM EDTA (pH 8.0), 1.2 M NaCl, 1 mM DTT), washing at 50 °C with 0.1× NHE buffer (0.5 mM HEPES-KOH (pH 7.5), 0.25 mM EDTA (pH 8.0), 20 mM NaCl, 0.5 mM DTT) and elution at 72 °C with 0.1× NHE buffer. Eluted tRNAs were recovered by ethanol precipitation. Mature and precursor tRNAs were separated by 10% denaturing PAGE and stained with SYBR Gold. Visualized bands of mature and precursor tRNAs were cut out and eluted from the gel slices with elution buffer, followed by filtration to remove the gel pieces and precipitation with ethanol.

To crystallize native tRNA bearing ψ^47^, we conducted large-scale isolation of *S. tokodaii* tRNA^Ψ47^ using CCC (29). The *S. tokodaii* tRNA fraction (2,000 A260 units) was subjected to CCC with tandem affinity chromatography (TACC) (30). Approximately 200 absorbance at 260 nm (A260) units of the *S. tokodaii* tRNA fraction was subjected to CCC. The isolation procedure was carried out as follows: hybridization and elution at 66 °C in 6× NHE buffer (30 mM HEPES-KOH (pH 7.5), 15 mM EDTA (pH 8.0), 1.2 M NaCl, 1 mM DTT), washing at 50 °C with 0.1× NHE buffer (0.5 mM HEPES-KOH (pH 7.5), 0.25 mM EDTA (pH 8.0), 20 mM NaCl, 0.5 mM DTT) and elution at 72 °C with 0.1× NHE buffer. Eluted tRNAs were recovered by isopropanol precipitation. The sequences of the DNA probes are shown in Supplementary Table 6. The isolated tRNA was further purified by anion exchange chromatography to completely remove tRNA, as described below.
RNA mass spectrometry
For tRNA mass fragment analysis by RNA-MS, 30 ng (900 fmol) of the isolated tRNA or 150 ng (4.5 pmol) of tRNA mixture was digested with RNase T1 (Epicentre or Thermo Fisher Scientific) or RNase A (Ambion) and analysed with a linear ion trap–Orbitrap hybrid mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific) equipped with a custom-made nanospray ion source and a sputterless nanoHPLC system (DiNa, Kyla Technologies) as described previously25–27. To analyse W sites, tRNA was treated with acrylonitrile to cyanoethylate W35 and subjected to RNA-MS. For dephosphorylation of the U47-containing fragment (Extended Data Fig. 4a, b), RNase T1 digestion was performed in the presence of 0.01 U ml−1 bacterial alkaline phosphatase (BAP C75, Takara Bio). To precisely map tRNA modifications, RNA fragments were decomposed by CID in the instrument. The normalized collision energy of LTQ Orbitrap XL was set to 40%. Mango Oligo Mass Calculator v 2.08 (https://mods.rna.albany.edu/masspec/Mongo-Oligo) was used for assignment of the product ions in CID spectra.

For nucleoside analysis, 800 ng (24 pmol) of the isolated tRNA was digested with 0.09 U nuclease P1 (Fujifilm Wako Pure Chemical Corporation) in 20 mM ammonium acetate (pH 5.2) at 50 °C for 1 h and mixed with 1/8 volume of 1 M trimethylamine-HCl (TMA-HCl) (pH7.2) and 0.06 U phosphodiesterase I (Worthington Biochemical Corporation), followed by incubation at 37 °C for 1 h. To this mixture, 0.08 U BAP was added, and the sample was incubated at 50 °C for 1 h. After that, 9 volumes of acetonitrile were added, followed by LC–MS/MS analysis as described in refs. 5,25 with some modifications as follows. The samples were chromatographed with a ZIC-chiLIC column (3-µm particle size, 2.1 × 150 mm; Merck) and eluted with 5 mM ammonium acetate (pH 5.3) (solvent A) and acetonitrile (solvent B) at a flow rate of 100 µl min−1 with a multistep linear gradient: 90–50% solvent B for 30 min, 50% solvent B for 10 min, 50–90% solvent B for 5 min and then initialization with 90% solvent B. The chromatographed eluent was directly introduced into the electrospray ionization source of the Q Exactive Hybrid Quadrupole–Orbitrap mass spectrometer (Thermo Fisher Scientific).

For nucleoside analysis, 800 ng (24 pmol) of the tRNA fraction or individual tRNA was digested with 0.09 U nuclease P1, in 20 mM ammonium acetate (pH 5.2) at 50 °C for 1 h and then mixed with 9 volumes of acetonitrile for LC–MS. The digests were chromatographed with a ZIC-chiLIC column and analysed by Q Exactive Hybrid Quadrupole–Orbitrap mass spectrometer (Thermo Fisher Scientific) or LTQ Orbitrap XL (Thermo Fisher Scientific) with a multistep linear gradient: 90–50% solvent B for 30 min, 50% solvent B for 10 min, 50–90% solvent B for 5 min and then initialization with 90% solvent B. The acquired LC–MS data were analysed using Xcalibur 4.1 (Thermo Fisher Scientific) and were visualized with Canvas X (Nihon poladigital k.k).

Isolation and detection of pN324p
Five A260 units of the S. tokodaii tRNA fraction was completely digested with nuclease P1. Digests containing pN324p in vivo were subjected to periodate oxidation with 10 mM NaO4 for 1 h on ice in the dark. The reaction was stopped by addition of 1 M rhamnose and incubation for 30 min. For β-elimination, an equal volume of 2 M lysine-HCl (pH 8.5) was added, and the sample was incubated at 45 °C for 90 min. The product containing pN324p was then subjected to anion exchange chromatography with a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with 20 mM triethylammonium bicarbonate (TEAB) (pH 8.2). The eluate with 2 M TEAB was collected and dried by evaporation in vacuo. The pellet was dissolved with water and mixed with an equal volume of chloroform, followed by centrifugation at 20,000g for 5 min at 4 °C. The supernatant was recovered and dried again. This process was repeated five times. The resultant digest was mixed with 9 volumes of acetonitrile and subjected to LC–MS/MS using an LCQ-Advantage ion trap mass spectrometer (Thermo Scientific), equipped with an electrospray ionization source and an HPI100 LC system (Agilent Technologies). For LC, the digest was chromatographed with a ZIC-HILIC column (3.5 µm; pore size: 100 Å; internal diameter, 2.1 × 150 mm; Merck) and eluted with 5 mM formic acid (pH 3.4) (solvent A) and acetonitrile (solvent B) at a flow rate of 100 µl min−1 with a multistep gradient: 90–70% solvent B for 25 min, 70–10% solvent B for 15 min, 10% solvent B for 5 min and then initialized with 90% solvent B.

Expression and purification of recombinant proteins
Synthetic genes for arki from T. kodakarenensis, Methanocaldococcus fervens, P. oguniense, Aquifex aeolicus, Nautilia profundicola and Leptolyngbya sp. PCC7376 were designed with codons optimized for E. coli expression and synthesized by GENEWIZ or Thermo Fisher Scientific. Each gene was cloned into the pE-SUMO-TEV vector by the SLICE method28. N. viennensis arki was PCR amplified from genomic DNA with a set of primers (Supplementary Table 6) and cloned into the BamHI and NotI sites of pE-SUMO-TEV.

E. coli BL21(DE3) or Rosetta2(DE3) cells transformed with the pE-SUMO-TEV vector carrying each arki were cultured in 250 ml or 1 l of LB containing 50 µg ml−1 kanamycin and 20 µg ml−1 chloramphenicol when necessary. His−, SUMO-tagged recombinant protein was expressed at 37 °C for 3–4 h by induction with 0.1 or 1 mM IPTG or 2% (wt/vol) lactose when the cells reached OD600 = 0.4–0.6. P. oguniense Arki was expressed in cells cultured overnight at 18 °C. The collected cells were resuspended in lysis buffer (50 mM HEPES-KOH (pH 8.0), 150 mM KCl, 2 mM MgCl2, 20 mM imidazole, 12% (vol/vol) glycerol, 1 mM 2-mercaptoethanol and 1 mM PMSF) and disrupted by sonication, followed by centrifugation at 15,000g for 15 min at 4 °C. The supernatant was boiled at 60 °C for 20 min (for Arki homologues from T. kodakarenensis, M. fervens, P. oguniense and A. aeolicus) and centrifuged at 15,000g for 15 min at 4 °C. The recombinant protein was affinity captured on an Ni-Sepharose 6 Fast Flow column (GE Healthcare) and then eluted with lysis buffer containing 300 mM imidazole, followed by gel filtration with a PD-10 column (GE Healthcare) to remove the imidazole. The recombinant protein for N. viennensis Arki was purified using a HisTrap column (GE Healthcare) with a linear gradient of 0–500 mM imidazole, followed by dialysis using a Slide-A-Lyzer Dialysis Cassette (Thermo Fisher Scientific) to remove imidazole. The purified protein was subjected to Ulp1 digestion at 4 °C overnight to cleave the His−, SUMO tag and then passed through a Ni-Sepharose 6 Fast Flow column to remove the tag. Because Arki homologues from M. fervens (MfArki) and Leptolyngbya sp. PCC7376 (LrArki) aggregated following tag removal, His−, SUMO tag-fused proteins of these homologues were used for the phosphorylation assay. Purified protein was quantified by Bradford method using BSA as a standard.

For large-scale preparation of T. kodakarenensis Arki for crystallization, the E. coli BL21(DE3) strain carrying pE-SUMO-TkArki was cultured in 21 of LB containing 50 µg ml−1 kanamycin and TkArki was expressed at 25 °C overnight by induction with 0.1 mM IPTG when the cells reached OD600 = 0.4. The cells were collected and disrupted by sonication in lysis buffer (50 mM HEPES-KOH (pH 8.0), 150 mM KCl, 2 mM MgCl2, 20 mM imidazole, 12% (vol/vol) glycerol, 1 mM 2-mercaptoethanol and 1 mM PMSF). The protein was purified using a HisTrap column with a linear gradient of 20–520 mM imidazole. Fractions containing TkArki were pooled and subjected to Ulp1 digestion at 4 °C overnight to cleave the tag, followed by passage through a Ni-Sepharose 6 Fast Flow column to remove the tag fragment. The flow-through fraction was filtered through a 0.45-µm PVDF membrane to remove the resin. The protein was further purified by affinity chromatography with a HisTrap Heparin HP column (GE Healthcare) using a linear gradient of 150–1,150 mM KCl. TkArki was further purified by size exclusion chromatography using a Superdex 75/10/300 GL column (GE Healthcare) with buffer containing 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10 mM 2-mercaptoethanol and then concentrated to 5.74 mg ml−1 and stored at −80 °C.
The T. kodakarensis kptA gene was PCR amplified from genomic DNA from T. kodakarensis with the primers listed in Supplementary Table 6 and cloned into pE-SUMO-TEV to give pE-SUMO-TEV-TkKptA. The E. coli Rosetta2(DE3) strain carrying pE-SUMO-TEV-TkKptA was cultivated in 1 L LB containing 50 μg ml⁻¹ kanamycin and 20 μg ml⁻¹ chloramphenicol, and TkKptA was expressed at 37°C for 3 h by induction with 0.1 mM IPTG when the cells reached OD₆₀₀ = 0.6. The recombinant TkKptA was purified as described above. The gene encoding Tpt1p was PCR amplified from the genomic DNA of S. cerevisiae BY4742 with the set of primers listed in Supplementary Table 6 and was cloned into pET21b (Merck) between the Ndel and Xhol sites. Recombinant Tpt1p was purified as described above.

Removal of the 2'-phosphate of U47 by Tpt1p
Removal of the 2'-phosphate of U47 by yeast Tpt1p was performed as described. Individual tRNAs or the tRNA fraction was incubated for 3 h at 30 °C in a reaction mixture (25 μl) consisting of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl and 1 mM MgCl₂ and incubated at 80 °C for 5 min, followed by cooling to 25 °C at a rate of 0.1 °C s⁻¹. The samples were placed onto a Type 8 multi-micro UV quartz cell (path length, 10 mm). The hyperchromicity of tRNA was monitored on a UV–visible light spectrophotometer (V-630, JASCO). The gradients were as follows: 25 °C for 30 s, 25–40 °C at 5 °C min⁻¹, 40 °C for 5 min and 40–105 °C at 0.5 °C min⁻¹. The Tₘ was calculated using Spectra Manager v2 (JASCO). Melting curves were analysed by LC–MS. The tracer molecules were prepared by dephosphorylation of [15N]adenosine (10 pmol) and [15N]guanosine (10 pmol) as tracer molecules, followed by centrifugation at 15,000 g for 1 min at 4 °C. The supernatant was dried in vacuo and dissolved in 20 μl water. Half of the extract was treated with ethanol. The pellet was dissolved in water to a concentration of 0.1 A₂₆₀ units per μl. For the RNA degradation assay, the labelled tRNA (0.1 A₂₆₀ units, 10,000 c.p.m.) was incubated at 65 °C in a reaction mixture consisting of 10 mM HEPES-KOH (pH 7.6), 0.5 mM MgCl₂, 100 mM NaCl and 0.1 U μl⁻¹ RNase I (Promega). At time points of 1, 3, 5, 10, 15 and 30 min after starting the reaction, aliquots were taken from the mixture and mixed well with chilled phenol/chloroform/isoamyl alcohol (25:24:1, pH 7.9) to stop the reaction, followed by centrifugation at 15,000 g for 15 min at 4 °C. The supernatant was collected and treated with an equal volume of chloroform, followed by centrifugation at 15,000 g for 5 min at 4 °C. The supernatant was mixed with 2 x loading solution (2 x TBE, 7 M urea, 13.3% (wt/vol) sucrose, 0.05% (wt/vol) xylene cyanol and 0.05% (wt/vol) bromophenol blue) and subjected to 10% denaturing PAGE. The gel was exposed to an imaging plate and radioactivity was visualized by using an FLA-7000 imaging analyser (FujiFilm). Graphs were generated using Microsoft Excel.

Crystallization of S. tokodaii tRNA
S. tokodaii tRNA (500 μg) isolated as described above was refolded in annealing buffer (50 mMHEPES-KOH (pH 7.6), 5 mM MgCl₂ and 1 mM DTT) by incubation for 5 min at 80 °C and cooling to 25 °C with a rate of 0.1 °C s⁻¹. tRNA₄₅ was further purified by anion exchange chromatography using a Mono Q 5/50 GL column (GE Healthcare) with a linear gradient of 200–1,000 mM NaCl. The major peak was collected, precipitated with isopropanol, dissolved in water and precipitated with ethanol. Tpt1p treated tRNA₄₅ was prepared with the same procedure as described above. The purified tRNA was dissolved in buffer consisting of 10 mM Tris-HCl (pH 7.1) and 2 mM MgCl₂ to a concentration of 50 μM. One microlitre of tRNA solution was mixed with 1 μl Natrix 2 no. 32 (80 mM NaCl, 12 mM spermine-4HCl, 40 mM sodium cacodylate-3H₂O (pH 7.0) and 30% (vol/vol) MPD) (Hampton Research) on silicon-coated glass and crystallized by the hanging drop vapor diffusion method at 20 °C.

Crystallization of T. kodakarensis ArkI
The concentration of TkArkI was adjusted to 5 mg ml⁻¹ before crystallization. One microlitre of the protein solution was mixed with 0.5 μl reservoir solution, containing 25% (vol/vol) ethylene glycol. TkArkI was crystallized by the hanging drop vapor diffusion method at 20 °C.

Data collection and crystal structure determination
The datasets were collected at beamline BL-17A at the Photon Factory at KEK, Japan. For data collection for the T. kodakarensis crystals, the crystals were cryoprotected with a portion of the reservoir solution. For data collection for the native TkArkI crystal, the crystal was cryoprotected with solution containing 25% (vol/vol) ethylene glycol, 2 mM MgCl₂ and 1 mM ATP. For data collection for the iodide-derivative TkArkI crystal, the crystal was briefly soaked in and cryoprotected with solution containing 300 mM potassium iodide and 22.5% (vol/vol) ethylene glycol, and the diffraction dataset was collected at a wavelength of 1.5 Å. The datasets were indexed, integrated and scaled using xds. The initial phase of tRNA₄₅ was determined by molecular replacement with Phaser. The structure of T. thermophilus tRNA₄₅ (PDB, IVS) was used for the model. The initial phase of TkArkI was determined by the SAD method using the anomalous signal of iodide ions. The iodine sites were located by SHELX, and the initial phase was calculated by Phaser. Subsequent density modification and initial model building were performed with SOLVE. The model was further modified with Coot and refined with Phenix. Crystal structures and their electron density maps were visualized using PyMOL, CeuMol or Coot. Torsion angles of the tRNAs were analysed with DSSR software.

Analysis of ligands bound to TkArkI
TkArkI purified by affinity chromatography with a HiTrap Heparin HP column (GE Healthcare) (100 pmol) was mixed with [¹⁵N]adenosine (10 pmol) and [¹⁵N]guanosine (10 pmol) as tracer molecules, followed by addition of 4 volumes of methanol, an equal volume of chloroform and 3 volumes of water and vigorous mixing. The denatured protein was removed by centrifugation at 15,000 g for 1 min at 4 °C. The supernatant was dried in vacuo and dissolved in 20 μl water. Half of the extract was analysed by LC–MS. The tracer molecules were prepared by dephosphorylation of [¹⁵N]ATP and [¹⁵N]GTP as follows: 1,000 pmoles each of [¹⁵N]ATP (Silantes) and [¹⁵N]GTP (Silantes) was treated with 0.04 U alkaline phosphatase (PAP, from Shewanella sp. SIB1, BioDynamics Laboratory) in 20 mM ammonium acetate (pH 8.0) at 60 °C for 30 min. After dephosphorylation, PAP was heat denatured at 95 °C for 5 min.

Construction of gene knockout strains of T. kodakarensis
Knockout strains of T. kodakarensis were constructed by pop-in/pop-out recombination as described previously. The 5' and 3' flanking regions (about 1,000 bp) of T. kodakarensis kptA and kptA were PCR amplified from genomic DNA with a set of primers (Supplementary Table 6) and inserted into the pUD3 vector carrying the pyrf marker to yield pUD3-ark1 and pUD3-kptA. The T. kodakarensis KU216 strain (Δpyrf) was transformed with pUD3-ark1 or pUD3-kptA, and the...
T. kodakarensis strains used in this study are listed in Supplementary Table 7.

Growth phenotype analysis

T. kodakarensis KU216 (wild type), FFH05 (queE::Tn), ΔarkI and ΔarkI/queE::Tn strains were precultured in MA-YT-Pyr medium at 83 °C overnight and inoculated into 8 ml fresh MA-YT-Pyr medium with an initial OD600 of 0.01. The cells were cultured at 83 °C, 87 °C or 91 °C, and cell growth was monitored every 2 h by measuring OD600 with a SI200 diode array spectrophotometer. Graphs were generated using Microsoft Excel.

In vitro transcription of tRNA

For in vitro transcription of T. kodakarensis tRNAval and its G5–C68 variants by T7 RNA polymerase, template DNAs were constructed by PCR using synthetic DNA (Supplementary Table 6). The tRNAs were transcribed at 37 °C overnight in a reaction mixture consisting of 40 mM Tris-HCl (pH 7.5), 24 mM MgCl2, 5 mM DTT, 2.5 mM spermidine, 0.01% (vol/vol) Triton X-100, 0.8 μg/ml T7 RNA polymerase, 1 μg/ml pyrophosphatase, 30 mM DNA template, 2 mM ATP, 2 mM CTP, 2 mM UTP, 2 mM GTP and 10 mM GMP, followed by extraction with phenol/chloroform treatment and desalting with PD-10 columns (GE Healthcare). In vitro transcripts prepared in this way were separated by 10% denaturing PAGE, followed by staining with toluidine blue. The stained bands were cut out and eluted from the gel slice with elution buffer, followed by filtration to remove the gel pieces and ethanol precipitation.

In vitro phosphorylation of tRNA by ArkI

U47 formation by TkArkI was carried out at 70 °C for 20 min in a reaction mixture (30 μl) containing 50 mM HEPES-KOH (pH 7.5), 1 mM MgCl2, 1 mM MnCl2, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM ATP, 0.9 μM tRNA fraction (from the T. kodakarensis ΔarkI strain) and 1 μM TkArkI. After the reaction, the tRNA was extracted by acidic phenol/chloroform, desalted on a NAP-5 column (GE Healthcare) and precipitated with isopropanol. For RNA-MS, the prepared tRNA was desalted by drop dialysis as described above.

Formation of U47 by other ArkI homologues was carried out at 70 °C for 30 min in a reaction mixture (30 μl) containing 50 mM Pipes-NaOH (pH 6.9), 125 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM DTT, 10% (vol/vol) glycerol, 500 μM MATP, 0.05 mg/ml BSA (Takara), 1 μM tRNA transcript and 0.5 μM ArkI protein. For NvArkI, the reaction temperature was set to 45 °C. For ArkI homologue from N. profundicola (NpArkI), the reaction was carried out at 50 °C for 60 min. After the reaction, tRNA was prepared as described above. For PAGE analysis, U47 formation was carried out in a reaction mixture (8 μl) containing 50 mM Pipes-NaOH (pH 6.9), 125 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM DTT, 10% (vol/vol) glycerol, 100 μM [γ-32P]ATP (3,000 Ci mmol−1; PerkinElmer), 0.1 mg/ml BSA (Takara), 0.75 μM recombinant ArkI homologue (NpArkI, NvArkI or LeArkI) and 50 ng–1 μg total RNA. Then, the reaction mixture was mixed with 2× loading solution, resolved by 10% denaturing PAGE and visualized as described above.

In vitro dephosphorylation of tRNA by T. kodakarensis KptA

Dephosphorylation of U47 by TkKptA was carried out at 60 °C for 1 h in a reaction mixture (30 μl) containing 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA (pH 8.0), 1 mM NAD+, 0.1 mM DTT, 0.9 μM T. kodakarensis tRNA fraction and 0.1 μg µl−1 recombinant TkKptA. After the reaction, the tRNA was extracted by acidic phenol/chloroform, desalted on a NAP-5 column (GE Healthcare) and precipitated with isopropanol. For RNA-MS, the prepared tRNA was desalted by drop dialysis as described above.

Kinetic studies of T. kodakarensis ArkI and KptA

TkArkI-mediated U47 formation was quantified by γ-phosphate transfer from [γ-32P]ATP to tRNA. For kinetic measurement of the tRNA substrate, tRNA phosphorylation was performed at 70 °C in a reaction mixture (25 μl) consisting of 50 mM Pipes-NaOH (pH 6.9), 125 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM DTT, 10% (vol/vol) glycerol, 100 μM [γ-32P]ATP (1,500 Ci mmol−1; PerkinElmer), 0.05 mg/ml BSA (Takara), 0.05 μM TkArkI and 0.1–5.0 μM of in vitro-transcribed T. kodakarensis tRNAval. For kinetic measurement of the ATP substrate, the ATP concentration was altered from 15.6 to 1,000 μM [γ-32P]ATP (750 Ci mmol−1; PerkinElmer) and the tRNA concentration was increased to 1.0 μM. At each time point (2 and 5 min), 8-μl aliquots were taken and mixed with an equal volume of 2× loading solution (7 M urea, 0.2% (wt/vol) bromophenol blue, 0.2% (wt/vol) xylene cyanol and 50 mM EDTA (pH 8.0)) to quench the reaction. Each sample was subjected to 10% denaturing PAGE. The gel was exposed on an imaging plate to measure radiolabelled tRNAs using an FLA-9000 imaging analyser. Kinetic parameters were calculated using Prism 7 (GraphPad).

TkKptA-mediated dephosphorylation of U47 was quantified by measuring the reduction in radioactivity for tRNA. In vitro-transcribed T. kodakarensis tRNAval was phosphorylated by TkArkI with [γ-32P]ATP as described above and then purified by gel extraction and isopropanol precipitation. In addition, the same tRNA was phosphorylated by TkArkI with unlabelled ATP. By mixing labelled and unlabelled tRNAs, the specific activity of the labelled tRNA was adjusted to 6,250 c.p.m. per pmol in buffer consisting of 50 mM Pipes-KOH (pH 7.6), 5 mM MgCl2 and 1 mM DTT. The labelled tRNA was incubated at 80 °C for 5 min and then cooled at room temperature, followed by isopropanol precipitation. The labelled tRNA was dissolved in water to a concentration of 8 μM (50,000 c.p.m. per μl). Dephosphorylation of the labelled tRNA by TkKptA was performed at 70 °C in a reaction mixture (30 μl) consisting of 50 mM Pipes-NaOH (pH 6.9), 125 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, 1 mM DTT, 10% (vol/vol) glycerol, 1 mM NAD+, 0.05 mg/ml BSA (Takara), 1 μM TkKptA and 12.5–800 nmol [32P]-labelled tRNA. At each time point (2 and 5 min), 8-μl aliquots were spotted on Whatman 3MM filter paper, which was immediately soaked in 5% (wt/vol) trichloroacetic acid. The filter paper was washed three times for 15 min with ice-cold 5% (wt/vol) trichloroacetic acid, rinsed for 5 min with ice-cold ethanol and dried in air. Radioactivity on the filter paper was measured by liquid scintillation counting.
counting (Tri-Carb 2910TR, PerkinElmer). Kinetic parameters were calculated using Prism 7.

In vivo dephosphorylation of U^47 by Kpta

_N. viennensis arki_ was PCR amplified and cloned into pMW118 (Invitrogen) under the control of the synthetic constitutive J23106 promoter^2^, followed by insertion of sequences encoding a His_6_ tag and a 3xFlag tag at the C terminus of the _N. viennensis arki_ gene, yielding pMW-J23106-nvarkf (Supplementary Table 7). _T. kodakaraensis kpta_, _E. coli kpta_ and _S. cerevisiae tpt1_ were PCR amplified and cloned into pQE-80L (Qiagen). The ampicillin resistance cassette (Amp^r_) was replaced with a chloramphenicol resistance cassette (Cam^r_), yielding pQE-80LC-tkktA, pQE-80LC-ekptA and pQE-80LC-scpt1, respectively (Supplementary Table 7). The _E. coli ΔtmRNAtapT_ (Kan^r_) strain was transformed with pMW-J23106-nvarkf and further transformed with pQE-80LC-tkktA, pQE-80LC-ekptA or pQE-80LC-scpt1. The transformants were inoculated in 3 ml LB supplemented with 20 μg ml^-1_ chloramphenicol, 50 μg ml^-1_ kanamycin and 100 μg ml^-1_ ampicillin and cultured at 37 °C until mid-log phase. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 10 or 100 μM to induce expression of the Kpta/Tpt1 homologue and cells were cultured for 3.5 h. A 1.5-ml aliquot of the culture was taken, and the tRNA fraction was extracted and analysed by shotgun RNA-MS as described above. Primers, _E. coli_ strains and plasmids used are listed in Supplementary Tables 6, 7. Bar graphs with independent plots were prepared with R (R Foundation).

**Drawing of chemical structures**

Chemical structures were drawn with chemical structure drawing tools, including ACD/ChemSketch (ACD/Labs) or ChemDraw (PerkinElmer).

**Reporting summary**

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

**Data availability**

Coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 7VNW, 7VNQ and 7VNX. Source data are provided with this paper.

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50. Sato, T., Fukui, T., Atomi, H. & Imanaka, T. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon Thermococcus kodakaraensis KOD1. _J. Bacteriol_. **185**, 210–220 (2003).
51. Robb, F. T. & Place, A. R. Media for Thermophiles (Cold Spring Harbor Laboratory Press, 1995).
52. Suzuki, T. & Suzuki, T. Chaplet column chromatography: isolation of a large set of individual RNAs in a single step. _Methods Enzymol._ **425**, 231–239 (2007).
53. Møgel-Jørgensen, J. & Kirpekar, F. Detection of pseudouridine and other modifications in tRNA by cyanoethylation and MALDI mass spectrometry. _Nucleic Acids Res._ **30**, e135 (2002).
54. Miyachi, K., Kuma, S. & Suzuki, T. A cyclic form of N^6-ethenylcarbamoyladenosine as a widely distributed tRNA hypermodification. _Nat. Chem. Biol._ **9**, 105–111 (2013).
55. Zhang, Y., Werling, U. & Edelmann, W. SUC.E: a novel bacterial cell extract-based DNA cloning method. _Nucleic Acids Res._ **40**, 12 (2012).
56. Kabsch, W. XDS. Acta Crystallogr. D **66**, 123–132 (2010).
57. McCoy, A. J. et al. Phaser crystallographic software. _J. Appl. Crystallogr._ **40**, 658–674 (2007).
58. Fuku, S. et al. Mechanism of molecular interactions for tRNA(pyrimid) recognition by valyl-tRNA synthetase. _RNA_ **9**, 100–111 (2003).
59. Sheldrick, G. M. A short history of SHELX. _Acta Crystallogr._ A **64**, 112–122 (2008).
60. Terwilliger, T. C. Maximum-likelihood density modification. _Acta Crystallogr._ D **56**, 965–972 (2000).
61. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. _Acta Crystallogr._ D **66**, 486–501 (2010).
62. Adams, P. D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. _Acta Crystallogr._ D **66**, 213–221 (2010).
63. Lu, X. J., Bussemaker, H. J. & Olson, W. K. DSSR: an integrated software tool for dissecting the spatial structure of RNA. _Nucleic Acids Res._ **43**, e142 (2015).
64. Sato, T., Fukui, T., Atomi, H. & Imanaka, T. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon Thermococcus kodakaraensis. _Appl. Environ. Microbiol._ **71**, 3889–3899 (2005).
65. Kubon, H. et al. Characterization of NADH-oxidase/NADPH polysulfolide oxidoreductase and its unexpected participation in oxygen sensitivity in an anaerobic hyperthermophilic archaeon. _J. Bacteriol._ **192**, 5192–5202 (2010).
66. Sampson, J. R. & Uilenberg, O. C. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed in vitro. _Proc. Natl Acad. Sci._ USA **85**, 1033–1037 (1988).
67. Anderson, C. J. Anderson promoter collection (2007).
68. Kelly, J. R. et al. Measuring the activity of Biobrick promoters using an in vivo reference standard. _J. Biol. Eng._ **3**, 4 (2009).

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Author contributions

K. Minowa, T.O. and K.S. mainly performed the series of experiments. K.S. and T.O. conducted LC-MS analyses and biochemical and thermodynamic analyses of _S. tokodaii_ RNA assisted by K. Miyachi and Y.S. Crystal structure analysis of RNA was performed by K.S., T.O. and K. Minowa with support from K.T. Gene identification by comparative genomics was conducted by K. Minowa and T.O. Biochemical characterization of ArkI proteins was performed by K. Minowa and R.K. Minowa performed genetic work assisted by A.K., I.O. and T.F. K. Minowa performed genetic work assisted by K. Miyachi and Y.S. All authors discussed the results and revised the manuscript. T.O., K. Minowa and T.S. designed the studies and wrote the manuscript. T.S. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Takayuki Ohira, Kozo Tomita or Tatsuro Suzuki.

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Extended Data Fig. 1 | Isolation of *S. tokodaii* tRNAs. tRNAs for Val, Ile2, and Phe were isolated by CCC\(^3\). The other tRNAs were isolated by RCC\(^2\). The isolated tRNAs and total RNA as a maker were resolved by electrophoresis on a 10% (w/v) polyacrylamide gel containing 7 M urea and 1 × TBE, and stained with SYBR Gold. Smearing of bands is due to the high GC content of tRNAs. The precursor tRNAs for Ile2 and Phe containing an intron are indicated by red triangles. 5S rRNA, Class II tRNAs, and Class I tRNAs are indicated. We confirmed the reproducibility of this result. The unprocessed gel images are provided in Supplementary Fig. 10.
Extended Data Fig. 2 | Nucleoside analysis and RNA-MS of *S. tokodaii* tRNA\(^{\text{Val2/3}}\). (a) Nucleosides of *S. tokodaii* tRNA\(^{\text{Val2/3}}\) were subjected to LC/MS analysis. Top panel shows the UV trace at 254 nm. Second to bottom panels show XICs detecting proton adducts of modified nucleosides as indicated. N\(^{324}\) nucleoside was not detected in the bottom panel. m’G is derived from tRNA\(^{'\text{Val}}\), which was co-isolated with tRNA\(^{'\text{Val}}\) (Supplementary Fig. 1). Asterisks indicate unassigned ions. (b) RNA-MS of RNase T\(_1\) digests of the isolated *S. tokodaii* tRNA\(^{\text{Val2/3}}\). The upper panel shows the BPC of RNase T\(_1\)-digested fragments. The sequence and molecular mass of each RNA fragment numbered in BPC are listed in Supplementary Table 1a. The lower panel shows the XIC of the divalent negative ion of the fragment containing N\(^{324}\). (c) RNA-MS of RNase A digests of isolated *S. tokodaii* tRNA\(^{\text{Val2/3}}\). The upper panel shows the BPC of RNase A-digested fragments. The sequence and molecular mass of each RNA fragment numbered in BPC are listed in Supplementary Table 1b. The lower panel shows the XIC of the divalent negative ion of the fragment containing N\(^{324}\).
**Extended Data Fig. 3** N\textsuperscript{324} is present in various class I tRNAs. (a) RNA-MS of RNase T\textsubscript{1} digests of the isolated *S. tokodaii* tRNAs. XICs show negative ions of RNA fragments derived from V-loop containing N\textsuperscript{324} (red line) or U (black line) at position 47. Sequence and \textit{m/z} value of each fragment are provided in Supplementary Table 2. Modification frequency of N\textsuperscript{324} indicated in each tRNA was calculated from relative peak intensities of the modified and unmodified fragments. Unassigned fragments are indicated by asterisks. (b) CID spectrum of the RNA fragments detected in (a). The negatively-charged ion of each fragment was used as a precursor ion for CID analysis. The product ions in the CID spectrum are assigned on the sequences. N\textsuperscript{324} is shown in red. (c) N\textsuperscript{324} is introduced in precursor tRNAs. RNA-MS of RNase T\textsubscript{1} digests of the precursor tRNAs for Ile\textsubscript{2} and Phe isolated from *S. tokodaii* (Extended Data Fig. 1). XICs show negative ions of the RNA fragments derived from V-loop containing N\textsuperscript{324} (red line) or U (black line) at position 47. Modification frequency of N\textsuperscript{324} indicated in each tRNA was calculated from relative peak intensities of the modified and unmodified fragments.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Chemical structure determination of N₃²⁴. (a) RNA-MS of the N₃²⁴-containing fragment of tRNAVal3 digested with RNase T₁, with (+) or without (-) BAP treatment. XICs show the divalent negative ions of N₃²⁴m⁵Cm⁵CUGp (m/z 845.59, z = -2) and Um⁵Cm⁵CUGp (m/z 765.52, z = -2). Two phosphates were removed by this treatment. (b) CID spectrum of the N₃²⁴-containing fragment treated with BAP. The product ions are assigned on Um⁵Cm⁵CUGp. (c) Preparation scheme of pN₃²⁴p. S. tokodaii tRNA fraction is digested with nuclease P₁, yielding dinucleotide pN₃²⁴m⁵C. The digests were subjected to periodate oxidation and β-elimination to remove the 3’ terminal residue. The resultant pN₃²⁴p was purified by anion exchange chromatography and subjected to LC/MS/MS analysis. (d) LC/MS nucleotide analysis of the nuclease P₁ digest of S. tokodaii tRNA fraction. UV trace at 254 nm (upper panel) and XIC of the negatively charged ion of pN₃²⁴m⁵C (m/z 722, z = -1) (lower panel) are shown. (e) CID spectrum of pN₃²⁴m⁵C. The product ions were assigned on the predicted chemical structure of pN₃²⁴m⁵C. The phosphate group of N₃²⁴ is shown in red. (f) CID spectrum of the N₃²⁴ nucleotide (pN₃²⁴p; m/z 483, z = -1). The product ions are assigned in the predicted chemical structure of pN₃²⁴p. (g) RNA-MS of the V-loop-containing RNA fragment with (+) or without (-) Tpt1p treatment before RNase T₁ digestion. XICs show the divalent negative ions of U²⁴m⁵Cm⁵CUGp (m/z 845.59, z = -2) and Um⁵Cm⁵CUGp (m/z 805.60, z = -2). (h) CID spectrum of the dephosphorylated fragment by Tpt1p. The Tpt1p-treated tRNA Val3 was digested with RNase T₁ and analyzed by RNA-MS. The V-loop-containing fragment was selected as a precursor for CID. The product ions are assigned on the sequence as indicated.
Extended Data Fig. 5 | Crystal structures of S. tokodaii tRNA<sup>Val</sup>. 
(a,b) Schematic views of Mol. A (a) and B (b) of S. tokodaii tRNA<sup>Val</sup>. Each residue is shown as a box. Color codes for the base pairs and base triples are the same as those in Fig. 2b,c. Tertiary interactions are shown as blue dashed lines.
(c,d) Simulated annealing-omit F<sub,o</sub>-F<sub,c</sub> map contoured at 3.0 sigma around 2′-phosphate of Mol. A (c) and Mol. B (d).
(e) LeVitt base pair of G<sup>15</sup> and m<sub>5</sub>C<sub>48</sub> with neighboring residues shown in stick representation with electron density map. 2F<sub,o</sub>-F<sub,c</sub> electron density map contoured at 0.76 sigma around G<sup>15</sup>–m<sub>5</sub>C<sub>48</sub> is shown in the lower panel.
(f) Close-up view of the tRNA core around the G<sup>15</sup>–m<sub>5</sub>C<sub>48</sub> base pair. G<sup>15</sup>, m<sub>5</sub>C<sub>48</sub>, and neighboring residues are shown in stick representation. Other residues are indicated as lines. Backbones are shown as cartoons. Hydrogen bonds are indicated by yellow dash lines.
(g) Base pair of ac<sub>4</sub>C<sub>6</sub> with G<sub>67</sub> in stick representation with electron density map. 2F<sub,o</sub>-F<sub,c</sub> electron density map contoured at 0.76 sigma around ac<sub>4</sub>C<sub>6</sub>–G<sub>67</sub> is shown in the lower panel.
(h) Close-up view of the acceptor stem including ac<sub>4</sub>C<sub>6</sub>, G<sub>67</sub>, and neighboring base-pairs and nucleotides.
(i) Electron density map of m<sub>1</sub>I<sub>57</sub> and m<sub>1</sub>A<sub>58</sub>. 2F<sub,o</sub>-F<sub,c</sub> electron density maps contoured at 0.76 sigma for m<sub>1</sub>I<sub>57</sub> and 1.02 sigma for m<sub>1</sub>A<sub>58</sub> are shown.
(j) Close-up view of T-loop including m<sub>1</sub>I<sub>57</sub>, s<sub>5</sub>U<sub>54</sub>, m<sub>1</sub>A<sub>58</sub>, and neighboring base-pairs and nucleotides.
(k) Base pair of m<sub>2</sub>G<sub>26</sub> with A<sub>44</sub> in stick representation with electron density map. 2F<sub,o</sub>-F<sub,c</sub> electron density map contoured at 0.76 sigma around m<sub>2</sub>G<sub>26</sub>–A<sub>44</sub> is shown in the lower panel.
(l) Close-up view of D- and anticodon-stems including m<sub>2</sub>U<sub>10</sub>, m<sub>2</sub>G<sub>26</sub>, and neighboring base-pairs and nucleotides.
Extended Data Fig. 6 | Torsion angles of nucleotides around position 47 of tRNAs. (a) Key to torsion angles of nucleic acid backbone. Nomenclature of each angle is shown next to its direction, depicted as a black curved arrow. (b) Comparison of torsion angles at positions 44–48 of *S. cerevisiae* tRNA<sup>Phe</sup> (PDB: 1EHZ) (leftmost), and *S. tokodaii* tRNA<sup>Val</sup> Mol. A (left), Mol. B (right), and Tpt1p-treated tRNA Val3 (rightmost). Torsion angles of each tRNA are listed in Supplementary Table 3. Torsion angle changes from yeast tRNA<sup>Phe</sup> to Mol. A are shown on Mol. A as curved arrows. Torsion angle changes from Mol. A to Mol. B are shown on Mol. B as curved arrows. Torsion angle changes from Mol. A to Tpt1p-treated tRNA (Mol. A) are shown on Tpt1p-treated tRNA as curved arrows. The lower panels show the 90 degree-rotated models.
Extended Data Fig. 7 | Crystal structure of Tpt1p-treated *S. tokodaii* tRNA*val*<sup>3</sup>. (a) Overviews of crystal structure of Tpt1p-treated *S. tokodaii* tRNA*val*<sup>3</sup> with stick representation. Molecules A (left) and B (right) are shown in stick representation in pink and orange, respectively. U47 is colored in red. (b) Close-up views of the core structure of Mol. A (left) and B (right). Color code is the same as in Fig. 2b. (c) Schematic views of the core structure of Mol. A (left) and B (right). (d, e, f) Atomic structures of the base triples s<sup>1</sup>U8–A14–A21 (top), Ψ13–G22–G46 (middle) and C12–G23–C9 (bottom), in the core region of Mol. A (left) and B (right). Dashed lines indicate predicted interactions, with bond length in Å. U47 is shown in red.
Extended Data Fig. 8 | Phylogenetic distribution of U^p47 in archaeal species. (a) LC/MS nucleotide analyses of tRNA fractions from S. tokodaii, S. acidocaldarius, S. solfataricus, A. pernix, T. kodakarensis, and M. acetivorans. UV trace at 254 nm (upper panels) and XICs of the proton adducts of pN^324m5C (m/z 724, z = 1) (lower panels) are shown. Asterisks indicate unassigned ions. (b) RNA-MS shotgun analysis of N. viennensis tRNA fraction treated with (right panel) or without (left panel) Tpt1p before RNase T_1 digestion. XICs show the RNA fragments containing U^p47 (UpCGp; m/z 1053.08, z = -1). The product ions in the CID spectrum are assigned on U^pCGp. (c) RNA-MS shotgun analysis of P. oguniense tRNA fraction treated with (right panel) or without (left panel) Tpt1p before RNase T_1 digestion. XICs show the RNA fragments containing U^p47 (Upm5Cm5Cac4CGp; m/z 866.10, 1733.20, z = -2, -1). The product ions in the CID spectrum are assigned on U^m5Cm5Cac4CGp. (d) RNA-MS shotgun analysis of T. acidophilum tRNA fraction digested by RNase T_1. XICs show the expected RNA fragments containing U^p47 (left panels) or U47 (right panels) as indicated.
Extended Data Fig. 9 | Sequence alignment of ArkI family (COG2112). ArkI homologs and PRKACA (PRKACA_MOUSE, PDB: 1ATP) as a canonical ePK are aligned based on structure comparison using DALI (http://ekhidna2.biocenter.helsinki.fi/dali/). Bacterial and archaeal homologs of ArkI are added using MAFFT (https://mafft.cbrc.jp/alignment/server/). Black and gray boxes indicate the degree of sequence similarity. Residues mutated in TkArkI are indicated as red letters. The alpha helices and beta strands observed in the TkArkI structure are depicted on top of alignments as helices and arrows, respectively. Those observed in PRKACA are depicted under the alignments, as well. Subdomains (I to XI) and representative motifs (P-loop, HRD, DFG, and APE) in ePK are underlined and featured. Abbreviations for organisms: Tk, Thermococcus kodakarensis; PAB, Pyrococcus abyssi; STK, Sulfurisphaera tokodaii; Mefer, Methanocaldococcus fervens; Pogu, Pyrobaculum oguniense; aq, Aquifex aeolicus.
Extended Data Fig. 10 | Dephosphorylation of U^47 by KptA/Tpt1p homologs in *E. coli*. (a, c) *In vivo* dephosphorylation of U^47 by EcKptA (a) or ScTpt1p (c). XICs show U^47-containing fragments from various *E. coli* tRNA species (Supplementary Table 5) isolated from *E. coli* ΔtrmB/ΔtapT strain expressing *N. viennensis* ArkI; U^CGp (top panels), U^CUGp (second panels), U^CACGp (third panels), U^CACAGp (fourth panels), and m^5UΨCGp as a control fragment (bottom panels). Relative abundance of the U^47-containing fragments was measured in *E. coli* strain in which EcKptA (a) or ScTpt1p (c) is not expressed (left panels) or induced by 10 μM (middle panels) or 100 μM IPTG (right panels). (b, d) Quantification of U^47 dephosphorylation in *E. coli* by EcKptA (b) or ScTpt1p (d). Peak intensity is shown for each U^47-containing fragment detected in tRNA fractions from *E. coli* strain cultured with 0, 10, or 100 μM IPTG. Data represent average values of technical triplicates ± s.d.
## Extended Data Table 1 | Data collection and refinement statistics.

|                  | Wild-type St tRNA<sup>Val</sup> | Dephosphorylated St tRNA<sup>Val</sup> | TkArk1_K1 | Native TkArk1 |
|------------------|---------------------------------|---------------------------------------|------------|--------------|
| **Space group**  | *P*<sub>2</sub>                 | *P*<sub>2</sub>                       | *P*<sub>6</sub> | *P*<sub>6</sub> |
| **Cell dimensions** |                                |                                       |            |              |
| *a, b, c* (Å)    | 32.65, 116.62, 57.24            | 32.56, 116.68, 56.14                  | 68.03, 68.03, 99.45 | 66.85, 66.85, 98.28 |
| *α, β, γ* (°)    | 90, 100.97, 90                  | 90, 101.34, 90                        | 90, 90, 120 | 90, 90, 120 |
| **Wavelength (Å)** | 0.98                           | 0.98                                  | 1.5        | 1.0          |
| **Resolution (Å)** | 50.1-1.9                       | 50.2-1.9                              | 50.2-1.9   | 50.1-1.8     |
| *R*<sub>sym</sub>* | 0.068 (1.552)                  | 0.111 (1.090)                         | 0.213 (2.887) | 0.069 (1.810) |
| *<I/σI>*          | 15.7 (1.6)                      | 14.5 (2.0)                            | 20.5 (2.9) | 35.2 (2.7)   |
| *CC<sub>1/2</sub>* | 0.998 (0.609)                  | 0.997 (0.649)                         | 0.999 (0.787) | 1.000 (0.882) |
| **Completeness (%)** | 98.8 (97.5)                    | 99.8 (99.5)                           | 100.0 (99.4) | 100.0 (99.8) |
| **Redundancy**    | 6.7 (6.8)                      | 6.7 (6.2)                             | 39.0 (38.9) | 39.6 (40.5)  |
| **Phasing**       |                                 |                                       |            |              |
| I sites          |                                  |                                       | 10         |              |
| FOM              |                                  |                                       | 0.322      |              |

### Refinement

|                  | Wild-type St tRNA<sup>Val</sup> | Dephosphorylated St tRNA<sup>Val</sup> | TkArk1_K1 | Native TkArk1 |
|------------------|---------------------------------|---------------------------------------|------------|--------------|
| Resolution (Å)   | 50.1-1.9                        | 50.2-1.9                              |            | 50.1-1.8     |
| No. reflections  | 32414                           | 12511                                 |            | 23014        |
| *R*<sub>work</sub> / *R*<sub>free</sub> (%) | 19.47 / 21.98 | 22.49 / 26.23 |            | 17.10 / 20.49 |
| No. atoms        |                                 |                                       |            |              |
| RNA              | 3368                            | 3360                                  |            | 1701         |
| Ligand           | -                               | -                                     |            | 20           |
| Water            | 92                              | 67                                    |            | 77           |
| *B*-factors (Å<sup>2</sup>) |                                |                                       |            |              |
| RNA              | 53.8                            | 51.3                                  |            | 42.18        |
| Ligand           | -                               | -                                     |            | 53.32        |
| Water            | 39.9                            | 39.0                                  |            | 47.48        |
| R.m.s. deviations |                                |                                       |            |              |
| Bond lengths (Å) | 0.007                           | 0.005                                 |            | 0.012        |
| Bond angles (°)  | 0.95                            | 1.06                                  |            | 1.11         |
| Estimated mean coordinate error |                  |                                       |            |              |
| Phenix maximum likelihood (Å) | 0.24                           | 0.43                                  |            | 0.17         |

*Values in parentheses are for the highest-resolution shells.*
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- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

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Data collection

- Gel images were obtained by FLA-7000 and FLA-9000.
- Melting curves were obtained by V-630.
- MS data were obtained by LTQ Orbitrap XL, Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer, LCQ Advantage Ion-trap Mass Spectrometer.
- Radioactivities were measured by Tri-Carb 2910TR1
- Cell densities were monitored by S1200 diode array spectrophotometer.
- X-ray diffraction data were obtained by Dectris Eiger X16MS detector and UGUA control system with BL17A beamline at the Photon Factory.
Data analysis

Canvas X (version 20), ACD/ChemSketch (Freeware, 2018.2.1), ChemDraw (20.1.1), Excel (2016, 2019) and R (4.1.2) were used to draw figures.

Xcalibur (4.1) was used for mass spec analysis.

Spectra Manager (v2) was used for Tm measurement.

Multi Gauge (V3.0) was used for graphical analysis.

Prism 7 was used for kinetic analysis

XDS/XSCALE (VERSION Feb 5, 2021), SHEXL (2016/1), Phaser (2.8.3), RESOLVE (2.15), APBS (1.5), Coot [0.8.9.1], and Phenix (Version 1.18.2.3874) were used for structural analyses.

Coot (0.8.9.1), Pymol (2.4.0), CueMol (2.2.3.443) were used for draw structure data.

RECOG (1.1.32) was used for comparative genome.

x3dna-dssr (v1.9.10) was used for analysis of torsion angles.

PhyIoT (v2) and iTOL [6.5] were used for analysis of phylogenetic distribution.

DALI (v.5) and MAFFT (Version 7) were used for sequence alignment.

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Public databases: Microbial Genome Database (MGDB), NCBI database, COG database, BacDive, Genome Online Database, IMG database, Mongo Olgo Mass Calculator v2.0, Genomic tRNA database, Modomics, Protein Data Bank (PDB) [1EHZ, 1LVS, and 1ATP]. Coordinates and structure factors have been deposited in PDB under accession code 7VNV, 7VNW, and 7VNX.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

3 data points were used in student t-test.

Data exclusions

No data were excluded.

Replication

All attempts to replicate experiments succeeded.

I1m measurement, RNAse probing, growth comparison, in vitro biochemical studies, were technically or biologically triplicated.

Randomization

Randomization was irrelevant for this basic study because it did not involve clinical trials or population studies.

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No blinding was required because the results of measurement or analysis was not affected by knowledge of sample identities.

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