Published in final edited form as:

FEBS Lett. 2015 July 22; 589(16): 2124–2130. doi:10.1016/j.febslet.2015.06.028.

In vitro substrate specificities of 3'-5' polymerases correlate with biological outcomes of tRNA 5'-editing reactions

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Abstract

Protozoan mitochondrial tRNAs (mt-tRNAs) are repaired by a process known as 5'-editing. Mt-tRNA sequencing revealed organism-specific patterns of editing G-U base pairs, wherein some species remove G-U base pairs during 5'-editing, while others retain G-U pairs in the edited tRNA. We tested whether 3'-5' polymerases that catalyze the repair step of 5'-editing exhibit organism-specific preferences that explain the treatment of G-U base pairs. Biochemical and kinetic approaches revealed that a 3'-5' polymerase from *A. castellanii* tolerates G-U wobble pairs in editing substrates much more readily than several other enzymes, consistent with its biological pattern of editing.

Keywords

tRNA editing; mitochondrial tRNA; Thg1-like proteins; *Acanthamoeba castellanii*; *Dictyostelium discoideum*; 3'-5' polymerase

A family of 3'-5' polymerases has been discovered, members of which catalyze nucleotide addition in the reverse direction to all known DNA/RNA polymerases (1, 2). Based on sequence similarity, 3'-5' polymerases are further divided into two distinct sub-families: the tRNA^{His} guanylyltransferases (Thg1) and the Thg1-Like Proteins (TLPs) (3). Thg1 enzymes are strictly eukaryotic and their biological function, addition of an essential 5'-guanosine (G₋₁) to tRNA^{His}, is well-established (4-6). In contrast, TLPs, found in a limited number of bacteria but more widespread in archaea and protozoan eukaryotes, are less well-characterized (3, 7-11). However, recent data identified four TLPs as the first candidate mitochondrial-tRNA (mt-tRNA) 5'-editing enzymes (12, 13).

Mitochondrial 5'-editing was discovered in *Acanthamoeba castellanii*, but has since been demonstrated to occur in many eukaryotic protozoa, including *Dictyostelium discoideum* (14-20). During editing, mt-tRNA with mismatched nucleotides encoded in their aminoacyl-acceptor stems undergo a 2-step process that first removes 5'-mismatched nucleotides,

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followed by 5'-end repair of the resulting 5'-truncated tRNA (Figure 1) (3, 21, 22). The second step of the reaction requires a 3'-5' polymerase to extend the 5'-truncated RNA using the 3'-half of the acceptor stem as a template. However, no enzyme with this activity was known until bacterial TLPs were shown to catalyze a similar 5'-end repair activity with model tRNA substrates (9). Subsequently, two *D. discoideum* TLPs (DdiTLP3 and DdiTLP4) and two *A. castellanii* TLPs (AcaTLP1 and AcaTLP2) were each demonstrated to repair multiple 5'-truncated mt-tRNA in vitro, suggesting that these enzymes could catalyze 5'-editing (12, 13).

Investigation of mt-tRNA 5'-editing patterns revealed additional complexity in the treatment of G-U wobble base pairs. Direct sequencing of mt-tRNA from 8 different organisms revealed significant differences in the extent of G-U base pair editing (14, 16-19). For example, in *D. discoideum* a U₃-G₇₀ base pair in Ile_{GAU(1)} is 100% edited to a Watson-Crick (WC) C₃-G₇₀ base pair (Figure 2A) (14). However, editing of a U₃-G₇₀ base pair in an identical context in *Polysphondylium pallidum* is barely detected (in only 6% of sequences) (Figure 2B) (14). Other examples of partially edited or unedited G-U base pairs directly adjacent to edited base pairs are observed in *Monoblepharella* and *Harposchyzium* (19). In *A. castellanii*, editing of a U₃-U₇₀ mismatch in mt-tRNA^{Ala}_{UGC} occurs immediately adjacent to a U₄-G₆₉ base pair that is retained after editing (Figure 2C) (18). Thus, *A. castellanii* and *D. discoideum* exhibit disparate abilities to tolerate U-G base pairs in the context of editing substrates. This observation prompted us to use the four previously characterized TLPs (from *A. castellanii* and *D. discoideum*) to test whether organism-specific preferences of TLPs could explain the G-U editing patterns observed in vivo. We hypothesized that if a TLP efficiently extends a substrate mt-tRNA with a G-U base pair at its terminus, then the G-U base pair could remain part of the final edited mt-tRNA. In contrast, if the TLP does not efficiently repair these substrates, then the nucleotide involved in the G-U base pair would necessarily be removed (by the as of yet unidentified nuclease(s) prior to action of the TLP in repairing the 5'-end), resulting in replacement of G-U wobble base pairs with WC base pairs during editing.

To test this, we developed a new assay for 3'-5' polymerase activity, which has significant advantages over previously utilized assays. We measured single-turnover kinetic parameters for single and multiple nucleotide additions to two different 5'-truncated substrates based on *D. discoideum* mt-tRNA^{Ile}_{GAU(1)}. The two substrates differ by the identity of their terminal base pair, with one terminating in a WC C-G base pair, while the other terminates in a wobble U-G base pair. Here we show that among the four 3'-5' polymerase repair enzymes, three (DdiTLP3, DdiTLP4 and AcaTLP1) catalyze inefficient repair of the wobble-terminating mt-tRNA relative to the WC-containing substrate. However, one of the *A. castellanii* TLPs (AcaTLP2) exhibits a substantially improved tolerance for the substrate that terminates with a U-G base pair. This result is consistent with biological patterns of 5'-editing, in which the 5'-end repair reaction leaves a U-G base pair intact adjacent to edited mismatches in *A. castellanii*, while no examples of this are observed in *D. discoideum*.

Materials and methods

Preparation of tRNA substrates and 3'-5' polymerases

Sequence-verified plasmids were used as templates for in vitro T7 transcription with a 5'-hammerhead ribozyme engineered to generate the desired 5'-start site for each transcript (C_{+4} or U_{+3}) (12). Hammerhead-cleaved transcripts were either 5'-end labeled by T4 polynucleotide kinase and γ - ^{32}P -ATP or uniformly labeled by inclusion of $[\alpha$ - ^{32}P]-ATP during transcription. TLPs from *D. discoideum* and *A. castellanii* were expressed as N-terminal His₆-tagged proteins in *E. coli* and purified as previously described (12, 13).

Enzyme titration assay for 3'-5' addition to tRNA^{Ile}_{GAU(1)}

5'-truncated mt-tRNA^{Ile}_{GAU(1)} (C_{+4} or U_{+3} -start, as indicated) substrates were incubated at room temperature with five-fold serial dilutions of TLP enzymes (2.7 μM to 0.2 nM), 1 mM CTP and/or UTP and 0.1 mM ATP in reaction buffer (25 mM HEPES, pH 7.5, 10 mM MgCl₂, 3 mM DTT, 125 mM NaCl and 0.2 mg/mL BSA). Reactions were quenched after 2 hours by addition of 12.5 mM ethylenediamine tetraacetic acid (EDTA), 5 U/ μL RNase T1 (Ambion), 20 mM sodium acetate, pH 5.2 and 100 ng/ μL carrier yeast tRNA; RNase T1 cleavage was at 37°C for 30 minutes. An equal volume of formamide loading dye was added and reactions were resolved by 12 % polyacrylamide, 7 M urea, 1X tris-borate-EDTA gel. Dried gels were visualized using a Typhoon imager. For uniformly-labeled substrates, after RNase T1 cleavage, 0.1 U/ μL CIAP (Invitrogen) and 1X dephosphorylation buffer were added and reactions were incubated at 37 °C for 30 minutes prior to resolution and visualization as described above.

Single-turnover kinetic measurements of 5'-end repair

TLP enzymes and uniformly-labeled 5'-truncated tRNA^{Ile}_{GAU(1)} (initiating with C_{+4} or U_{+3}) were added to reactions in a ratio of at least 50:1 with varied concentrations for each enzyme (1, 2 or 4 μM), the 5'-activating ATP (0.1 or 0.25 mM) and added CTP and/or UTP (0.25, 0.5 and 1 mM). Time courses ranged from 10 sec – 2 hr for U_{+3} -start and from 10 sec – 1 hr for C_{+4} -start. Reactions were resolved by 20 % polyacrylamide, 8 M urea, 1X tris-borate-EDTA gel. Percent product conversion at each time point (P_t) was plotted vs. time and fit to the single exponential rate equation (eq. 1), where P = maximal product formation.

$$Pt = \Delta P (1 - \exp(-k_{obs} \times t)) \quad (\text{eq. 1})$$

For very slow reactions, (i.e., DdiTLP3/4 on U_{+3} -start substrate), the method of initial rates was used to calculate k_{obs} from the slope of the line fit to the product formation plot. Rates from three independent measurements at 0.5 mM CTP and/or UTP, as indicated, were averaged and reported errors are standard derivations.

Results

A gel-based assay to detect multiple nucleotide addition to mt-tRNA

Previous characterization of 3'-5' polymerases utilized a phosphatase protection assay developed to study single nucleotide addition catalyzed by *S. cerevisiae* Thg1 (6). Although

the assay has some advantages in resolution and direct quantification of both substrate and product for kinetics, the need for defined standards and sequence incompatibility with some substrates presents challenges for studying multiple nucleotide addition reactions, such as those that occur during 5'-editing.

We sought an alternative approach using polyacrylamide gel electrophoretic (PAGE) analysis, with two different substrates based on *D. discoideum* mt-tRNA^{Ile}_{GAU(1)} (Figure 3). The first substrate (C₊₄-start) mimics the 5'-truncated tRNA after removal of the first three 5'-nucleotides (including the wobble base-paired U₊₃). The second substrate (U₊₃-start) mimics a 5'-truncated tRNA resulting from removal of only the first two mismatched A nucleotides, while retaining the terminal wobble base pair. Each 5'-³²P labeled substrate was incubated with varied concentrations of purified *A. castellanii* or *D. discoideum* TLPs in the presence of ATP (to activate the 5'-monophosphate, as required for the first nucleotide addition, see Figure 1) and CTP and UTP (to be incorporated into the tRNA according to the 3'-end template sequence) (Figure 3). After two hours, reactions were quenched by addition of EDTA and RNase T1, generating a labeled 5'-oligonucleotide from cleavage at G₁₅, and products were resolved by denaturing PAGE (Figure 4).

For the C₊₄-start tRNA, bands consistent with expected 3'-5' addition products were observed (Figure 4A). For all four enzymes, a major oligonucleotide product (indicated by red arrows) was observed at moderate concentrations of enzyme corresponding to addition of the missing C₊₃, U₊₂ and C₊₁ nucleotides, based on the presence of much lighter, but visible, intermediate bands that migrate in between unreacted substrate and major product bands. At high enzyme concentrations (>100 nM), DdiTLP4 and AcaTLP1 also form a major product that contains one additional 5'-nucleotide (indicated by dashed arrow), consistent with addition of 5'-U₋₁ to base pair with A₇₃, as reported previously for bacterial TLPs (9). Strikingly, although addition beyond U₋₁ by AcaTLP1 and DdiTLP4 is detectable, albeit relatively inefficient, AcaTLP2 efficiently adds many additional 5'-nucleotides, apparently corresponding to addition beyond the predicted 3'-end template of the tRNA.

With the U₊₃-start tRNA, we observed similar 5'-end repair of the missing 2 nucleotides (U₊₂ and C₊₁), and also further addition past the +1 position at high enzyme concentrations (Figure 4B). However, three TLPs (DdiTLP3, DdiTLP4 and AcaTLP1) exhibited much less efficient overall activity with the U₊₃-start tRNA, as judged by significantly weaker intensity of products generated at the same enzyme concentration. In contrast, AcaTLP2 formed similar amounts of product with both RNAs.

Uniformly-labeled tRNA to eliminate migration differences based on differences in 5'-phosphorylation

The chemical nature of the 3'-5' nucleotide addition reaction introduces the possibility of additional complexity, since after each nucleotidyl transfer step, the 5'-triphosphate of the added NTP can remain intact to provide the activated 5'-end for subsequent nucleotide additions (Figure 1) (2). Under some conditions, *S. cerevisiae* Thg1 hydrolyzes the 5'-triphosphate to a 5'-monophosphate (23), and oligonucleotides bearing different numbers of 5'-phosphates would predictably migrate differently by PAGE. Although 5'-pyrophosphate

removal by TLPs has not been tested, we considered the possibility that the slower-migrating reaction products observed at the highest enzyme concentrations (interpreted to correspond to addition of an extra U_{-1} nucleotide above) could instead correspond to 5'-monophosphorylated versions of the same C_{+1} -containing reaction products produced at intermediate enzyme concentrations.

To test this, we generated the same two in vitro transcripts, instead using α - ^{32}P -ATP in the transcription reactions to generate uniformly-labeled tRNA, with only internal phosphodiester bonds ^{32}P -labeled (Supplemental Figure 1) thus allowing treatment with alkaline phosphatase to remove all terminal phosphates from digestion products prior to electrophoresis. PAGE analysis ruled out 5'-end phosphate heterogeneity as the cause of multiple major product bands (Figure 5). Instead, we saw the same pattern of bands observed previously with the 5'-end labeled RNAs, with the majority of products at moderate/low enzyme concentrations corresponding to repair up to addition of the C_{+1} nucleotide, while at higher enzyme concentrations, addition of further nucleotides (U_{-1} and beyond) were observed. Moreover, the same enzyme-substrate preferences were observed, with DdiTLP3, DdiTLP4 and AcaTLP1, but not AcaTLP2, exhibiting much less efficient 5'-repair of the U_{+3} -start tRNA than the C_{+4} -start substrate.

Quantification of substrate-specific preferences exhibited by 3'-5' polymerases from *D. discoideum* vs. *A. castellanii*

To quantify activities of the four 3'-5' polymerases, we performed a single-turnover kinetic analysis. Reactions with C_{+4} -start or U_{+3} start uniformly-labeled RNAs were initiated by addition of excess enzyme and resolved by PAGE as above (Figure 6 and Supplemental Figures 2, 3 and 4). Product band intensities were quantified and plotted as a function of time to determine k_{obs} (Supplemental Figure 5). To ensure that maximal concentration-independent rates (with respect to enzyme and NTPs) were measured, measurements were repeated at varied concentrations of each enzyme, the 5'-activating ATP and added CTP and/or UTP. Observed rates were independent of [enzyme] and [ATP] at all concentrations tested (data not shown), and maximal k_{obs} were observed at 0.5 mM CTP and/or UTP (Supplemental figure 6).

For consistency between reactions involving addition of different numbers of added nucleotides, we first measured k_{obs} for a single nucleotide addition. To do this, we included only the appropriate NTP for the first missing nucleotide from each tRNA (CTP for C_{+4} -start or UTP for U_{+3} start). Although the single nucleotide addition product was clearly observed in all reactions, we also saw more nucleotides added at longer reaction times, suggesting that incorrect addition can occur (with different efficiencies) in the absence of the correct WC base-pairing NTP (top panels, Figure 6, Supplemental Figures 2, 3 and 4), as observed previously for DdiTLPs (12). Nonetheless, in agreement with the earlier assays, the k_{obs} for single nucleotide addition by DdiTLP3, DdiTLP4 and AcaTLP1 to the WC-terminating C_{+4} substrate were consistently ~100-fold faster than for addition to the U_{+3} tRNA (Table 1 and Figure 7). In contrast, the difference in k_{obs} for AcaTLP2 was 10-fold.

To rule out effects due to the identity of the added NTP (since the first missing nucleotide is different for each substrate), we also determined k_{obs} for the complete 5'-end repair reaction

in the presence of both CTP and UTP (bottom panels, Figure 6, Supplemental Figures 2,3, and 4). Partially repaired intermediates were no longer observed in reactions once both NTPs were included. Bands corresponding to full-length (C_{+1} -containing) and longer nucleotide addition products were quantified to determine k_{obs} . We observed the same pattern of WC preference as for the single nucleotide addition reactions, with DdiTLP3, DdiTLP4 and AcaTLP1 all exhibiting a much faster rate with the WC substrate than with its wobble-base paired counterpart (Table 1 and Figure 7). Interestingly, k_{obs} for complete extension were all strikingly similar to those for the corresponding single nucleotide addition reactions, suggesting that the first addition is the rate-determining step for multiple addition and consistent with a processive mechanism for 3'-5' addition of multiple nucleotides by TLPs.

Discussion

Mitochondrial 5'-editing repairs mismatched base pairs in tRNA aminoacyl-acceptor stems. However, wobble (G-U) base pairs are abundant in tRNA, and comparisons of editing in a variety of organisms was puzzling in that sometimes G-U base pairs in mt-tRNA were “corrected” by editing, while in other cases G-U base pairs were retained or only partially corrected. Here we demonstrate that *A. castellanii* encodes a 3'-5' polymerase (AcaTLP2) that catalyzes relatively more efficient 3'-5' addition to a 5'-editing substrate that contains a G-U wobble base pair than several other tested TLPs (Table 1, Figure 7). Since 5'-editing in *A. castellanii* requires repair of mt-tRNA^{Ala}_{UGC} immediately adjacent to the U₊₄-G₆₉ base pair (Figure 2) (18), AcaTLP2 is a good candidate for the enzyme that catalyzes this repair reaction. In *D. discoideum*, however, all instances of G-U base pairs immediately adjacent to mismatched bases are edited to WC base pairs (14). This is consistent with the observation that *D. discoideum* TLPs discriminate against G-U wobble base pairs at the terminus of the 3'-5' polymerase substrate, and suggests that these G-U base pairs must be removed prior to repair by TLPs, consistent with the absence of retained G-U base pairs in *D. discoideum* mt-tRNA.

It remains to be demonstrated whether only one or both TLPs edit mt-tRNA in vivo in *A. castellanii* and *D. discoideum*. In previous in vitro assays, no obvious biochemical differences were observed between the candidate enzymes to imply preferential roles for one or the other in 5'-editing in vivo (12-14). Here, however, we identified distinct biochemical properties of the tested TLPs that are consistent with biological editing patterns. The ability of AcaTLP2 to efficiently repair the wobble-terminating substrate suggests it may be better-suited than AcaTLP1 to edit mt-tRNA^{Ala}_{UGC} in *A. castellanii*. In light of this proposed function, the extensive 5'-end elongation observed at high concentrations of AcaTLP2 in vitro must also be rationalized. Although the biochemical basis for this unexpected property of AcaTLP2 requires further investigation, it is possible that physiological concentrations of AcaTLP2 are low enough to limit activity to the correct number of nucleotides to repair the mt-tRNA, as observed in Figures 4 and 5. Alternatively, the complete editing enzyme might employ additional factors to counteract addition of extra nucleotides beyond the fully-repaired 5'-end of the mt-tRNA. Further biological characterization of the 5'-editing reaction in *A. castellanii* will be required to assess these, or other, possibilities. For the *D. discoideum* TLPs, DdiTLP3 correctly repairs mt-tRNA^{Ile}_{GAU(1)} even at high enzyme

concentrations, while DdiTLP4 consistently adds one or more nucleotides beyond the correct tRNA 5'-end. This could imply a preferential role for DdiTLP3 in 5'-editing, but regardless is the first observation of any biochemical difference between these two enzymes and may be functionally relevant. Interestingly, DdiTLP3 and AcaTLP2 are predicted to localize to the mitochondria where 5'-editing occurs, but whether these are unique 5'-editing enzymes in either organism awaits further biological characterization.

In this study we developed a new PAGE assay, which can be applied more broadly to study 3'-5' polymerase family enzymes. The decision to use 5'-end labeled vs. uniformly-labeled RNA can be flexible based on similar results of our enzyme titration assays. Although in this study a nuclease was used to generate smaller oligonucleotide reaction products that are better resolved by PAGE than full-length tRNA, nuclease treatment could be omitted, and full-length substrates resolved by PAGE if necessary. The PAGE assay is highly amenable to kinetic characterization of multiple nucleotide addition reactions as demonstrated here, and will be valuable for future investigation of the mechanism of these unusual polymerases. This study provided some kinetic insight into 3'-5' polymerization reactions, suggesting that the first nucleotide addition is rate-limiting. Based on earlier kinetic studies, this may be due to the slower kinetics of the 5'-adenylation step (which is only required for the first addition to a 5'-monophosphorylated RNA) (24). The PAGE assay opens the door to further investigating enzymatic properties of the unusual 3'-5' reverse polymerase family including substrate specificity, processivity and catalytic mechanism, all of which will advance understanding of the evolutionary connection between canonical and 3'-5' polymerases and the mysterious but versatile roles of these enzymes in biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this work was provided by NIH grant nos. GM087543 (to J.E.J.). Y.L. was supported by the Pelotonia Graduate Fellowship. The authors are grateful to S. Dodbele, A. Krishnamohan, Q. Liu and K. Patel for valuable comments on the manuscript.

Abbreviations

Thg1	tRNA ^{His} guanylyltransferase
TLP	Thg1-like protein
WC	Watson-Crick
mt-tRNA	mitochondrial tRNA
EDTA	ethylenediamine tetraacetic acid

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Highlights

1. Mitochondrial tRNA in many protozoan eukaryotes undergo 5'-editing.
2. TLPs use 3'-5' polymerase activity to repair 5'-truncated editing substrates.
3. *A. castellanii* TLP2 tolerates G-U pairs in editing substrates better than other TLPs.
4. TLP activities correlate with biological outcomes for G-U base pairs during editing.
5. Biochemical evidence suggests specialized functions for different eukaryotic TLPs.

Figure 1. Mechanism of mitochondrial tRNA 5'-editing

A representative example of mt-tRNA with encoded 5'-mismatches is repaired by 5'-editing. In the first step, mismatched nucleotides are removed from the 5'-end of the aminoacyl-acceptor stem by an unidentified nuclease(s), generating a 5'-truncated mt-tRNA. In Step 2, TLP enzymes repair the truncated 5'-end using their 3'-5' polymerase activity. For the first nucleotide addition to a 5'-monophosphorylated substrate, TLPs activate the 5'-end by adenylation with ATP (shown in blue). Subsequently, incoming NTPs (shown in red) are selected according to their ability to form WC base pairs with the 3'-end template sequence and added to the growing 5'-end by attack of the NTP 3'-hydroxyl on the activated 5'-end, as indicated. After the first addition, the 5'-triphosphate of the previously added NTP serves as the activated end for nucleotide addition, releasing pyrophosphate as a consequence of the reaction. For intermediates generated during the 3'-5' polymerase reaction, only the aminoacyl-acceptor stem sequence is shown for clarity.

Figure 2. Organism-specific outcomes for U-G base pairs during 5'-editing

Aminoacyl-acceptor stem sequences of (U-G)-containing mt-tRNA that undergo 5'-editing are shown for three different eukaryotes. Genomically-encoded mt-tRNA sequences are shown on the left, and experimentally-determined sequences of the 5'-edited tRNA are shown to the right of each arrow. The U-G base pairs immediately adjacent to mismatches that undergo editing are highlighted by red boxes; nucleotides added by TLPs during 5'-end repair are shown in red. **(A)** mt-tRNA^{Ile}_{GAU(1)} from *D. discoideum*; the U₃-G₇₀ base pair in is 100% edited. **(B)** mt-tRNA^{Phe}_{GAA} from *P. pallidum*; the U₃-G₇₀ base pair is unedited in 94% of sequences, while the adjacent A₂-A₇₁ mismatch is fully edited. **(C)** mt-tRNA^{Ala}_{UGC} from *A. castellanii*; the U₄-G₆₉ base pair adjacent to the edited U₃-U₇₀ mismatch is 100% unedited.

Figure 3. 5'-end repair substrates based on mt-tRNA^{Ile} GAU(1) from *D. discoideum*

To evaluate the 5'-repair activity of TLPs, two model tRNA substrates were based on the sequence of mt-tRNA^{Ile}. In vitro transcripts initiating with either C₊₄ (C₊₄-start) or U₊₃ (U₊₃-start) were generated and either 5'-³²P-labeled or internally labeled with [α -³²P]-ATP, as indicated. The U₊₃ and C₊₄ nucleotides are highlighted in blue and nucleotides expected to be added by TLPs to repair the 5'-aminoacyl-acceptor stem according to the 3'-template sequence are marked in red. The position of G₁₅, where RNase T1 cleaves to yield a labeled 5'-fragment to be resolved by PAGE, is indicated.

Figure 4. Enzyme titration assay for 5'-end repair with 5'-³²P-labeled tRNA^{Ile}_{GAU(1)}
 The *C*₊₄-start (**A**) or *U*₊₃-start (**B**) substrates were incubated with five-fold serial dilutions (2.7 μM to 0.2 nM) of each of the four TLP enzymes, as indicated, for two hours. Reactions were quenched by addition of EDTA and RNase T1 and the resulting oligonucleotides were resolved by 12% polyacrylamide, 7 M urea PAGE. The tRNA diagrams show the complete tRNA sequence, with the expected labeled oligonucleotide derived from unreacted substrate indicated by the green line; the band corresponding to this oligonucleotide is marked by the red (S) on the gels (as seen in lane -, which is the no enzyme control lane for each substrate). Reaction products corresponding to 3'-5' addition of the indicated NTPs are indicated by arrows, with expected sequences of the added nucleotides shown in red for each band. The red arrows indicate fully-repaired 5'-ends, and the dashed arrow indicates nucleotides added beyond the +1 (full-length) position. Lanes corresponding to reactions performed at 22 nM of the indicated TLP are marked with red stars for comparison between the two substrates.

Figure 5. 5'-repair assay with uniformly-labeled mt-tRNA^{Ile}_{GAU(1)}
Uniformly-labeled C₊₄-start (**A**) or U₊₃-start (**B**) mt-tRNA^{Ile}_{GAU(1)} were generated by in vitro transcription with [α -³²P]-ATP. The two RNA substrates were incubated with five-fold serial dilutions (2.7 μ M to 1 nM) of the indicated TLP enzymes for two hours. Reactions were quenched by addition of EDTA and RNase T1, and then treated with phosphatase prior to resolution by 12% polyacrylamide, 7 M urea PAGE. The tRNA diagram to the right of each panel shows expected labeled oligonucleotide fragments generated by RNase T1 digestion of the unreacted substrate. Bands were colored according to size: green – 12-mer (C₊₄-start) or 13-mer (U₊₃-start); yellow – 11-mer; purple – 6-mer; blue – 5-mer (2 different fragments) and brown – 4-mer (3 different fragments); specific sequences of labeled reaction products are shown in Supplemental Figure 1. Only RNA fragments that contain internal A-nucleotides are visible; for example, the A₂₃-G₂₄ dinucleotide (marked by a star) is not labeled because the 5'-³²P of A₂₃ is transferred to G₂₂ as a consequence of RNase T1 digestion, and then removed by phosphatase treatment. Bands corresponding to unreacted substrate are labeled with (S), and bands corresponding to nucleotide addition products are indicated by arrows as in Figure 4, with the red arrow corresponding to the fully-repaired

tRNA. On the 12% gels used for these assays, the sequence of the 11-mer (indicated in yellow) causes it to migrate slightly higher than the 12-mer from the 5'-end of unreacted C₊₄-start and co-migrate with the 13-mer from the 5'-end of unreacted U₊₃-start; higher percentage gels are required to resolve the 11-mer band, as demonstrated in Figure 6.

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DdiTLP3

Figure 6. Single-turnover kinetic analysis of DdiTLP3-catalyzed 5'-end repair of uniformly-labeled tRNA^{Ile}_{GAU(1)}
 DdiTLP3 (2 μ M) was used to initiate reactions with uniformly-labeled C₊₄-start (left) or U₊₃-start (right) mt-tRNA^{Ile}_{GAU(1)} in the presence of 0.1 mM ATP and 0.5 mM NTP (CTP or UTP or both, as indicated). Reactions were quenched at time points ranging from 10 s to 2 h by addition of EDTA and RNase T1, and then treated with phosphatase prior to resolution by 20% polyacrylamide, 8M urea PAGE. Bands corresponding to unreacted substrate (S) and 3'-5' addition products (arrows) are labeled according to the same colors shown in Figure 5 with the identity of the added nucleotide(s) shown in red. Smaller oligonucleotides (4-6-mers) produced from these reactions are not shown for clarity. The 11-mer (yellow labeled band) is well-resolved from unreacted substrate bands on this higher percentage gel and remains constant during the course of the reaction, as expected.

Figure 7. WC preferences of TLPs from *D. discoideum* and *A. castellanii*

The preference of each of the four TLPs to extend substrates with WC base pair vs. UG base pair termini was determined by calculating the ratio of the average k_{obs} for the C₊₄-start substrate to the average k_{obs} for the U₊₃-start substrate. Values for WC preference were calculated for both the first nucleotide addition (hatched bar) and complete extension (solid bar), as indicated for each enzyme.

Table 1

Single turnover kinetic analysis of 3'-5' addition catalyzed by TLPs

<i>Enzyme</i>	<i>k_{obs}</i> (min ⁻¹) First Addition ^a		<i>k_{obs}</i> (min ⁻¹) Complete extension ^a	
	<i>U</i> _{+3'-start}	<i>C</i> _{+4'-start}	<i>U</i> _{+3'-start}	<i>C</i> _{+4'-start}
AcaTLP1	0.022 ± 0.001	2.6 ± 0.3	0.018 ± 0.011	1.6 ± 0.4
AcaTLP2	1.2 ± 0.2	11 ± 4	0.79 ± 0.32	4.9 ± 2.2
DdiTLP3	0.0084 ± 0.0004 ^b	0.52 ± 0.18	0.0094 ± 0.0014 ^b	0.60 ± 0.21
DdiTLP4	0.0076 ± 0.0014 ^b	0.98 ± 0.27	0.0074 ± 0.0024 ^b	0.71 ± 0.05

^a Average of three independent measurements performed under saturating conditions with respect to enzyme, ATP and added NTP; errors are standard deviations.

^b Rates were calculated by the method of linear initial rates because of slow reaction rates.