*Published online 14 December 2012* *Nucleic Acids Research, 2013, Vol. 41, No. 3* ***1885–1894***
*doi:10.1093/nar/gks1242*

# Absence of a universal element for tRNA$^{His}$ identity in *Acanthamoeba castellanii*

**Bhalchandra S. Rao[1,2], Fuad Mohammad[1], Michael W. Gray[3] and Jane E. Jackman[1,2,*]**

[1]Department of Chemistry and Biochemistry and Center for RNA Biology, The Ohio State University, Columbus, OH 43210, USA, [2]Molecular, Cellular and Developmental Biology Graduate Program, The Ohio State University, Columbus, OH 43210, USA and [3]Department of Biochemistry and Molecular Biology, Centre for Comparative Genomics and Evolutionary Bioinformatics, Dalhousie University, Halifax, NS B3H 4R2, Canada

Received October 5, 2012; Revised October 23, 2012; Accepted November 1, 2012

## ABSTRACT

**The additional $G_{-1}$ nucleotide on tRNA$^{His}$ is a nearly universal feature that specifies tRNA$^{His}$ identity in all three domains of life. In eukaryotes, the $G_{-1}$ identity element is obtained by a post-transcriptional pathway, through the unusual 3′–5′ polymerase activity of the highly conserved tRNA$^{His}$ guanylyltransferase (Thg1) enzyme, and no examples of eukaryotic histidyl-tRNAs that lack this essential element have been identified. Here we report that the eukaryote *Acanthamoeba castellanii* lacks the $G_{-1}$ identity element on its tRNA$^{His}$, consistent with the lack of a gene encoding a bona fide Thg1 ortholog in the *A. castellanii* genome. Moreover, the cytosolic histidyl-tRNA synthetase in *A. castellanii* exhibits an unusual tRNA substrate specificity, efficiently aminoacylating tRNA$^{His}$ regardless of the presence of $G_{-1}$. *A. castellanii* does contain two Thg1-related genes (encoding Thg1-like proteins, TLPs), but the biochemical properties we associate here with these proteins are consistent with a function for these TLPs in separate pathways unrelated to tRNA$^{His}$ metabolism, such as mitochondrial tRNA repair during 5′-editing.**

## INTRODUCTION

Transfer RNA species are extensively processed before their use in translation. These processing events include various types of RNA editing and modification reactions that alter the sequence or chemical structure of the nucleotide bases from that originally encoded in the genome (1–4). Enzymes that carry out tRNA editing and modification are found in all three domains of life, and many comprise highly conserved enzyme families that catalyse similar reactions with tRNAs from multiple organisms. One of the most highly conserved tRNA modifications is the presence of an additional G residue ($G_{-1}$) at the 5′ end of tRNA$^{His}$, which is a unique distinguishing feature of tRNA$^{His}$ in all three domains of life. Only histidine tRNAs have the extra G nucleotide, and the presence of $G_{-1}$ is required for efficient recognition of the tRNA for aminoacylation by the histidyl-tRNA synthetase (HisRS) (5–8).

Although the $G_{-1}$ residue is a ubiquitous feature, it is obtained by different mechanisms depending on domain of life. In bacteria (and many archaeons), $G_{-1}$ is encoded in the 5′-leader sequence of the tRNA$^{His}$ gene and transcribed as part of the pre-tRNA, following which an alternative ribonuclease P (RNase P)-catalysed 5′-processing event results in retention of $G_{-1}$ in mature tRNA$^{His}$ (9). The presence of an encoded $G_{-1}$ is nearly 100% conserved throughout all sequenced bacterial genomes: only one small group of ~20 α-proteobacteria has a tRNA$^{His}$ that does not contain a $G_{-1}$ residue (10). In eukaryotes, however, the tRNA$^{His}$ gene does not have an encoded $G_{-1}$, and the identity element is added post-transcriptionally to the RNase P-processed tRNA$^{His}$ by the action of the tRNA$^{His}$ guanylyltransferase (Thg1) (Figure 1A) (11,12). First identified in *Saccharomyces cerevisiae*, Thg1 is a member of a superfamily of reverse (3′–5′) polymerase enzymes, and in eukaryotes, Thg1 uses this 3′–5′ nucleotide addition activity to add the single non-templated $G_{-1}$ residue, opposite $A_{73}$, to the 5′-end of tRNA$^{His}$ (16,17). Until now, there have been no examples of eukaryotic tRNA$^{His}$ species that lack this important recognition element.

Although addition of the $G_{-1}$ element to tRNA$^{His}$ was the first activity associated with the Thg1 superfamily, subsequent biochemical characterization of other Thg1-related enzymes revealed a functionally distinct group of enzymes known as Thg1-like proteins (TLPs). TLPs, which

*To whom correspondence should be addressed. Tel: +1 614 247 8097; Fax: +1 614 292 6773; Email: Jackman.14@osu.edu

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

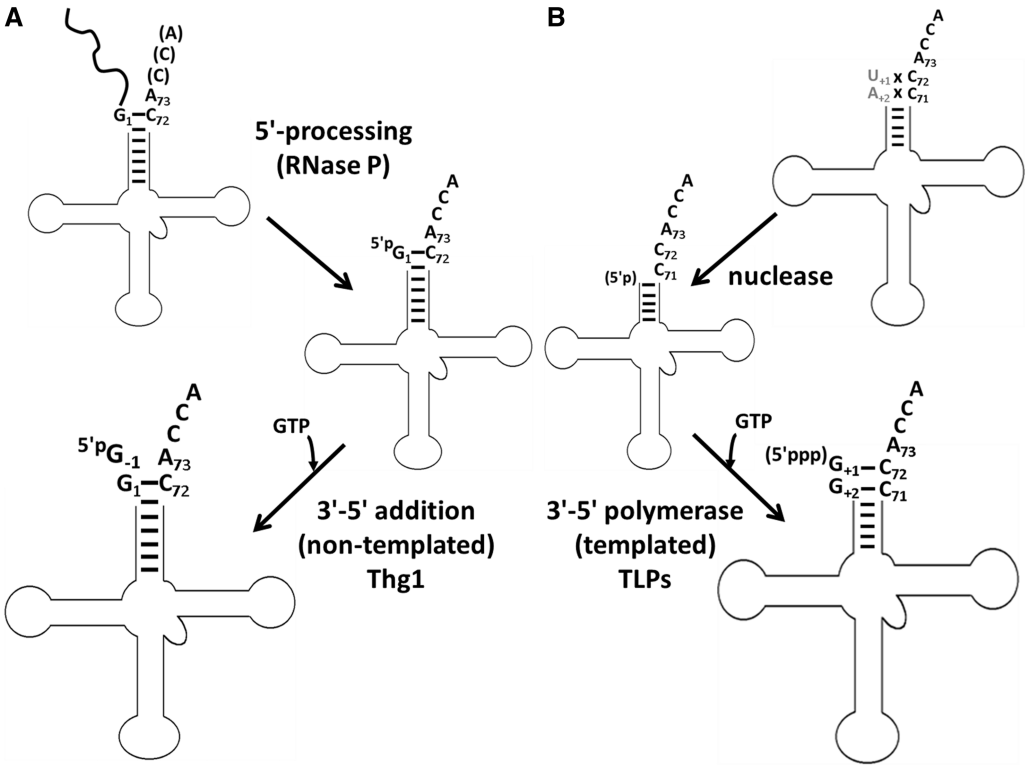


**Figure 1.** Multiple roles for 3′–5′ addition catalysed by Thg1 family enzymes. (**A**) Participation of Thg1 enzymes in $tRNA^{His}$ maturation in eukaryotes. In this reaction, which is highly selective for $tRNA^{His}$, Thg1 adds a single non-templated $G_{-1}$ residue to the 5′-end of the tRNA following processing of the pre-tRNA by RNase P (11,12). This essential function of Thg1 in eukaryotes serves to add the required $G_{-1}$ identity element for recognition by the HisRS. **(B)** Predicted participation of TLPs in mt-tRNA 5′-editing in protozoa. The process is a two-step reaction catalysed by an unidentified nuclease to remove mismatches from the 5′-end of the encoded tRNA, followed by repair of the 5′-truncated tRNA using 3′–5′ polymerase activity to restore a fully base-paired aminoacyl-acceptor stem (13). The 5′-tRNA repair reaction is efficiently catalysed by TLP members of the Thg1 superfamily (14,15).

are abundant in the domain Archaea, and more sporadically distributed throughout Bacteria and Eucarya, do not efficiently add a non-templated $G_{-1}$ nucleotide to $tRNA^{His}$, nor do they exhibit the rigorous specificity for $tRNA^{His}$ associated with eukaryal Thg1 enzymes (14,17–20). Instead, these enzymes selectively catalyse template-dependent 3′–5′ polymerization and exhibit a proficient tRNA 5′-end repair activity, suggesting alternative roles for these enzymes *in vivo* (14,15). Although the precise physiological role for TLPs in Bacteria and Archaea remains unknown, two TLP gene products from the eukaryotic slime mold *Dictyostelium discoideum* were recently implicated in a mitochondrial tRNA (mt-tRNA) 5′-editing reaction (Figure 1B), based on their ability to catalyse repair of 5′-truncated mt-tRNAs (15).

Consistent with the distinct biochemical properties associated with Thg1 and TLP enzymes investigated so far, a phylogenetic analysis of Thg1 and TLP gene sequences reveals that these genes segregate into distinct clades that can also be distinguished on the basis of conserved sequence motifs (17,21). The Thg1 clade contains genes closely related to *S. cerevisiae THG1* and the presence of a Thg1 gene is widespread in eukaryotes, consistent with the critical importance of the $G_{-1}$ identity element and the highly conserved mechanism of its post-transcriptional addition in all eukaryotes studied to date. As the vast majority of studied eukaryotes are not predicted to contain mt-tRNAs that require 5′ editing (and therefore do not encode any TLP genes), most eukaryotes encode only a single Thg1 gene that clusters as an ortholog to other bona fide Thg1 enzymes. However, a deeper investigation of the Thg1 family tree reveals several eukaryotes that lack any identifiable Thg1 homolog, and thus are notable exceptions to this general rule (12,17). These organisms can be classified further into two groups. The first group contains those that lack any identifiable Thg1 or TLP gene, a group that consists primarily of protists (eukaryotic microbes), including diatoms and several types of algae. The basis for the apparent lack of any Thg1-related gene in these species has not been determined. A second group consists of organisms that lack an identifiable Thg1 gene, but encode one or more TLPs. This group includes several fungi (*Phaeosphaeria nodorum* and *Spizellomyces punctatus*) as well as the amoebozoan *Acanthamoeba castellanii* (17). In the case of *A. castellanii*, we have previously emphasized that the lack of an identifiable Thg1 gene in this organism is unlikely to be due to incomplete genome data, given the extensive genomic and transcriptomic coverage in the genome project for this organism (http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-pubPreview.xsp?project_id = 163).

We chose to investigate the two TLP genes encoded in the genome of *A. castellanii* (designated AcaTLP1 and AcaTLP2) to answer several questions related to the biological function(s) of these genes and the apparently non-canonical requirements for $tRNA^{His}$ maturation in this species. Because, like all Thg1/TLP enzymes studied so far, the AcaTLPs contain all of the functional sequence motifs associated with the ability to catalyse 3′–5′ nucleotide addition (Supplementary Figure S1) (16,22,23), one obvious possibility was that, despite the inability of all previously characterized TLP enzymes to efficiently catalyse $G_{-1}$ addition to cytosolic $tRNA^{His}$, one or both of the AcaTLPs might exhibit this activity, and thus maintain a Thg1 family-dependent pathway for $tRNA^{His}$ maturation. Our data do not support this hypothesis, as here we show that neither of the AcaTLPs catalyses $G_{-1}$ addition to cytosolic $tRNA^{His}$ at significant levels. Instead, consistent with the lack of a predicted Thg1 gene in *A. castellanii*, cloning and sequencing of the cytosolic *A. castellanii* $tRNA^{His}$ reveals that this tRNA lacks the characteristic $G_{-1}$ element. Remarkably, unlike any other previously characterized eukaryotic HisRS, we also demonstrate that purified cytosolic HisRS from *A. castellanii* does not depend on the $G_{-1}$ identity element for aminoacylation of cytosolic $tRNA^{His}$. Thus, we have identified the first example of a eukaryotic organism that breaks the rule regarding presence of the $G_{-1}$ element for specifying $tRNA^{His}$ identity. These findings are the first demonstration of absence of the universal $G_{-1}$ identity element in eukaryotic $tRNA^{His}$, and open the door to investigation of other non-canonical mechanisms for specifying $tRNA^{His}$ identity in eukaryotes.

## MATERIALS AND METHODS

### Cloning and purification of *A. castellanii* TLPs and HisRS

*A. castellanii* cDNA library was synthesized from ~3 μg *A. castellanii* Neff total RNA (generously provided by Dr R. Gawryluk, Dalhousie University) using 2.5 μM $oligo(dT)_{20}$ primer and Superscript III reverse transcriptase (RT) (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. The resulting cDNA was treated with RNase H and RNase A, purified by phenol:chloroform extraction and used as the template for PCR with AcaTLP1, AcaTLP2 and AcaHisRS gene-specific primers (Supplementary Figure S2), using iProof DNA polymerase (BioRad, Hercules, CA). Gene, coding and protein sequences were assembled from genomic and EST data publicly available in GenBank, supplemented by unpublished RNAseq data kindly provided by Prof. Brendan Loftus (Conway Institute, Dublin) and Prof. Andrew Roger (Dalhousie University) (see Supplementary Figure S2). Gel-purified PCR products were cloned by ligation-independent cloning into a previously described vector (AVA421) that contains sequences for expression of an *N*-terminal $His_6$-tag for affinity purification of the overexpressed protein (24).

Available EST data for AcaTLP1 support the presence of a short poly-serine stretch at the extreme C-terminus of AcaTLP1 (Supplementary Figure S1). However, as the exact number of serine residues in this sequence is somewhat ambiguous (owing to differences between the genomic sequence and a few ESTs), and because the residues at the C-terminal end are not highly conserved and not essential for $G_{-1}$ addition activity with human Thg1 (16), we did not include these 7 terminal serine residues, or the preceding threonine, in the final clones.

Sequence-verified clones [GenBank accession numbers JX547005 (AcaTLP1), JX547006 (AcaTLP2) and JX547007 (AcaHisRS)] were transformed into Rosetta(pLysS) (AcaTLPs) or BL21(DE3)pLysS (AcaHisRS) *Escherichia coli* strains and induction of protein expression and purification of proteins was performed as described, except that induction was performed at 37°C for 6 h for the AcaTLPs and 18°C overnight for the HisRS proteins (15). All resulting proteins were dialysed into buffer containing 50% glycerol, 20 mM Tris, 4 mM $MgCl_2$, 1 μM ethylenediamine tetraacetic acid (EDTA), 1 mM dithiothreitol, pH 7.5 for storage at −20°C. AcaTLP dialysis buffer additionally contained 0.5 M NaCl, whereas AcaHisRS dialysis buffer also contained 0.05 M NaCl. Protein concentrations were determined using the BioRad protein assay, and recombinant proteins were judged to be ≥90% pure by sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis of purified proteins.

### $G_{-1}$ addition/5′-end repair activity assays

A previously described phosphatase protection assay was performed to determine the biochemical activity of the purified AcaTLPs (25). [5′-$^{32}$P]tRNAs (10–50 nM) were incubated with purified AcaTLPs (or SceThg1, bacterial TLP and/or DdiTLPs in control assays) in Thg1 reaction buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.2 mg/ml bovine serum albumin, 3 mM dithiothreitol, pH 7.5], additionally containing 10 mM $MgCl_2$ and 125 mM NaCl (standard SceThg1 assay conditions), or 10 mM $MnCl_2$ and 50 mM NaCl (as indicated for each figure) for 1–2 h at room temperature. Reactions were quenched by addition of EDTA, followed by digestion with RNase A to generate labeled oligonucleotide reaction products. After treatment of the digested reaction products with alkaline phosphatase, unreacted substrate tRNAs are visualized as inorganic phosphate, and 5′-nucleotide addition products are visualized as internally labeled oligonucleotides (as indicated in figure legends for each assay). Reaction products were resolved using silica thin-layer chromatography (TLC) plates in a 55:35:10 (v:v:v, 1-propanol: $NH_4OH$: $H_2O$) solvent system.

### Determination of steady-state kinetic parameters

Steady-state kinetic assays were performed with *A. castellanii* mt-$tRNA^{Leu}$ $\Delta G_{+2}$ substrate and purified AcaTLP1 or AcaTLP2-mito, as previously described (22). Linear initial rates were determined from time courses of assays containing 0.1–25 μM tRNA and

1–60 nM purified enzyme, maintaining a minimum excess of at least 5-fold substrate over enzyme in each reaction. Reactions were performed in standard Thg1 reaction buffer and time courses were selected to yield <10% conversion to product over the course of the measured reaction. Initial rates (normalized for [TLP]) were derived from at least three independent assays, plotted as a function of [tRNA] and fitted to the Michaelis–Menten equation for determination of steady-state kinetic parameters.

### Determination of $G_{-1}$ status of *A. castellanii* cytosolic $tRNA^{His}$

Total RNA was isolated from *A. castellanii* cells ($\sim 10^6$ cells, generously provided by Dr Greg Booton, Ohio State University) using Tri Reagent (Sigma) according to the supplier's instructions. Purified RNA (5–10 μg) was circularized using T4 RNA ligase (USB) as described in (26), and circularized RNA (circRNA) was purified by phenol:chloroform extraction, followed by ethanol precipitation using 5 μg glycogen. CircRNA (2 μg) was used as the template for cDNA synthesis with 2 pmol AcaHisRT primer (5′-GACCTGCAGACCAATGTACT ACCAC-3′); the primer sequence includes a PstI restriction site (underlined) for the subsequent cloning steps. After heating together and cooling the primer and circRNA (95°C for 4 min, followed by slow cooling to room temperature for 30 min), cDNA synthesis was performed with Superscript III RT according to the manufacturer's instructions, for 1 h at 50°C. cDNA reactions were treated with RNase A (0.5 mg/ml final concentration) to remove remaining RNA, and reactions were purified by phenol:chloroform extraction followed by ethanol precipitation. The purified cDNA was resuspended in 20 μl TE pH 8.0, and 3–5 μl of purified cDNA was used as the template for PCR with AcaHisRT and AcaHis3′ (5′-GCAGG ATCCCAACCCGGGTTCAATTCCCG-3′) primers. AcaHis3′ primer also contains a BamHI restriction site (underlined) for downstream cloning. PCR reactions were performed with iProof polymerase (BioRad) according to the manufacturer's instructions. Reverse transcriptase-polymerase chain reaction (RT-PCR) products of the expected size were purified by electrophoresis in 1% agarose gels, digested with BamHI and PstI (NEB) and subsequently ligated into a similarly digested pUC19 vector using 0.1 U/μl T4 DNA ligase (USB). Plasmid DNA was isolated from individual positive clones and sequenced.

### Histidylation assays

CCA labeling of tRNAs with [α-$^{32}$P]-ATP and histidylation assays were performed essentially according to reference (27), according to details provided in the Supplementary Data Methods. The 3′-labeled tRNA (labeled at the 5′-phosphate of $A_{76}$) was incubated with 250 μM histidine, 4 mM ATP and Sce or Aca cyto HisRS (2.5–0.1 μM; in 5-fold serial dilutions, as indicated), in aminoacylation buffer (27). P1-digested reactions were resolved by PEI-cellulose TLC using a glacial acetic acid: 1 M $NH_4Cl$: water (5:10:85, v:v:v) solvent system, and plates were visualized using a Typhoon trio imager.

## RESULTS

### *A. castellanii* TLP1 and TLP2 lack the ability to catalyse efficient $G_{-1}$ addition to cytosolic $tRNA^{His}$ *in vitro*

We cloned both of the AcaTLP coding sequences into a vector previously used for expression of Thg1-family proteins in *E. coli*, and used these vectors for purification of the proteins according to previous protocols (18) (Supplementary Figures S1 and S2). As AcaTLP2 is predicted to contain a 44-amino acid *N*-terminal mitochondrial targeting signal, expression clones were generated that both contain and lack this *N*-terminal sequence, designated AcaTLP2 and AcaTLP2-mito, respectively (Supplementary Figure S1).

The three proteins (AcaTLP1, AcaTLP2 and AcaTLP2-mito) were overexpressed in *E. coli* and purified using immobilized metal-ion affinity chromatography. The resulting purified proteins were tested for ability to add $G_{-1}$ to a cytosolic $tRNA^{His}$ substrate, which contains the $A_{73}$ discriminator nucleotide that is present in all eukaryotic cytosolic $tRNA^{His}$ species. Reactions with any of the purified AcaTLP proteins exhibited little detectable $G_{-1}$ addition product, with a maximum yield of 0.1% and 1% $G_{-1}$ addition product in reactions containing the highest concentration AcaTLP1 and AcaTLP2, respectively, as compared with 40% in the control reaction with *S. cerevisiae* Thg1 (SceThg1) (Figure 2A). Although the $tRNA^{His}$ used for these assays was derived from *S. cerevisiae*, not *A. castellanii*, and thus the possibility of minor kinetic differences between this tRNA and the native substrate cannot be excluded, the complete lack of observable $G_{-1}$ addition, coupled with the fact that the $tRNA^{His}$ GUG anticodon is the major determinant for recognition of the tRNA for $G_{-1}$ addition by yeast Thg1 (25), indicate that the AcaTLPs are not efficient $G_{-1}$ addition enzymes.

In these same assays, each of the AcaTLP enzymes also produced small, but detectable amounts of products whose migration is consistent with previously identified activated intermediates resulting from addition of a 5′-adenylate or guanylate to the 5′-end of the tRNA during the first step of 3′–5′ nucleotide addition (18). These products are evident in reactions with high concentrations of AcaTLPs, suggesting a similar block to nucleotide addition following the activation step of the reaction to that observed previously for other TLPs (Figure 2A). The presence of the mitochondrial targeting peptide on AcaTLP2 does not appear to substantially affect the pattern or amount of product produced.

### AcaTLP1 and AcaTLP2 catalyse template-dependent 3′–5′ polymerase activity

A hallmark of other studied TLPs is their ability to efficiently catalyse template-dependent Watson–Crick 3′–5′ polymerase activity, but not non-templated 3′–5′ addition (18). This property explains the general inability of TLPs to catalyse $G_{-1}$ addition to wild-type cytosolic

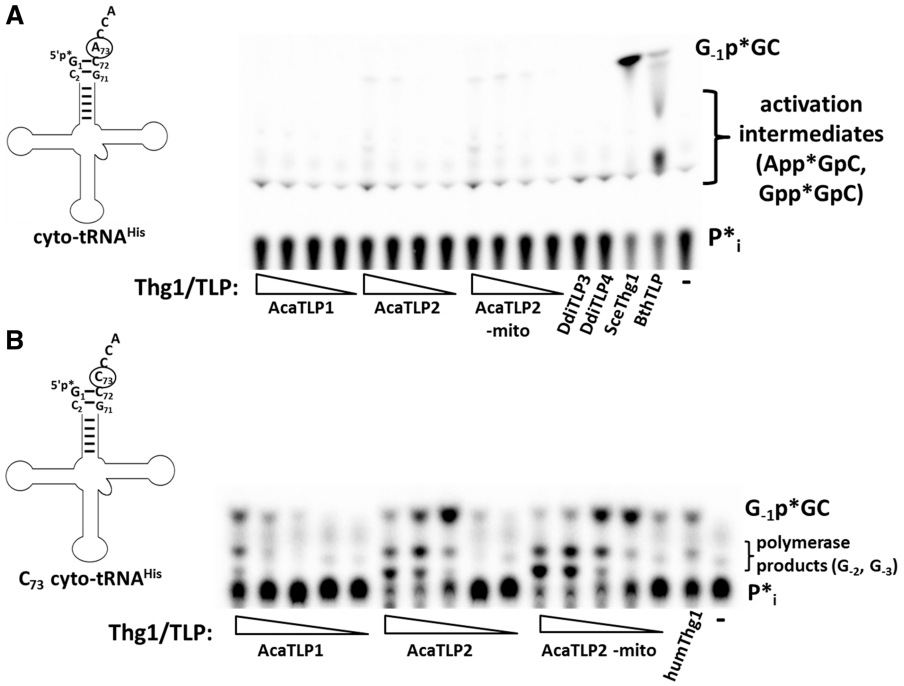


**Figure 2.** AcaTLPs catalyse templated, but not non-templated, $G_{-1}$ addition to tRNA$^{His}$. (**A**) $G_{-1}$ addition assay to wild-type yeast tRNA$^{His}$. Phosphatase protection assays were used to determine whether either of the AcaTLPs catalyse addition of $G_{-1}$ to 5′-$^{32}$P labeled tRNA; addition of $G_{-1}$ results in production of a labeled $G_{-1}$p*GpC oligonucleotide. Reactions contained 5-fold serial dilutions of purified AcaTLPs (0.4 mg/ml–3 μg/ml AcaTLP1 and AcaTLP2, 1.2 mg/ml–10 μg/ml AcaTLP2-mito), or 1 mg/ml other purified TLP/Thg1 enzymes as indicated; −, no enzyme control. Reactions shown were performed in Thg1 assay buffer containing 125 mM NaCl, 10 mM $MgCl_2$. **(B)** $G_{-1}$ addition assay with $C_{73}$-tRNA$^{His}$ variant. Phosphatase protection assays were performed with serial dilutions of purified enzyme as in (A) (0.4 mg/ml–0.6 μg/ml AcaTLP1 and AcaTLP2, 1.2 mg/ml–2 μg/ml AcaTLP2-mito); addition of $G_{-1}$ is indicated by $G_{-1}$p*GpC reaction product, and additional polymerase products (due to subsequent addition of templated $G_{-2}$ and $G_{-3}$ nucleotides) are indicated. Reactions shown were performed in Thg1 reaction buffer containing 50 mM NaCl, 10 mM $MnCl_2$.

tRNA$^{His}$ in eukaryotes, as addition of $G_{-1}$ would result in formation of a non-canonical $G_{-1}$-$A_{73}$ base pair (see Figure 1B). However, when a tRNA$^{His}$ variant that contains a $C_{73}$-discriminator nucleotide is used as the substrate for $G_{-1}$ addition, TLPs from Bacteria, Archaea and *D. discoideum* efficiently add $G_{-1}$ (and sometimes multiple G-residues), reflecting their preference for Watson–Crick base pair formation (14,15,18).

To determine whether AcaTLPs exhibit similar behavior, we tested activity of each enzyme with a $C_{73}$-containing variant of cytosolic tRNA$^{His}$, using a similar assay with 5′-$^{32}$P-labeled tRNA. In the assays, AcaTLP1 and AcaTLP2 both catalysed 3′–5′ addition of $G_{-1}$ as well as formation of additional products, which migrate according to the positions of 3′–5′ polymerase reaction products (corresponding to $G_{-2}$ and $G_{-3}$ additions). AcaTLP2 is apparently more active than AcaTLP1 under these assay conditions, and again, the presence of the mitochondrial targeting signal in the purified protein has little discernible effect on activity (Figure 2B). The ability to efficiently add $G_{-1}$ to the $C_{73}$-containing, but not $A_{73}$-containing, tRNA$^{His}$ affirms that the AcaTLPs exhibit biochemical properties similar to other TLPs characterized so far, and thus are unlikely to participate in cytosolic tRNA$^{His}$ maturation *in vivo* in *A. castellanii*. Moreover, neither of the AcaTLPs is able to support growth of the *S. cerevisiae thg1Δ* strain when provided as the sole source of Thg1 activity in the cell (Supplementary Figure S3), consistent with the results of the activity assays. This observation helps to rule out the possibility that a low level of non-templated $G_{-1}$ addition activity is exhibited by the enzymes, which is apparently sufficient to support tRNA$^{His}$ maturation *in vivo* as observed previously for one bacterial TLP (14,20).

## Cytosolic tRNA$^{His}$ in *A. castellanii* lacks the canonical $G_{-1}$ identity element

As the *A. castellanii* genome does not encode a bona fide Thg1 ortholog, and neither of the AcaTLPs appears to be able to replace this enzymatic activity, we considered two possibilities for the maturation of tRNA$^{His}$ in *A. castellanii*. First, it is possible that another enzyme in *A. castellanii*, unrelated to the Thg1 family, catalyses this essential activity. Second, the tRNA$^{His}$ in *A. castellanii* might be unusual, lacking the $G_{-1}$ identity element that is the hallmark of all other studied eukaryotic tRNA$^{His}$ species (28). To distinguish between these possibilities, we determined the $G_{-1}$ status of the cytosolic tRNA$^{His}$ in *A. castellanii*. Total RNA was purified from *A. castellanii* cells and subjected to circularization using T4 RNA ligase, followed by RT-PCR to amplify the junction region formed between the mature 5′- and 3′-ends of the tRNA, then cloning and sequencing of the resulting PCR products. Sequences were obtained for 12 full-length clones, and for 11 of 12 sequences, the clones lacked a $G_{-1}$ nucleotide and instead resulted

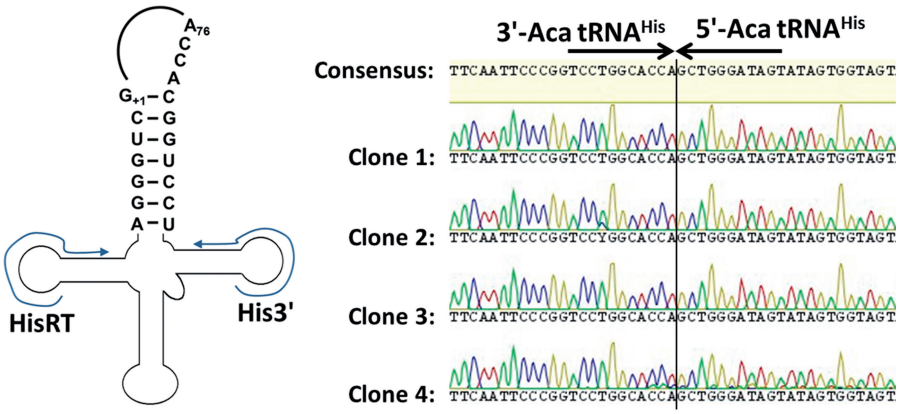


**Figure 3.** Sequencing the 5′-end of *A. castellanii* cytosolic $tRNA^{His}$. Isolated total RNA from *A. castellanii* was circularized with T4 RNA ligase to join 5′- and 3′-ends as indicated on the tRNA diagram, and subjected to RT-PCR. Sequencing traces are shown from four representative clones (out of 12 total sequenced); arrows indicate the direction of the 5′- and 3′-end sequences corresponding to the sequence on the tRNA diagram.

from ligation of the predicted 5′ and 3′-ends without $G_{-1}$ addition (Figure 3). For the remaining sequenced clone, the RT-PCR product similarly lacked a $G_{-1}$ nucleotide, but an additional C-residue between the 5′- and 3′-ends (the origin of which is unclear) was evident from the sequence. Thus, the requirement for $G_{-1}$ addition for cytosolic $tRNA^{His}$ maturation in *A. castellanii* appears to be different from that of all other known eukaryotes, resulting in $tRNA^{His}$ that lacks this otherwise essential identity element.

## Cytosolic HisRS in *A. castellanii* is a non-canonical eukaryotic HisRS

Previous studies with cytosolic HisRS from Bacteria and Eucarya have demonstrated a substantially decreased (~100–1000-fold in catalytic efficiency) ability of these enzymes to aminoacylate $tRNA^{His}$ species that lack the $G_{-1}$ identity element (5,7,29). This strong dependence on $G_{-1}$ is similarly observed *in vivo*, and in *S. cerevisiae*, the major cause of the growth defect of the *thg1Δ* strain has been attributed to loss of aminoacylated $tRNA^{His}$ as a consequence of loss of the $G_{-1}$ residue from the tRNA (30,31). To determine whether the lack of $G_{-1}$ on $tRNA^{His}$ is accommodated differently in *A. castellanii*, we cloned, expressed and purified the cytosolic *A. castellanii* HisRS (AcaHisRS) from the cDNA library, as described above for the AcaTLPs (see Supplementary Figure S2). The purified AcaHisRS was tested for its ability to aminoacylate two $tRNA^{His}$ substrates that are otherwise identical, differing only by the presence or absence of $G_{-1}$. Aminoacylation assays were performed as previously described with substrates labeled with $^{32}P$ at the 5′-phosphate of $A_{76}$; following digestion of the reactions with nuclease P1, the ester-linked histidyl-$A_{76}$ product is resolved from the unreacted 3′-terminal nucleotide by TLC (27). Control reactions with purified *S. cerevisiae* HisRS (SceHisRS) demonstrate the expected strict dependence on the presence of $G_{-1}$, as only the $G_{-1}$-containing $tRNA^{His}$ is aminoacylated to detectable levels in the *in vitro* assay (Figure 4). In contrast, the

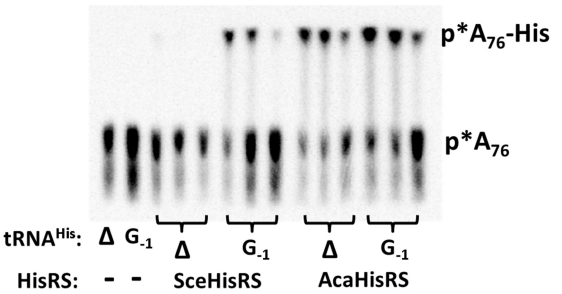


**Figure 4.** The $G_{-1}$ nucleotide is not an identity element for AcaHisRS. Aminoacylation assays were performed with two yeast $tRNA^{His}$ transcripts, one lacking the $G_{-1}$ residue (Δ) and one in which the $G_{-1}$ residue is included as a part of the *in vitro* transcript ($G_{-1}$). Both tRNAs were $^{32}P$-labeled at the 5′-phosphate of the terminal $A_{76}$ nucleotide according to previously described protocols. After incubation of the labeled tRNA with three dilutions of purified HisRS enzymes (2.5–0.1 μM from *S. cerevisiae* or *A. castellanii*, as indicated) in the presence of ATP and histidine, the reactions were digested with nuclease P1 to release the labeled 3′-terminal $A_{76}$ nucleotide, and TLC was used to resolve the histidylated-AMP (product of the reaction) from the AMP generated by digestion of unreacted substrate. Lanes indicated by a minus sign (–) contain no enzyme in the aminoacylation reactions.

AcaHisRS generated similar amounts of aminoacylated $tRNA^{His}$ from both the $G_{-1}$-containing and $G_{-1}$-lacking $tRNA^{His}$ substrates. Thus, the $G_{-1}$ residue is not a critical identity element for recognition of the tRNA by cytosolic AcaHisRS.

## Both AcaTLP1 and AcaTLP2 exhibit tRNA repair of 5′-edited mt-tRNAs

As our data do not suggest a role for either of the AcaTLPs in $tRNA^{His}$ maturation, we finally tested whether either of the AcaTLPs catalyses 5′-end repair of truncated tRNAs, as would be expected of enzymes that participate in 5′-editing of mt-tRNA, which occurs with 12 of 15 mt-tRNAs in *A. castellanii* (32–34). The substrate used for these assays was a mitochondrially encoded $tRNA^{LeuUAA}$ from *A. castellanii*. This tRNA undergoes editing to remove $A_{+1}$ and $U_{+2}$ nucleotides that form

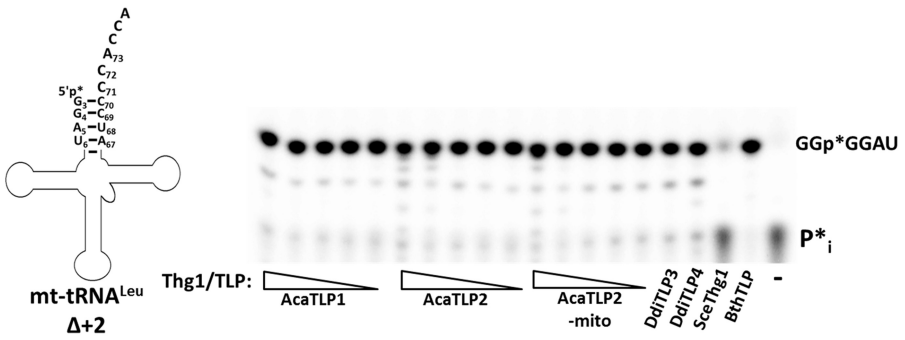


**Figure 5.** AcaTLPs both catalyse efficient repair of 5′-truncated *A. castellanii* mt-tRNA. Phosphatase protection assays were performed with mt-tRNA$^{Leu}\Delta_{+2}$ substrate to detect repair by addition of missing $G_{+2}$ and $G_{+1}$ nucleotides, which generates a $G_{+1}G_{+2}$p*GGAU oligonucleotide, as indicated on the TLC plate. Reactions contained 5-fold serial dilutions of AcaTLPs (0.4 mg/ml–0.6 μg/ml AcaTLP1 and AcaTLP2, 1.2 mg/ml–2 μg/ml AcaTLP2-mito), or 1 mg/ml of other purified TLP/Thg1 enzymes, as indicated; −, no enzyme control.

mismatches at the 5′-end of the aminoacyl acceptor stem, and to replace those positions with the correctly base-paired $G_{+1}$ and $G_{+2}$ (33). This second reaction requires 3′–5′ polymerase activity, as recently associated with two TLPs encoded in *D. discoideum* (DdiTLP3 and DdiTLP4) (15). We used a tRNA (mt-tRNA$^{Leu}\Delta_{+2}$) that mimics the presumed *in vivo* substrate for 5′-end repair, in which the two 5′-mismatched nucleotides have been removed and the resulting tRNA transcript begins with $G_{+3}$ (Figure 5). For these assays, we used the 5′-$^{32}$P-labeled tRNA, and looked for production of a protected oligonucleotide fragment (corresponding to GGp*GGAU) after incubation with TLPs followed by RNase A digestion and phosphatase treatment. In sharp contrast to the $G_{-1}$ addition assays described above (Figure 2), both of the AcaTLPs catalyse robust addition of the missing 5′-G nucleotides to this substrate (Figure 5); in fact, we observed nearly 100% conversion to product even at the lowest concentration of enzyme added in the enzyme titrations, which are the same concentrations tested in Figure 2. As previously demonstrated for other 5′-truncated tRNA species, SceThg1 catalysed little detectable 5′-end repair, and other TLPs (including DdiTLP3, DdiTLP4 and BthTLP—a bacterial TLP from *Bacillus thuringiensis*) catalysed efficient repair, generating the same products as the AcaTLPs (14). Measurement of steady-state kinetic parameters for the repair activity demonstrates that AcaTLP1 and AcaTLP2 both catalyse repair of the 5′-truncated mt-tRNA$^{Leu}$ with similar overall catalytic efficiencies ($1.4 \pm 0.4 \times 10^4\,M^{-1}s^{-1}$ versus $1.2 \pm 0.5 \times 10^4\,M^{-1}s^{-1}$, respectively) (Supplementary Table S1). Thus, either of these enzymes is biochemically competent to participate in the 5′-end repair reaction that occurs during mt-tRNA editing *in vivo* in *A. castellanii*.

## DISCUSSION

Repeated negative searches of extensive genomic and transcriptomic sequence data effectively demonstrate that the amoeboid protozoan *A. castellanii* lacks a canonical Thg1 ortholog that is otherwise ubiquitous among previously studied eukaryotes. Although *A. castellanii* encodes genes specifying two related members of the Thg1 superfamily (AcaTLP1 and AcaTLP2), neither of these TLP enzymes is able to substitute for Thg1 by catalysing the $G_{-1}$ addition reaction that is canonically required to specify tRNA$^{His}$ identity in eukaryotes. This conclusion is based on lack of *in vitro* $G_{-1}$ addition activity exhibited by purified AcaTLP1 and AcaTLP2 with cytosolic tRNA$^{His}$ substrates, as well as inability of these enzymes to complement the growth defect of the yeast *thg1Δ* strain (Figure 2 and Supplementary Figure S3). In fact, sequencing of tRNA$^{His}$ reveals that the mature tRNA$^{His}$ in *A. castellanii* lacks the $G_{-1}$ element (Figure 3), and that the cytosolic HisRS in *A. castellanii* does not require the presence of this $G_{-1}$ nucleotide for aminoacylation of tRNA$^{His}$ (Figure 4). Thus, we have identified a cytosolic eukaryotic tRNA$^{His}$–HisRS pair that does not rely on the canonical $G_{-1}$ identity element to maintain fidelity of translation. Interestingly, the two *A. castellanii* TLPs exhibit biochemical activities that are similar to other studied TLPs (from Bacteria, Archaea and *D. discoideum*) in that they preferentially catalyse template-dependent 3′–5′ addition and use this activity to efficiently repair 5′-truncated tRNAs (Figure 5, Supplementary Table S1). Therefore, as observed previously with the slime mold *D. discoideum*, there are two distinct gene products in *A. castellanii* that could potentially play a role in 5′-editing of mt-tRNA species *in vivo*.

Although this is the first example of absence of the critical $G_{-1}$ identity element from tRNA$^{His}$ in eukaryotes, there has been one other demonstration of a similar phenomenon in ~20 α-proteobacterial species from the RC-clade (including *Rhizobiales* and *Caulobacterales*) (35). Normally, bacterial tRNA$^{His}$ genes contain an encoded $G_{-1}$ residue that is predictably retained from the precursor tRNA transcript after processing by the 5′-maturation enzyme RNase P (9). However, in these α-proteobacterial species, the tRNA$^{His}$ genes do not contain the encoded $G_{-1}$, and consequently the mature tRNA$^{His}$ (as in *A. castellanii*) lacks $G_{-1}$. The ability of these organisms to carry out aminoacylation of the tRNA$^{His}$ lacking the $G_{-1}$ identity element is owing to changes both in tRNA$^{His}$ and the HisRS. However, there are significant differences between the bacterial case and the situation in *A. castellanii*, suggesting that absence of the identity element arose from independent events at least twice during evolution.

Recognition of $tRNA^{His}$ by *Caulobacter crescentus* HisRS (CcHisRS) does not require a $G_{-1}$ nucleotide and, in fact, the presence of $G_{-1}$ is slightly inhibitory for histidylation (36). Instead, CcHisRS relies heavily on the nucleotides of the GUG anticodon, and to a more modest extent on nucleotides in the aminoacyl-acceptor stem, particularly a $G_{+1}$–$U_{72}$ wobble base pair and an unusual bacterial $A_{73}$ discriminator nucleotide. Although *A. castellanii* $tRNA^{His}$ contains the $A_{73}$ that is typical of eukaryotic $tRNA^{His}$ as well as the GUG anticodon sequence, it does not contain the +1–72 GU wobble base pair, and histidylation by AcaHisRS is not inhibited by the presence of the $G_{-1}$ nucleotide (Figure 4). Therefore, absence of the $G_{-1}$ identity element is accommodated by distinct mechanisms in these two examples from separate domains of life.

In terms of the synthetase enzymes themselves, several amino acid replacements and small insertions in α-proteobacterial HisRS of the RC-clade (including CcHisRS) appear to have co-evolved with the loss of this identity element from the tRNA. One of the most important of these is the replacement of an otherwise highly conserved Q224 in Motif IIb with G224 in the α-proteobacterial HisRS enzymes (35). Q224 interacts with the $G_{-1}$–$C_{73}$ base pair that is the canonical feature of bacterial $tRNA^{His}$, and thus this Q-to-G change in the RC-clade of α-Proteobacteria is likely to play a role in tRNA recognition. Again, it is unlikely that AcaHisRS uses a similar mechanism to achieve $\Delta G_{-1}$-$tRNA^{His}$ recognition, as AcaHisRS shares overall sequence similarity with other eukaryotic HisRS enzymes and accordingly does not have the same conserved motif II sequence (including Q/G224) as a typical bacterial HisRS. Further investigation of the molecular basis for the non-canonical $tRNA^{His}$ recognition by AcaHisRS will be important and may lead to the ability to use this second example of an orthogonal HisRS/$tRNA^{His}$ pair for additional applications, such as incorporation of histidine amino acid analogs into proteins in a site-selective manner.

Interestingly, the mitochondrial HisRS in *S. cerevisiae* (which is encoded by the same gene as the cytosolic HisRS and uses an alternative translation start site to generate the mitochondrially targeted protein) does not depend on the presence of $G_{-1}$ for aminoacylation of the mt-$tRNA^{His}$ encoded in yeast (37). However, we note that the mt-$tRNA^{His}$ in *S. cerevisiae* retains the encoded $G_{-1}$ residue, so despite the ability of the mt-HisRS to aminoacylate the tRNA that lacks $G_{-1}$, the identity element is apparently still present in the mature tRNA species. Moreover, this same SceHisRS enzyme remains highly dependent on the presence of $G_{-1}$ for aminoacylation of cytosolic $tRNA^{His}$, suggesting that although there may be unique features of mt-$tRNA^{His}$ that result in alternative tRNA recognition properties for this particular tRNA, the fundamental nature of the SceHisRS in terms of its $G_{-1}$ dependence remains as expected for a canonical eukaryotic HisRS, and is distinct from the lack of dependence on $G_{-1}$ observed with AcaHisRS in this work. Intriguingly, at least one other case of mt-$tRNA^{His}$ species that are only partially modified with $G_{-1}$ have been identified, suggesting that there may be additional complexity related to the presence of the identity element in organelles, although again, in these cases, the HisRS enzyme depends on the presence of $G_{-1}$, as expected, and the role of the $\Delta G_{-1}$-$tRNA^{His}$ in the mitochondria remains unknown (38).

The aberrant nature of $tRNA^{His}$ in *A. castellanii* and the resulting effects on $tRNA^{His}$ identity described here have implications for other eukaryotic species. Although a bona fide Thg1 ortholog is identifiable in the majority of eukaryotes for which genomic sequences are available, there are other species that similarly lack an evident Thg1 gene (17). Some of these species, like *A. castellanii*, may obviate the need for Thg1-catalysed $G_{-1}$ addition by use of a $\Delta G_{-1}$-type $tRNA^{His}$. Identification of other organisms for which this is the case might provide clues to the evolutionary pathway of this unusual tRNA identity feature. It is also possible that in some Thg1-deficient eukaryotes that encode one or more TLPs, one that catalyses the cytosolic $G_{-1}$ addition activity may yet be identified, thereby allowing retention of the canonical identity element. Identification of TLPs of this type may again provide important data to help understand the evolution of the non-templated $G_{-1}$ addition activity that is a unique feature of eukaryotic Thg1 enzymes. In this regard, it will be interesting to investigate organisms (such as the fungus *P. nodorum*) that encode TLPs that do not contain mitochondrial targeting signals and whose mt-tRNAs (as inferred from secondary structure modeling) do not require 5′-editing (17). Finally, there may be alternative non-Thg1-dependent pathways that remain to be identified whereby the $G_{-1}$-residue on $tRNA^{His}$ might be acquired. The results described here, however, clearly challenge the previously accepted paradigm that $tRNA^{His}$ identity and the presence of a $G_{-1}$ residue are inextricably linked in eukaryotes; as such, these observations represent an important step toward understanding the multiple pathways to $tRNA^{His}$ identity in biology.

The question of the precise biological role of the two encoded TLPs in *A. castellanii* remains to be addressed completely. Biochemically, as with other TLPs studied previously, both AcaTLPs possess efficient 3′–5′ polymerase activity when acting on truncated tRNAs, suggestive of a role in tRNA repair (Supplementary Table S1, Figure 1). As tRNA 5′-repair is a required activity for 5′-editing of mt-tRNAs, one or both of these enzymes may participate in this process, which has now been either predicted or demonstrated to occur in the mitochondria of a number of other unicellular eukaryotes in addition to *A. castellanii* (26,39,40). The predicted mitochondrial localization of AcaTLP2 suggests that it is likely to localize to the correct compartment to participate in this activity, but the actual subcellular location of TLPs has not been demonstrated in any organism. It is intriguing that *A. castellanii*, like *D. discoideum*, has two separate genes whose protein products possess the biochemical functionality to participate in the editing process, only one of which contains a predicted mitochondrial targeting signal. Whether in protozoa these enzymes form heteromeric 5′-editing complexes during editing, or whether the two enzymes function in an altogether

different cellular compartment (e.g. mitochondria and cytosol) remains an interesting question that is currently being addressed.

## ACCESSION NUMBERS

JX547005, JX547006, JX547007.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1, Supplementary Figures 1–3 and Supplementary Methods.

## ACKNOWLEDGMENTS

We are grateful to Dr Mom Das and Dr Karin Musier-Forsyth for providing reagents and assistance with aminoacylation assays, to Dr Ryan Gawryluk for initially providing *A. castellanii* RNA for cDNA library construction, to Monica Crary and Dr Greg Booton for providing *A. castellanii* cells for isolation of RNA and to Prof. Brendan Loftus and Prof. Andrew Roger for provision of unpublished RNAseq data. Thanks also to Juan Alfonzo for helpful comments on the manuscript.

## FUNDING

National Institutes of Health (NIH) [GM087543 to J.E.J.]; The Ohio State University Graduate School Presidential Fellowship [to B.S.R.]; The Ohio State Department of Biochemistry Summer Undergraduate Research Program Fellowship [to F.M.]. Funding for open access charge: NIH [R01 GM087543].

*Conflict of interest statement:* None declared.

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