The structural basis of transfer RNA mimicry and conformational plasticity by a viral RNA

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RNA is arguably the most functionally diverse biological macromolecule. In some cases a single discrete RNA sequence performs multiple roles, and this can be conferred by a complex three-dimensional structure. Such multifunctionality can also be driven or enhanced by the ability of a given RNA to assume different conformational (and therefore functional) states¹. Despite its biological importance, a detailed structural understanding of the paradigm of RNA structure-driven multifunctionality is lacking. To address this gap it is useful to study examples from single-stranded positive-sense RNA viruses, a prototype being the tRNA-like structure (TLS) found at the 3' end of the turnip yellow mosaic virus (TYMV). This TLS not only acts like a tRNA to drive aminoacylation of the viral genomic (g)RNA²–⁴, but also interacts with other structures in the 3' untranslated region of the gRNA⁵, contains the promoter for negative-strand synthesis, and influences several infection-critical processes⁶. The TLS is thus structured to perform several functions and interact with diverse binding partners, and we demonstrate its ability to specifically bind to ribosomes.

The TYMV TLS RNA (hereafter termed 'the TLS') is a tRNA mimic, a subject of broad biological and evolutionary importance⁷, as highlighted by the fact that some tRNA mimics are linked to disease⁸–¹⁰. Like tRNA, the aminoacylated TLS binds to eukaryotic elongation factor 1A (eEF1A) and is a substrate for tRNA-modifying enzymes⁶. These activities and other data suggest a tRNA-like structure¹¹–¹⁶. However, the topology of the TLS differs from tRNA, mandated by its location on the 3' end of the gRNA (Fig. 1a, b and Extended Data Fig. 1). In addition to affecting many viral processes¹⁷–¹⁹, the TLS may regulate the activities of ribosomes and replicases on the gRNA⁶,¹⁰. This function could be conferred by its ability to readily transition between folded and unfolded states. Simple tRNA mimicry is insufficient to explain these phenomena; although tRNAs flex while transiting through the ribosome they do not unfold and refold. To explore the paradigms of tRNA mimicry and RNA structural and functional plasticity, we solved the structure

Figure 1 | Function and structure of the TYMV TLS. a. The TLS (dashed box) at the 3' end of the gRNA, with the UPD upstream. AARS (red) valylates the TLS, which can interact with the RDRP (yellow) or eEF1A (blue). Ribosome binding (green) was suspected but untested. aa, amino acid. b. Topology of tRNA and the TLS in rainbow colours. 5' Ends are blue and 3' ends are red. Attached amino acid is labelled 'aa' or Val (for valine). AC, anticodon loop; D, D loop; T, T loop; V, variable loop. c. Secondary structure of the crystallized RNA. Lowercase letter indicates the single mutation. Numbering is from the 5' end of the crystallized sequence. d. Three views of the structure, coloured to match scheme used in c. The conformation of the 3'CCA and the AC loop differ from tRNA, probably owing to crystal packing (Extended Data Fig. 8).
of the TYMV TLS RNA by X-ray crystallography to 2.0 Å resolution (Fig. 1c and Extended Data Fig. 2), comparable to the highest-resolution structure of free tRNA, which we used here for comparison (1.93 Å)21. The TLS assumes the classic L-shaped tRNA conformation (Fig. 1d), but achieves this in a way that diverges from tRNA and from previous predictions3,22. The topology (Fig. 1b and Extended Data Fig. 3) and the intramolecular interactions that form the structure are different from those in tRNA (Fig. 2a). Although the TLS pseudoknot (the first recognized RNA pseudoknot14) is in the position of the acceptor stem of the tRNA, and elements analogous to the D loop, T loop and V loop are positioned as in tRNA, their interactions are not tRNA-like. In the elbow region of tRNA, the V loop interacts with the D stem, stabilizing the L-shaped tRNA structure (Fig. 2b). In contrast, the V-loop bases in the TLS point away from the D stem to interact with the 5' end and pseudoknot of the TLS (Fig. 2b). G4 adopts a syn conformation (Extended Data Fig. 4), forming a long-range base pair with C76 in a loop of the pseudoknot. The unexpected G4–C76 base pair is stabilized by stacking interaction of the D loop (cyan) with the T loop (red) of tRNA (left) and the TLS (right). Lines with embedded symbols30, single hydrogen bonds with dashed lines. Lines with embedded grey bar indicates the long-range linchpin interaction. Dashed lines indicate the C76–G4 base pair. The unexpected G4–C76 base pair is stabilized by stacking and interaction of the D loop (cyan) with the T loop (red) of tRNA (left) and the TLS (right). Dashed lines indicate the C76–G4 base pair.

The stabilizing intramolecular interactions of the TLS show how it can adopt different folded states, potentially to organize infection-important activities, achieving structural and functional plasticity. Disruption of the linchpin would lead to a loss of the L-shape fold and a propagated loss of interactions extending from the V loop to the D/T-loop interface. This effect is observed when the base pair and adjacent nucleotide that stack on and stabilize this pair are eliminated by truncating the TLS from the 5' end11 (Extended Data Fig. 5). This disruption could be induced by loading of the virally encoded RNA-dependent RNA polymerase (RDRP) at the 3' end15,23. The subsequent destabilization would create a favourable template for the RDRP and effectively remove competition between the RDRP and the proteins that require the stable fold (for example, aminoacyl tRNA synthetase (AARS)).

The TLS structure has two distinct 'faces'. The tRNA-deviating features are on one side of the structure, where the upstream pseudoknot domain (UPD) and the gRNA connect to the TLS (Fig. 1b and Extended Data Fig. 7). The structure reveals that the UPD is positioned to interact with the 'divergent face' of the TLS. The opposing side of the TLS, the 'tRNA-like face', interacts with the valyl-AARS when the TLS structure is modelled into a tRNAVal–AARS complex structure25 (Fig. 3a, b). The TLS structure is accommodated by the AARS, including the acceptor stem pseudoknot, which has a different structure to that shown by NMR (Extended Data Fig. 6). Like tRNA, the TLS has high crystallographic B factors in its anticodon (AC) loop and 3' CCA, suggesting that these can readily undergo structural changes (Fig. 3c, d and Extended Data Fig. 8). In the case of the AC loop, this is important to dock the valine-specifying identity elements in the AC loop onto the protein26. Modelling of the TLS structure onto an elongation factor structure also reveals an interface similar to that formed with tRNA and no obvious steric clash (Extended Data Fig. 6). Because the divergent face does not contact the AARS or eEF1A, the 5' end of the TLS is not occluded by interaction with either protein. Thus, the UPD and viral genome do not interfere with binding (Extended Data Fig. 9), and the precise mimicry of the tRNA-like face explains how the TLS can achieve tRNA-like valylation efficiencies and eEF1A binding affinities27.

The interactions of the TLS with AARS and eEF1A suggest that it could bind to the ribosome, as previously suggested28. Ribosome binding would require accommodating the entire TLS structure between the subunits, including elements that deviate from tRNA within the TYMV 3' untranslated region (UTR). We measured binding of TLS-containing RNAs to Thermus thermophilus 70S ribosomes, a valid model for tRNA binding assays given the interchangeability of eukaryotic and bacterial tRNAs29. In vitro transcribed Arabidopsis thaliana tRNAVal bound to the 70S (dissociation constant (Kd) = 0.27 ± 0.05 nM) whereas a 75-nucleotide-long negative control RNA (from bacteriophage phi29pRNA) did not (Kd > 1,000 nM) (Fig. 4a and Extended Data Fig. 1). Mutation of the tRNAVal D loop to disrupt the global tRNA fold resulted in a 28-fold loss of affinity (Kd > 7.6 ± 0.8 nM) (Fig. 4b), consistent with binding
being dependent on the global conformation of the tRNA. A TLS RNA containing the 5′-UUAG sequence bound with tRNA-like affinity ($K_d = 0.31 \pm 0.07\) nM), and mutation of this RNA’s D loop decreased binding ninefold ($K_d = 2.7 \pm 0.2\) nM) (Fig. 4c). Likewise, truncation of the 5′ end of the TLS to abrogate the linchpin interaction reduced binding approximately threefold ($K_d = 1.1 \pm 0.3\) nM) (Extended Data Fig. 5).

Figure 3 | tRNA mimicry and AARS binding. a, Backbone traces of superimposed tRNA (cyan) and TLS (red). The tRNA-like face is shown. b, Superposition of the TLS onto tRNA$^{\text{Val}}$ bound to valyl-AARS (Protein Data Bank accession 1GAX)$^{25}$. c, The AC loop of the TLS (red) must swing into position to match that of tRNA (cyan). d, TLS structure coloured by relative crystallographic $B$ factor (high, red; low, blue).

Figure 4 | Binding of tRNA and TLS to ribosomes. a, Binding curves of tRNA$^{\text{Val}}$ (positive control) and pRNA (negative control) to 70S ribosomes, fit by a Langmuir isotherm (for RNA sequences see Extended Data Fig. 1). b, Binding of wild-type tRNA and tRNA with mutated D loop (D-loop knockout (KO)). c, Diagram of the UUAG TLS (UUAG sequence in cyan) and binding curves of this TLS and versions with the D loop mutated and with the UUAG removed (0G). d, Diagram of the UPD TLS (UPD shown in green) and binding curves of this UPD TLS and a D-loop mutant. Error bars are 1 standard deviation from mean of 3 replicates.
Remarkably, an RNA containing the TLS, the UUAG and the 23-nucleotide-long UPD also bound to ribosomes (TMV UPD; Kd = 0.24 ± 0.11 nM), and binding of this RNA was reduced 100-fold by D-loop mutation (Kd > 24 ± 8 nM) (Fig. 4d). Thus, the folded TLS can bind the ribosome even in the context of the entire 3’ UTR and binding depends on native structure. The affinity is consistent with binding to the P site, although binding to other sites is possible. The ability of the entire TMV 3’ UTR to dock within ribosomes may relate to its functions as a regulatory switch, a translation enhancer and a means to protect the 3’ end of the genomic RNA17–19.

**METHODS SUMMARY**

*In vitro* transcribed RNA was crystallized by vapour diffusion. Crystals grew to full size in 1–2 days, were derivatized with iridium (III) hexamine and cryo-protected. Diffraction data were collected at Advanced Light Source beamline 4.2.2 and used in single-wavelength anomalous dispersion (SAD) phasing. Crystal diffraction data, phasing and refinement statistics are contained in Extended Data Table 1. Ribosome binding was measured by filter binding.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Author Contributions** J.A.H. and G.M.R. designed the crystallization RNAs and identified initial crystals. T.M.C. and D.A.C. improved and grew the crystals. Crystals were harvested by T.M.C., who also solved and refined the structure. J.C.N. collected and processed synchrotron diffraction data. G.M.R. conducted the ribosome binding experiments. J.S.K. provided overall supervision and guidance, and together with T.M.C. and D.A.C. wrote the manuscript.

**Author Information** Atomic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank under accession number 4PSJ. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.S.K. (jeffrey.kell@ucdenver.edu).
RESULTS


table 1. Further analysis of the structure was completed using MolProbity36,39. Summary of the output: clashscore = 14.19; probably wrong sugar puckers: 2; bad backbone conformations: 7; bad bonds: 0; bad angles: 3. Areas of concern were examined in the structure and generally fell within areas of the structure with unusual conformations, but the density and model agreed well in these regions. 

Mutagenesis for ribosome binding. Mutations to the DNA templates were made using a PCR-based site-directed mutagenesis protocol (Agilent) with primers designed to modify the D-loop nucleotides. The nucleotides comprising the D loops of RNA E20, the TYMV UUA G TLS and the TYMV UPP TLS were replaced with stable UUCG tetraloop sequences. For RNA, the primer sequence was 5’-GGG TGGTGTA CTTCCAGGCGGCTAGTC TCTC-3’. The UPD primer had the sequence 5’-CTTTTTAAATGCTTAGCTGCTGCGCGGAGTCTGTC TCCCC-3’. The UUA G primer sequence was 5’-CGGTTCAGTCCTGC TCGGAGGTCTG TCCCC-3’. 70S ribosome preparation. Preparation of 70S ribosomes was done by the Noller laboratory as previously described44.

Filter binding. The filter binding protocol used was modified from previously published methods44,45. Fifty-microlitre reactions contained 25 mM Tris–HCl, 50 mM KCl, 10 mM MgCl2, 2 mM Spermine at pH 7.8, 100 counts per minute of 35P-labelled RNA. The reactions were incubated at 37 °C for 30 min then passed through a sandwich of filters (pre-soaked in matching buffer) in a vacuum manifold. Filters: size exclusion (Tuffryn) filter (Pall), nitrocellulose filter (BioRad), Hybond N+ charged nylon filter (GE BioSciences), and filter paper (Whatman). The filters were washed three times with wash buffer (25 mM Tris–HCl, 100 mM KCl, 25 mM MgCl2, pH 7.5) and allowed to dry for 3 h. Reactions were quantified by phosphorimaging and data were fit using KaleidaGraph software.

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Extended Data Figure 1 | Sequences and structures of RNAs. Top left, sequence and secondary structure of the complete TYMV TLS and the UPD (green dashed box). The UPD is just upstream of the UUAG sequence that is important for stabilizing the L-shaped structure and the UPD is known to be able to pack against the TLS\(^5\,^{11}\). Interestingly, the stop codon for the Coat protein is within the UPD (magenta). Right and bottom, sequences and secondary structures of all additional RNAs used in ribosome binding assays or discussed in the text. Yellow highlights indicate the location of mutation.
Extended Data Figure 2 | Representative electron density and bound trivalent ions. a, Unbiased, density-modified electron density from SAD phasing using data to 2.5 Å (grey mesh, 2σ), superimposed on the final model. The T loop and part of the D loop is shown. For simplicity, the density and structure of water and ions is not shown. b, Final 2F o − F c electron density map after model building and refinement to 1.99 Å (2σ). c, Structure with the location of 12 iridium (III) hexammine ions. Although many of these hexammine binding sites may also be Mg^{2+} binding sites important for stabilizing the fold, the trivalent hexammine was present at 8 mM and thus many weaker Mg^{2+} binding sites could have been occupied. For this reason, and because there is not a one-to-one correlation of Mg^{2+} binding sites and trivalent hexammine sites, we do not make conclusions about Mg^{2+} binding on the basis of this structure.
Extended Data Figure 3 | Topologies and three-dimensional structures of tRNA and the TYMV TLS.  

**a**, Top, the topology of a canonical tRNA is shown in rainbow colours with the 5' end in blue and the 3' end in red. The attached amino acid is shown (labelled 'aa' or Val) and structural features are labelled: AC, anticodon loop; D, D loop; T, T loop; V, variable loop. The 5' and 3' ends of the RNA are shown. Bottom, ribbon representation of the backbone of tRNA\textsuperscript{Phe} coloured roughly to match the cartoon diagram.  

**b**, Same as **a**, but for the TYMV TLS. The location of the UPD (grey dashed box) and gRNA (grey dashed line connected to the 5' end) are shown on the top diagram.
Extended Data Figure 4 | Assignment of bases to the syn conformation. a, Nucleotide G4, which forms the long-range base pair with C76 in the pseudoknot, is in a syn conformation. Top, placement of the base into an anti conformation results in positive and negative density (green and red, respectively) in the $F_o - F_c$ map (left, contoured at 3σ), and the $2F_o - F_c$ map (right) shows the base is incorrectly placed (blue density, contoured at 1.5σ). In contrast, placement of the base into the syn conformation (bottom) results in a flat $F_o - F_c$ map (left, contoured at 3σ) and a good fit to the $2F_o - F_c$ map (right, blue density contoured at 1.5σ). Base A11 is also in a syn conformation; the same analysis was performed to verify this (data not shown). b, $2F_o - F_c$ map surrounding bases A3–C5. The C4′–C5′ bond of G4 is best modelled in the trans conformation.
Extended Data Figure 5 | Effect of breaking the linchpin interaction.

a. Small-angle X-ray scattering (SAXS) analysis of TYMV TLS RNAs, adapted with permission from ref. 5. Left, ab initio SAXS reconstruction of the shape of the TLS when the 5' sequence that interacts with the pseudoknot (Fig. 2) is present. The RNA forms an L shape overall, illustrated by the black bars (stabilizing long-range interaction in grey). When these 5' nucleotides are removed (right), the L shape is lost and the RNA becomes more extended.

b. Hydroxyl radical probing of several TYMV TLS RNAs that indicate the effect of disrupting the long-range interaction, adapted with permission from refs 5, 11. Green and red indicate protection from cleavage by radicals and enhanced cleavage by radicals, respectively. Overall, the presence of green and red indicate tightly folded RNA. When the 5' nucleotides that form the long-range interaction are present, the RNA stably folds (TYMV UUAG, left). Removal of the 5' nucleotides destabilizes the fold (TYMV 0G, right). The presence of just G4 on the 5' end partially stabilizes the RNA fold (TYMV 1G, middle), confirming its importance in folding and also indicating that the nucleotides adjacent to G4 further stabilize the fold.
Extended Data Figure 6 | T loop and acceptor stems of the tRNA and TLS, and elongation factor binding. a, Superimposed structures of the TLS T loop (red) and part of the D loop (cyan) with the analogous structures in tRNA (grey). TLS bases A11 and A12 are shown; these bases match the interactions formed by analogous bases in tRNA. In the TLS, A11 is in a syn conformation, but the matching base in tRNA is not. This may be due to local differences in the backbone conformation. b, Superimposed structures of the TLS T loop (red) and pseudoknot (blue) with the T loop and acceptor stem elements in a tRNA (grey). View is from the ‘top’ of the molecule, down the axis of the D and AC stems. c, Top, the structure of the T loop (red) and acceptor stem pseudoknot (blue) in the TLS crystal structure. Bottom, structure of these elements isolated from the rest of the TLS and solved by NMR (Protein Data Bank accession 1A60). d, Superposition of the TLS structure (red) onto the tRNA (cyan) of a tRNA bound to EF-Tu (yellow), the bacterial homologue of eEF1A (Protein Data Bank accession 1TTT). Binding is probably facilitated by the fact that the RNA backbone conformation of the TLS pseudoknot and T stem/loop matches that of a tRNA.
Extended Data Figure 7 | The ‘two-faced’ architecture of the TYMV TLS and connection with the UPD. Several views of the TLS (red) superimposed on tRNA^{Phe} (cyan) are shown, rotated 90° relative to each other. The dashed line bisects the structure into its two faces. The backbones are very similar on the tRNA-like face, but differ on the divergent face. Locations where the two structures diverge most markedly are shaded grey. The 5’ end of the TLS, where the UPD connects, is indicated.
Extended Data Figure 8 | The AC loop: structures and crystal packing.

**a**, Structure of the AC loop of tRNA^{Phe}, solved to 1.93 Å (ref. 21). The loop is coloured to reflect relative B factors, with red as the highest and blue as the lowest. **b**, Structure of the AC loop of the TYMV TLS, coloured identically to **a**. The asterisk marks the C30 base that was mutated to G to enhance crystallization. This was the only mutation made to the TLS for crystallization and does not inhibit aminoacylation\(^3\). Overall, the loop structures are similar and both have high crystallographic B factors compared with other parts of the structures, a common feature of tRNAs. There is no evidence that the TYMV TLS AC loop is post-transcriptionally modified, yet it has structural features and conformation flexibility similar to the AC loop of a tRNA (which is often modified; Fig. 2a). **c**, Crystal packing involving the AC loop of the TYMV TLS. Two interacting copies of the RNA are shown in red and magenta, with the C30G mutation in yellow. This mutation, although not appearing to alter the overall AC-loop structure compared to a tRNA, induces intermolecular base pairing in the crystal (pattern shown to the right), suggesting why this mutation aided crystallization. **d**, Crystal packing of the 3’ CCA of the TLS (red, labelled) against an adjacent molecule (magenta) probably causes the CCA to adopt a folded-back conformation.
Extended Data Figure 9 | Models of protein binding to the TLS and the location of the UPD. a, Model of the TLS (red, backbone ribbon shown) on the valine of AARS (green; Protein Data Bank accession 1GAX), similar to Fig. 3b, but viewed from the top and with the tRNA\(^{\text{Val}}\) not shown. The location of the UPD directly 5′ of and against the TLS is shown as a grey oval. The viral genomic RNA is 5′ of the UPD. Note that the strategy used by the TYMV TLS to interact with this protein is probably very different from that used by the TLSs that are histidylated or tyrosylated, which are very different in terms of their secondary structure and fold\(^{6,9}\). b, Same as a, but with the TLS modelled onto the bacterial homologue of eEF1A (EF-Tu) as in Extended Data Fig. 6. tRNA\(^{\text{Thr}}\) is not shown. In both complexes, the location of the 5′ end, the UPD, and viral genome would not interfere with protein binding. This would not be true if the TLS had a tRNA-like topology with the 5′ end paired to the 3′ end.
Extended Data Table 1 | Crystallographic data collection, phasing and refinement statistics

|                             | Iridium (III) hexammine |
|-----------------------------|-------------------------|
| **Data collection**         |                         |
| Space group                 | I 222                   |
| Cell dimensions             |                         |
| $a$, $b$, $c$ (Å)           | 55.3, 101.6, 111.6      |
| $\alpha$, $\beta$, $\gamma$ (°) | 90, 90, 90          |
| Resolution (Å)              | 28.87-1.99 (2.06-1.99)* |
| $R_{\text{sym or } R_{\text{merge}}}$ | 5.4 (82.3)            |
| $R_{\text{meas}}$          | 5.8 (89.5)              |
| $I_{\text{obs}}$           | 21.71 (2.19)            |
| $CC(1/2)$ †                | 99.9 (83.3)             |
| Completeness (%)            | 99.4 (94.7)             |
| Redundancy                  | 7.5 (6.5)               |
| **Refinement**              |                         |
| Resolution (Å)              | 28.9-1.99               |
| No. reflections             | 308254 (18783)          |
| $R_{\text{work}}$ / $R_{\text{free}}$ | 20.6 (29.5) / 24.0 (33.3) |
| No. atoms                   | 2011                    |
| RNA                         | 1785                    |
| Ligand/ion                  | 100                     |
| Water                       | 126                     |
| B-factors                   | 43.9                    |
| RNA                         | 44.1                    |
| Ligand/ion                  | 48.7                    |
| Water                       | 38.5                    |
| R.m.s deviations           |                         |
| Bond lengths (Å)            | 0.017                   |
| Bond angles (°)             | 1.83                    |

One crystal was used.
* Highest-resolution shell is shown in parentheses.
† $R_{\text{meas}}$ is $R_{\text{meas}}$ as reported by XDS in.44.
‡ CC(1/2) is the percentage of correlation between intensities from random half-data sets as defined in ref. 45.