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Mechanism of U-insertion RNA Editing in Trypanosome Mitochondria: Characterization of RET2 Functional Domains by Mutational Analysis

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Abstract

The 3' terminal uridylyl transferases (TUTases) selectively bind UTP and catalyze the addition of UMP to the 3' hydroxyl of RNA substrates in a template-independent manner. RNA Editing TUTase 1 (RET1) and 2 (RET2) play central roles in uridine insertion/deletion RNA editing, which is an essential part of mitochondrial RNA processing in trypanosomes. Although the conserved N-terminal (catalytic) and C-terminal (nucleotide base recognition) domains are readily distinguished in all known TUTases, the nucleotide specificity, RNA substrate preference, processivity, quaternary structures and auxiliary domains vary significantly among enzymes of divergent biological functions. RET2 acts as a subunit of the RNA editing core complex (RECC) to carry out guide RNA-dependent U-insertion into mitochondrial mRNA. By correlating mutational effects on RET2 activity as recombinant protein and as RECC subunit with RNAi-based knock-in phenotypes, we have assessed the UTP- and RNA-binding sites in RET2. Here we demonstrate functional conservation of key UTP binding and metal ion coordinating residues and identify amino acids involved in RNA substrate recognition. Invariant arginine residues 144 and 435 positioned in the vicinity of the UTP binding site are critical for RET2 activity on single- and double-stranded RNAs as well as function *in vivo*. Recognition of a double-stranded RNA, which resembles a gRNA/mRNA duplex, is further facilitated by multi-point contacts across the RET2-specific middle domain.

Keywords

Trypanosoma; mitochondria; RNA editing; TUTase; RNAi

Introduction

3' uridylylation and adenylation are phylogenetically widespread modification reactions essential for processing, stability, and function of many RNAs. Although nucleotidyl transfer is catalyzed by several enzyme superfamilies, these reactions follow a universal two-metal-mediated mechanism in which active site acidic residues coordinate divalent metal ions, most

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often Mg^{2+} , to position the α -phosphate of the incoming NTP for in-line nucleophilic attack by a partially deprotonated 3' hydroxyl group of a nucleic acid.¹ Members of the DNA polymerase β -type nucleotidyl transferases (Pol β NTs) possess a conserved catalytic domain with three invariant carboxylates and a signature helical turn motif hG(G/S)X9-13Dh(D/E)h (x – any amino acid, h – hydrophobic).² Within this superfamily, archaeal CCA-adding enzymes, eukaryotic nuclear poly(A) polymerases (PAPs), non-canonical Trf4/5-type PAPs, 2'-5' oligo synthetases,^{3–5} and terminal uridylyl transferases (TUTases) constitute an apparently monophyletic sub-family.⁶ Two cytoplasmic (TUT37 and TUT48) and three mitochondrial (RET1, RET2, and MEAT1) TUTases have been described in trypanosomes. Multifunctional RET1 associates with heterogeneous high molecular mass complexes and participates in uridylylation of small mitochondrial RNAs, including guide RNAs (gRNAs), ribosomal RNAs, and mRNAs^{9, 10} while RET2 is responsible for the mRNA U-insertion editing activity and is apparently confined to the RNA editing core complex (RECC).^{9, 11}

Prior to translation, twelve out of eighteen trypanosomal mitochondrial mRNAs require various degrees of post-transcriptional U-insertion/deletion RNA editing.^{12–17} In this process, trans-acting gRNAs anneal to pre-mRNAs thus defining sites of pre-mRNA cleavage, mediating the number of U's inserted or deleted, and promoting ligation of mRNA fragments (reviewed in 12, 18, 19). RET2 TUTase adds Us to the 3'-end of the mRNA cleavage fragment according to the number of guiding purine nucleotides in the gRNA. Both purified recombinant protein and the RECC-embedded RET2 are capable of inserting U's into gapped double-stranded RNAs *in vitro*.^{11, 20} However, the substrate requirements and processivity of gap-filling reactions differ substantially for individual and complex-bound enzymes.²¹

Crystallographic studies of DNA Pol β ,^{22, 23} bovine and yeast PAPs,^{24, 25} vaccinia virus PAP,²⁶ CCA-adding enzymes,²⁷ terminal deoxynucleotidyltransferase,²⁸ kanamycin nucleotidyl transferase,²⁹ and trypanosomal RET230 and TUT4⁸ TUTases revealed a conserved topology of the N-terminal catalytic domain consisting of a five-stranded mixed β -sheet flanked by two or three α -helices. The RET2 and TUT4 structures typify an unusual domain organization of TUTases in which the NTD and CTD are engaged in an extensive interaction essentially forming a compact bi-domain. Within this bi-domain, the NTD's anti-parallel β -sheet and the CTD's two helices form a deep cleft bearing the UTP binding site. The RET2-specific 110 amino acid insertion within NTD, termed “middle domain” (MD), extends into solvent to form a compact domain and then re-enters the NTD approximately 2 Å away from the exit site. Deletion of the middle domain abolished both RET2's activity and its MP81-mediated association with RECC.²¹

In vitro, RET2 is exclusively U-specific and is active on both single-stranded (ssRNA) and double-stranded (dsRNA) RNAs while TUT4 uridylylates ssRNA and displays limited CTP-incorporation activity. Accordingly, superpositioning of RET2 and TUT4 UTP-bound structures revealed a highly-conserved UTP-binding site, but also highlighted subtle differences in putative RNA-binding sites.⁸ In terms of nucleic acid substrate structure, RET2 is similar to DNA Pol β which recognizes gapped DNA, an intermediate in nucleotide excision repair (reviewed in 31). Furthermore, both RET2 and DNA Pol β require a 5' phosphorylated 3' fragment for *in vitro* activity. Indeed, tight DNA binding by Pol β can be achieved without the 5' fragment, suggesting that Pol β recognizes the 5'-phosphorylated 3'-fragment annealed to a DNA bridge.³² Intriguingly, the phosphorylation of the 3' fragment is essential for U-insertion activity of the recombinant RET2, but not that of RECC.²¹ RET2 and Pol β 's recognition of gapped substrates and requirement of a 5' phosphate group on the 3' cleavage fragment suggest a shared mechanism of nucleic acid recognition.

To determine the functional roles of amino acids which are conserved among trypanosomal TUTases and to locate RET2-specific residues required for the guided U-insertion activity,

mutated recombinant RET2 proteins and RNA editing core complexes harboring mutated RET2 subunits were analyzed *in vitro*. A comparison of *in vitro* data with inducible knockdown/knock-in phenotypes allowed us to distinguish substitutions that affect RET2 activity and function *in vivo* from mutations that only inhibit the activity of the recombinant protein. To that end, mutagenesis of several putative RNA binding amino acids had either no appreciable effects (R151 and R265), or severely inhibited *in vitro* editing activity without affecting cell viability (R134, R242 and R245). Alanine substitutions in NTD (R144) and CTD (R435) positions spatially adjacent to the UTP binding site and combinatorial substitution (R182/R245) within the electropositive cavity which traverses the middle domain, affected both RET2 activity and cell viability. These data demonstrate that recognition of the RNA substrate is accomplished by multi-point contacts with NTD and CTD amino acids that are conserved among TUTases and those located in the RET2-specific middle domain.

Results

UTP-binding and metal ion-coordinating residues are conserved among trypanosomal TUTases

Mutagenesis studies of *LtRET1* and *TbTUT4* identified three carboxylates that coordinate magnesium ions essential for catalysis and a key tyrosine residue necessary for stacking interaction with the uracil base.^{8, 33} Multiple sequence alignment of trypanosomal uridylyl transferases and superimposition of *TbTUT4* and *TbRET2* structures further illustrate the invariant nature of these residues (Fig. 1). To confirm functional roles of the Mg^{2+} - and UTP-binding residues in RET2, alanine substitution were introduced into positions D97 and Y319. To assess effects of RET2 mutagenesis on U-addition to ssRNA substrates and U-insertion into gapped double-stranded RNAs, a 5'-radioactively-labelled 24-mer RNA was either employed directly in the reaction or assembled with two other fragments to form a pre-cleaved RNA editing substrate³⁴ (Fig. 2a). As expected, the D97A and Y319A mutations resulted in complete inactivation of uridylyl transfer by rRET2 (Fig. 2b).

To establish whether corresponding mutations abolish the *in vitro* U-insertion activity of the RNA editing core complex, RECCs bearing RET2 variants were isolated via tandem affinity purification.³⁵ Protein profiles and abundances of RET2, as determined by Sypro Ruby gel staining and Western blotting with RET2-specific antibodies, respectively, were virtually identical in complexes purified from iCODA/RNAi cells expressing mutant (not shown) and wild-type²¹ versions. In agreement with the loss of rRET2's activity, U-addition to ssRNA and guided U-insertion into dsRNA activities by the purified complexes were not detected (Fig. 2c).

To determine effects of corresponding mutations on cell viability, the mutant proteins were expressed in the iCODA *in vivo* knock-in/knock-down system, which utilizes co-expression of an RNAi-resistant gene and gene-specific double stranded RNA.²¹ Upon tetracycline induction, iCODA/RNAi cells carrying D97A and Y319A mutations exhibited a severe growth-inhibitory phenotype similar to that observed with RET2 RNAi cells. This demonstrates an essential role of UTP- and Mg^{2+} -binding residues for RET2's enzymatic activity and cell viability (Fig. 2d).

Collectively, the loss of activity for recombinant protein and purified RNA editing core complex *in vitro* and the growth-inhibitory phenotype caused by active site mutations demonstrate conservation of key metal ion and UTP binding determinants among trypanosomal TUTases. In addition, a clear correlation between mutational effects on the U-addition and U-insertion activities of rRET2 and purified complexes and cell viability provides an internal control for investigating RNA binding features of RET2 in the context of the RNA editing core complex.

RNA binding residues adjacent to the catalytic site are essential for RET2 function

Previous mutagenesis studies of *TbTUT4* have uncovered a triad of arginine residues (positions 121, 126 and 307) in the vicinity of UTP binding site which are essential for ssRNA binding and TUTase activity.^{8, 36} Superposition of TUT4 and RET2 crystal structures showed that in RET2 corresponding positions are occupied by positively charged amino acids R144, K149 and R435 (Fig. 1d and Ref. 36). In RET2 crystals soaked in UTP and UMP, side chains of arginines 144 and 435 form contacts with UMP occupying the secondary binding site (site B) further implicating these residues in positioning of the RNA's 3'-end.³⁰ To assess the functional roles of putative RNA-binding residues in site B, alanine scanning mutagenesis was performed. Recombinant proteins were assayed for U-addition to single-stranded RNA substrates and guided U-insertion into double-stranded substrates programmed for +2 U's (Fig. 3a). The R435A and R144A mutations inhibited the catalytic activity beyond the dynamic range of the pre-cleaved assay while the K149A mutant retained partial activity on the ssRNA substrate.

Because the gap size in dsRNA has a profound effect on the recombinant RET2's activity,²¹ we next determined the steady-state kinetic parameters for UMP incorporation into a more efficient editing substrate programmed for a single U insertion (Table 1). A closer spacing between the 3' end of the 5'-fragment and the phosphate group of the 3'-fragment (Fig. 2a) partially rescued the activity of K149A mutant (~20 fold drop in catalytic efficiency, Table 1). However, editing activity of rRET2 variants with R144A and R435A substitutions was reduced beyond the sensitivity of the assay. These data indicate that positions R144 and R435, which are invariant among all trypanosomal TUTases (Fig. 1a), provide essential binding determinants for both single-stranded and double-stranded RNA substrates, whereas a mutation in the less conserved position, K149, predominantly affects dsRNA recognition by rRET2.

Incorporation of RET2 into the core editing complex stimulates processivity of the U-insertion reaction, most likely due to RECC's increased RNA-binding affinity.²¹ Therefore, we next analyzed the effects of corresponding mutations on U-addition and U-insertion activities of purified core editing complexes. Substitution of arginines at positions 144 and 435 (data not shown for the latter) led to inactivation of both activities while RECC bearing the K149A mutation exhibited partial U-addition and U-insertion activities (Fig. 3b). An apparent concurrence between data obtained for recombinant RET2 and for RET2-containing complex provides evidence that RNA-binding residues R144 and R435 are indispensable for RET2's activity. The moderately-reduced U-insertion activity of RECC-K149A, on the other hand, indicates that complex incorporation may stimulate RET2's inherent RNA binding affinity or provide additional RNA binding contacts with other complex components.

To verify these conclusions *in vivo*, corresponding mutations have been introduced into parasites and complementation phenotypes were monitored for 192 hours after knockdown/knock-in induction. In agreement with partially reduced editing activity of RECC-K149A, a minor inhibition of cell growth rate was observed after ~100 hours of induction (Fig. 3c). Induced RET2 R141A and R435A iCODA/RNAi cells exhibited severe cell growth inhibition kinetics relative to mock-induced cells, suggesting disruption of RNA editing activity *in vivo* (Fig. 3c). Collectively, *in vitro* and *in vivo* results implicate these closely-positioned arginine residues in the immediate vicinity of the UTP binding site as key RNA binding determinants. Our data also suggest that the mechanism of terminal RNA nucleoside binding is conserved among TUTases active on ss- and dsRNAs. Based on *TbTUT4* crystallographic studies³⁶ and RET2 mutagenesis, it seems plausible that the mechanism of U-insertion by RET2 involves triple-stacking interaction between Y319, the uracil base of bound UTP and the terminal base of RNA substrate, in which the latter is stabilized by contacts with R141 and R435. These results also demonstrate a correlation of iCODA/RNAi-induced phenotypes with partial (K149A) or complete (R144A) loss of U-insertion activities of RNA editing core complexes bearing mutant RET2 subunits.

Selectivity for the 3'-nucleoside of the RNA substrate

Trypanosomal TUTases exhibit a range of single-stranded RNA specificities: RET1,³⁷ TUT4³⁶ and MEAT1³⁸ prefer substrates terminating with U's while RET2 preferentially adds a single U to RNAs ending with purines.³⁶ The *in vivo* RET2 substrate is produced by RNA editing endonuclease 2 (REN2)-catalyzed cleavage at U-insertion editing sites³⁹ in purine-rich pre-edited mRNAs. Consequentially, the first uracil is most often added to the 5' mRNA cleavage fragment terminating with G's or A's.^{11, 40} The majority of editing sites, however, require insertion of more than one U. Therefore, +2 and subsequent Us are added to an mRNA fragment ending with uracil nucleoside upon its base-pairing with gRNA41 (Fig. 3d). We previously showed that +1 and successive insertions occur in distributive and processive modes, respectively.²¹ This observation suggested that RNA recognition by RET2 and processivity of U-insertion differ for R/Y base pairs (Fig. 3d, upper panels) and Y/R (Fig. 3d, lower panels) formed by the 5'-cleavage fragment and gRNA.

Crystal structures of *Tb*TUT4-UTP-UMP ternary and *Tb*TUT4-UpU binary complexes allowed modeling of AMP into the UMP binding site, highlighting RET2-specific residues which may be responsible for the different terminal RNA base preferences observed between TUT4 and RET2. Resultant models suggested that F52 and R141 of TUT4 may stabilize productive binding of a terminal uracil base while RET2's corresponding residues C84 and V271 (depicted in Figure 1d) may accommodate the larger adenosine base for +1 addition.³⁶ Reciprocal mutagenesis of C84 and V271 into TUT4-like amino acids was performed to evaluate their roles in RET2-catalyzed reactions. Double-stranded RNA substrates programmed for +2 addition with either a terminal G or terminal U at the 3'-end of the 5' cleavage fragment were used in pre-cleaved RNA editing assays. In the case of terminal G, a moderate decrease in catalytic efficiency was observed for either C83F or V271R mutations (Fig. 3d, top panel). However, an approximately two-fold rate stimulation was observed for both mutant enzymes with substrates possessing a terminal U (Fig. 3d, bottom panel), which is consistent with hypothesis that a TUT4-like substitutions may stimulate RET2 processivity on RNA substrates terminating with Us.

To further verify participation of V271 and C83 in terminal base specificity, catalytic parameters for UMP incorporation into +1-programmed dsRNA were then measured (Table 1). The V271R mutation resulted in a ~12-fold and ~4-fold reduction in catalytic efficiency on substrates with a terminal G and terminal U, respectively, suggesting a minor decrease in general catalytic activity. A ~13 fold decrease and a ~4-fold increase in catalytic efficiency with terminal G and terminal U substrates, respectively, were observed with the C83F mutation. Addition of a single U stimulates RET2 activity, which suggests a two-mode model in which +1 and longer insertions are added as results of separate binding events.²¹ In agreement with this model, introducing interactions that favor U-binding, such as C83F or V271R, produced a moderate stimulating effect on pre-cleaved substrates programmed for more than a single insertion.

The C83F and V271R mutations were analyzed in the iCODA/RNAi system to assess their effects *in vivo*, but only a moderate inhibition of cell growth was observed (Fig. 3e). Overall, unlike invariant arginines in positions 144 and 435, which are most likely critical for RNA binding, positions C83 and V271 appear to play a role in fine-tuning of the hydrophobic and/or van der Waals environment for optimal positioning of the RNA's acceptor nucleoside.

RNA-binding interface spans across N-terminal, C-terminal and middle domains

The RET2 crystal structure revealed an extensive positively charged surface that spans the N-terminal, C-terminal, and middle domains on both sides of the deep cleft which encloses the UTP-binding site.³⁰ We have shown that deletion of the middle domain is not only detrimental

for RET2 association with MP81 structural protein, and therefore with the RNA editing core complex, but it also abolishes TUTase activity.²¹ Therefore, we next determined whether the putative RNA binding path includes a RET2-specific middle domain, which would implicate MD as a bifunctional module. Several surface arginine residues positioned within 9 Å (R265) to 41 Å (R182) from the UTP's α -phosphate, which is a site of nucleophilic attack, have been selected for alanine scanning mutagenesis followed by *in vitro* and *in vivo* analyses (Fig. 4a). Unlike essential invariant arginines 144 and 435 located in the immediate vicinity of the active site (Figs. 1, 3), arginines at positions 134, 151, 182, 242, 245 and 265 are not conserved among trypanosomal TUTases (Fig. 1). In fact, position 265 is occupied by a negatively charged aspartic acid in all single-stranded RNA-specific TUTases.

As inferred from the RET2 crystal structure, all candidate residues possess flexible, solvent-exposed guanidinium groups (Fig. 4a). Accordingly, their mutations had no apparent effects on the solubility of recombinant proteins or protein composition of purified RNA editing core complexes (not shown). Substitution of the N-terminal domain's residue R151 to alanine had a minor (~2-fold rate reduction) effect on U-addition and U-insertion activities of the recombinant RET2. Likewise, mutagenesis of position 265 to alanine had no appreciable effect while a charge reversal from R to D, which occupies this position in all ssRNA-specific RNAs (Fig. 1a), produced a minor inhibition (~3-fold rate reduction) only with dsRNA substrate. Consistent with a possible role of R265 in selective dsRNA binding, R265D mutation increased rate of U-addition to ssRNA substrate (Fig. 4b).

Mutations in positions R134, R182, R242 and R245 reduced the rate of U-addition activity by 2–8-fold on single-stranded RNA substrates while eliminating (positions 134 and 182) or significantly reducing (>10-fold, positions 242 and 245) the rates of U-insertion activity on double-stranded substrates (Fig. 4c). Trace +1 signals observed in the editing assay with dsRNA are likely to be products of non-guided U-insertion. An observed loss of catalysis on editing-like substrates in contrast to minor effects on ssRNA U-addition activity suggests that the middle domain residues R182 and R245, and N-terminal domain's R134 are indeed critical for double-stranded RNA binding by a recombinant RET2. These results provide additional evidence that the inhibition of RET2 activity upon deletion of the middle domain was caused by the loss of RNA binding contacts.²¹

The effects of mutations within the putative dsRNA binding path were analyzed in the iCODA complementation system by measuring cell growth kinetics, which reflects U-insertion activity of the RECC-embedded RET2. In contrast to recombinant proteins, all but one mutation, in position R182, had no appreciable effect on cell viability (Fig. 5), suggesting that RNA editing *in vivo* had not been affected. In agreement with analysis of the K149 mutation (Fig. 3), complex incorporation apparently enhanced RNA binding affinity thereby compensating for the loss of a single-residue RNA-binding contact. To test this hypothesis, we have generated an iCODA/RNAi cell line expressing a dual mutant bearing substitutions that had a minor phenotypic manifestation (R182) and a mutation with no discernable growth inhibition (R245). Indeed, loss of two contacts led to complete inactivation of the recombinant protein (not shown) and a growth-inhibitory phenotype which was more severe than either mutation alone (Fig. 5). To conclude, RNA editing TUTase 2 binds dsRNA via multipoint contacts spread throughout the conserved N-terminal domain and divergent middle domain; RNA binding is stimulated by RET2 incorporation into RECC likely because of additional RNA-protein interactions with the MP81 subunit of RECC.

Discussion

The aim of this study was to locate amino acid residues responsible for catalyzing nucleotidyl transfer, UTP binding, and RNA substrate recognition by a key RNA editing enzyme, RET2.

A combination of sequence and structural alignments of RET2 and minimal TUTase TUT4 was utilized to predict targets for site-specific mutagenesis. We examined the activities of purified recombinant proteins and affinity-purified RNA editing core complexes containing RET2 variants to assess the effect on both gRNA-dependent U-insertion into double-stranded RNA and U-addition to single-stranded substrates. Furthermore, iCODA/RNAi knockdown/knock-in cell lines, which proved to be a highly sensitive *in vivo* tool, have been used to investigate the effects of conditional complementation on cell viability. A combination of *in vitro* and *in vivo* approaches allowed us to distinguish key residues essential for RET2 function in editing from positions that affect TUTase activity *in vitro*, but whose mutations are inconsequential *in vivo*.

Despite differences in sub-cellular localization, RNA substrate preference, and interaction partners, the catalytic bi-domains formed by the N-terminal and C-terminal domains of UTP-bound RET2 and TUT4 superimpose with 1.6 Å RMSD, highlighting subtle differences within their active sites. Extensive structural and mutational analyses of TUT4 provided a basis for comparative investigation of positions that are conserved among all known TUTases and those that are divergent in RET2 and, therefore, may be involved in editing function-specific interactions. Mutational analysis of the RET2 active site established that the mechanisms of metal-ion coordination and UTP binding are largely conserved among trypanosomal TUTases. Furthermore, two invariant arginines at positions 144 and 435 which belong to N-terminal catalytic and C-terminal base-recognition domains, respectively, and are positioned within 5 Å of the bound uracil base, have been determined as essential for RET2 enzymatic activity and *in vivo* function (Fig. 2). Variations within the catalytic site, such as replacement of the highly-conserved phenylalanine within the Pol β signature sequence (position 83) for a smaller cysteine and charge removal at the position 271 may contribute to preferences displayed by RET2 toward a double-stranded pre-cleaved substrate in which the 5' fragment ends with uracil nucleoside²¹ (Fig. 3).

U-insertion substrates are believed to consist of mRNA fragments scaffolded by a guide RNA. We have shown that the MD is essential for RET2 docking into the RECC and enzymatic activity. However, the deletion of the middle domain does not affect protein expression and its grafting into a TUT4 partially confers a gain of MP81-binding function.²¹ This implicates the MD as an accessory domain that adapts a conserved catalytic module shared by all TUTases to perform a specialized role in editing. To dissect the middle domain's function in RECC association and dsRNA binding and to map the RNA binding path of RET2, we have investigated select surface-exposed arginine residues within the large electropositive surface spanning N-terminal and middle domains (Fig. 4). Although five out of six mutations inhibited recombinant RET2's enzymatic activity, only one amino acid substitution led to a discernable slow-growth phenotype. However, introduction of two mutations led to a synergistic effect, which produced a more severe inhibition of cell division. These findings lend further support to a hypothesis that the middle domain is involved in both interaction with RET2's binding partner MP81 and binding of dsRNA substrates. Furthermore, stimulation of RET2 activity upon incorporation into RECC is consistent with RET2's reported enhancement by *in vitro* synthesized MP81¹¹ and suggests that the RNA-recognition determinants are extended beyond RET2 and include other RECC components.

Materials and Methods

Cell cultures and iCODA/RNAi co-expression

Clonal cell lines for co-expressing wt or mutated RET2-iCODA and RET2 RNAi were generated as described²¹ by co-transfection or sequential transfection of genetic constructs into procyclic *T. brucei* strain 29-13⁴² and subsequent selection of cells resistant to blasticidin, phleomycin, hygromycin, and neomycin by limiting dilution. Concurrent RET2-iCODA-TAP

expression and RET2 RNAi was performed as described.²¹ iCODA expression and endogenous RET2 knockdown of iCODA/RNAi cell lines was verified by Western blotting analysis of whole cell lysates.

Protein purification and mutagenesis

Wild-type and mutant rRET2 and RECC purifications were performed as previously described.²⁰ Point mutations were introduced into the RET2-iCODA gene via the QuikChangeTM II XL site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Protein concentrations of recombinant and RECC-embedded RET2 proteins were normalized via Sypro Ruby gel staining or quantitative Western blotting, respectively.

Enzymatic assays

Pre-cleaved editing assays were performed with 5–20 nM of rRET2 or ~2 nM of RECC-embedded RET2.²⁰ Steady-state kinetic parameters of UMP incorporation were obtained as previously described.^{20, 21}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Domain organization of RNA uridylyl transferases from *T. brucei*. (a) Partial multiple sequence alignment of *TbRET1* (AAK38334), *TbRET2* (AAO63567), *TbTUT3* (XP_822966), *TbTUT4* (DQ923393) and *TbMEAT1* (FJ561337). N-terminal (NTD), middle (MD), and C-terminal (CTD) domains are indicated by purple, yellow and teal bars, respectively. The signature motif of the Pol β NT superfamily is boxed. Identical amino acids are white on black and blocks of similar amino acids are black on gray. Amino acid positions mutated in *TbRET2* are indicated by asterisks - putative contacts involved in metal ion coordination, UTP binding, and RNA binding colored in blue, green, and red, respectively. Metal-coordinating carboxylate residues are indicated by arrows. (b) Overall structures of *TbRET2* and *TbTUT4*. NTD, MD and CTD domains are colored in purple, yellow and teal, respectively. (c) Superposition of *TbRET2* (blue) and *TbTUT4* (yellow) crystal structures. Middle domain only found in *TbRET2*. (d) Catalytic site of *TbRET2*. RET2 amino acids involved in metal ion coordination, UTP binding, and putative RNA binding are indicated by blue, green, and red labels, respectively. Panels b, c, and d were generated using PyMOL [<http://www.pymol.org>].

Fig. 2. Mutagenesis of select UTP- and metal ion-binding determinants. (a) A diagram representing ssRNA and dsRNA substrates used in enzymatic assays. Asterisk indicates radiolabeled fragment. (b) U-addition (upper panels) and U-insertion pre-cleaved editing (lower panel) assays were carried out with 20 nM of rRET2, 100 μ M UTP and 100 nM of single-stranded RNA or editing substrates programmed for +2 insertion, respectively, for indicated periods of time. C – control reaction, no UTP added. (c) Same as (b) but with 2.8 nM of RECC-embedded RET2 mutants. (d) Growth kinetics of RET2-RNAi and RET2-iCODA/RNAi cells harboring indicated mutations. Open circles – mock induced; filled boxes – tetracycline induced.

Fig. 3.

Mutagenesis of putative RNA binding contacts adjacent to UTP binding site. (a) Pre-cleaved editing assays were carried out with 20 nM of rRET2 mutants, 100 nM of single-stranded 5' fragment (top panel) or editing substrates programmed for +2 insertion (bottom panel), and 100 μM UTP for indicated periods of time. C – no UTP added. (b) Same as (a) but with 2.8 nM of RECC-embedded RET2 mutants. (c) Growth kinetics of RET2-iCODA/RNAi cells harboring indicated mutations. Open circles – mock-induced. Filled boxes – tetracycline induced. (d) Pre-cleaved editing assays were performed as in (a) with rRET2 proteins harboring indicated mutations with RNA substrates assembled with 5' fragments with terminal G (top panel) or terminal U (bottom panel) bases. Substrates diagrammed on right. (e) Same as (c) with cell harboring indicated mutations.

Fig. 4.

Mutagenesis of putative dsRNA binding contacts. (a) Electropositive residues spanning the proposed RET2 RNA-binding surface. Domains and residue labels are depicted in purple (NTD), yellow (MD), and teal (CTD). Positive and negative regions are depicted in blue and red, respectively. UTP is shown as ball-and-stick. The figure was generated using PyMOL [<http://www.pymol.org>]. (b–c) Enzymatic assays were carried out with 20 nM of rRET2 mutants, 100 nM single-stranded 5' fragment (top panel) or editing substrates programmed for +2 insertion (bottom panel), and 100 μ M UTP for indicated periods of time. C – no UTP added. ND- not detected. Rates of +1 addition (top panels) and +2 insertion (bottom panels) were calculated in percentile relative to wild-type RET2 activity.

Fig. 5.

In vivo analysis of mutations within putative dsRNA binding path. Growth kinetics of RET2-iCODA/RNAi cells harboring indicated mutations. Open circles – mock induced. Filled boxes – tetracycline induced.

Steady-state kinetic parameters of UMP incorporation by rRET2 mutants and cell growth inhibition in corresponding RET2-iCODA knock-in cell lines. Apparent K_m and k_{cat} were measured with a dsRNAs diagrammed in Fig. 3d, but programmed for +1 insertion.

Table 1

Protein	terminal nucleoside (5'-fragment)	K_m , UTP (μM)	$\pm$	k_{cat} (min^{-1})	$\pm$	k_{cat}/K_m ($\text{min}^{-1} M^{-1}$)	$[k_{cat}/K_m]_{\text{mut}}/[k_{cat}/K_m]_{\text{wt}}$	Phenotype
WT	U	12.6	3.2	1.8×10^{-1}	7.0×10^{-7}	1.5×10^4	1.0	none
WT	G	2.5	1.9	1.8×10^{-2}	2.4×10^{-3}	7.0×10^3	1.0	none
D97A	G	Inactive	-	-	-	-	-	+++
Y319A	G	Inactive	-	-	-	-	-	+++
R144A	G	Inactive	-	-	-	-	-	+++
K149A	G	8.1	2.1	9.6×10^{-4}	1.5×10^{-4}	3.2×10^2	0.1	+
R435A	G	Inactive	-	-	-	-	-	+++
V271R	U	20.9	10.6	7.3×10^{-2}	2.6×10^{-6}	3.5×10^3	0.2	+
V271R	G	2.8	0.9	1.6×10^{-3}	9.1×10^{-5}	5.8×10^2	0.1	
C83F	U	2.6	0.9	9.0×10^{-2}	3.0×10^{-7}	3.4×10^4	2.3	+
C83F	G	0.6	0.6	3.2×10^{-3}	4.3×10^{-4}	5.2×10^3	0.7	
V271R/C83F	U	3.0	1.4	1.1×10^{-1}	5.2×10^{-7}	3.6×10^4	2.4	+
V271R/C83F	G	0.5	0.4	6.9×10^{-3}	7.0×10^{-4}	1.3×10^4	1.9	

Degree of growth inhibition: + (minor); ++ (moderate); +++ (severe).