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The Nucleic Acid-binding Domain and Translational Repression Activity of a Xenopus Terminal Uridylyl Transferase

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Background: TUT7 adds uridines to and regulates RNAs.

Results: Xenopus TUT7 (XTUT7) uridylates RNAs, possesses a basic region that binds nucleic acids, and represses translation of a polyadenylated RNA.

Conclusion: XTUT7 contains a nucleic acid-binding domain important for activity and can repress translation.

Significance: The basic region of XTUT7 may bind RNA in vivo. XTUT7 may control mRNAs by occluding poly(A).

Terminal uridylyl transferases (TUTs) catalyze the addition of uridines to the 3’ ends of RNAs and are implicated in the regulation of both messenger RNAs and microRNAs. To better understand how TUTs add uridines to RNAs, we focused on a putative TUT from Xenopus laevis, XTUT7. We determined that XTUT7 catalyzes the addition of uridines to RNAs. Mutational analysis revealed that a truncated XTUT7 enzyme, which contained solely the nucleotidyl transferase and poly(A) polymerase-associated domains, was sufficient for catalytic activity. XTUT7 activity decreased upon removal of the CCHC zinc finger domains and a short segment of basic amino acids (the basic region). This basic region bound nucleic acids in vitro. We also demonstrated that XTUT7 repressed translation of a polyadenylated RNA, to which it added a distinct number of uridines. We generated a predicted structure of the XTUT7 catalytic core that indicated histidine 1269 was likely important for uridine specificity. Indeed, mutation of histidine 1269 broadened the nucleotide specificity of XTUT7 and abolished XTUT7-dependent translational repression. Our data reveal key aspects of how XTUT7 adds uridines to RNAs, highlight the role of the basic region, illustrate that XTUT7 can repress translation, and identify an amino acid important for uridine specificity.

The addition of nontemplated uridines to the 3’ ends of RNAs is an emerging form of RNA control that can influence RNA stability and processing (1–4). Terminal uridylation regulates both messenger RNAs (mRNAs) and small, noncoding RNAs. Uridylation of specific mRNAs in Schizosaccharomyces pombe and mammalian cells promotes removal of the 5’ cap and subsequent degradation (5–11). Uridylation of microRNA precursors (pre-miRNAs) can either block or promote processing depending on the cellular context (12–18). In nematodes, plants, and algae, uridylation destabilizes microRNAs (miRNAs)3 and/or small-interfering RNAs (siRNAs) (19–22). Mammalian miRNAs are also uridylated (23–26). Despite the apparent pervasiveness of RNA uridylation, little is known about the enzymes that add uridines to RNAs.

Terminal uridylyl transferases (TUTs; also known as poly(U) polymerases) add uridines to RNAs (27–29). TUTs are noncanonical ribonucleotidyl transferases (rNTases) of the DNA polymerase-β superfamily, which contains enzymes that add nucleotides to a variety of substrates, including RNAs (28). The nucleotide specificity of a particular rNTase is difficult to predict by amino acid sequence and must be experimentally derived as determinants for specificity remain unclear. Additionally, TUTs possess several conserved domains: the nucleotidyl transferase domain (NTD), the poly(A) polymerase-associated domain (PAPD), and the nucleotide recognition motif (NRM) (3, 28). NTDs contain the conserved catalytic motif characteristic of rNTases and the catalytic triad of acidic residues, typically aspartates (28). PAPDs encode an NRM, which mediates nucleotide specificity by contacting the base in the active site (28, 30–33).

TUT7 orthologs are poly(U)-adding enzymes implicated in the regulation of let-7 miRNA biogenesis, a family of miRNAs critical during development and oncogenesis (6, 16, 17, 29, 34). Uridylation of let-7 precursor (pre-let-7) RNAs can either block or promote processing, depending on cell type. In mammalian stem cells, the paralogs TUT7 and TUT4 add several uridines to pre-let-7 after recruitment by the RNA-binding protein LIN28

3 The abbreviations used are: miRNA, microRNA; TUT, terminal uridylyl transferase; TUT7, either mammalian (species indicated) or generic TUT7 enzymes; XTUT7, Xenopus TUT7; rNTase, ribonucleotidyl transferase; NTD, nucleotidyl transferase domain; PAPD, poly(A) polymerase-associated domain; NTD*, cryptic nucleotidyl transferase domain; PAPD*, cryptic poly(A) polymerase-associated domain; NRM, nucleotide recognition motif; BR, basic region; ARM, arginine-rich motif; MBP, maltose-binding protein; r.m.s.d., root mean square deviation; pre-let-7, let-7 precursor.
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(12, 14–16). Uridylation blocks processing of pre-let-7 into mature miRNAs, as well as destabilizes pre-let-7 RNAs. In mammalian somatic cells, however, TUT7 acts independent of LIN28 and adds a single uridine to a subset of pre-let-7 RNAs (17). Monouridylation of these pre-miRNAs creates an optimal 3’ end for downstream processing into mature miRNAs.

To further understand TUT7-dependent RNA uridylation, we identified and focused on *Xenopus* TUT7 (XTUT7) as it may have key roles in the oocyte and/or embryo. We sought to better understand how XTUT7 adds uridines to RNAs and its potential role in mRNA regulation. We utilized *Xenopus* oocytes because uridylated RNAs are stable, and microinjected miRNAs are efficiently translated. With this approach, we identified XTUT7 domains important for catalytic activity, illustrated that XTUT7 can repress translation of a polyadenylated RNA, and pinpointed an important residue for uridine specificity. Our experiments also revealed a key role for a small region of basic amino acids that binds nucleic acids.

**EXPERIMENTAL PROCEDURES**

**MS2 Fusion Protein Plasmids**—The pCS2+3HA:MS2, pCS2+3HA:MS2:XP54, and pCS2+3HA:MS2:GLD2-D242A plasmids were previously described (35). Newly constructed MS2 fusion plasmids, and the primers and restriction sites used for their construction, are listed in *supplemental Table 1*. All MS2 fusion proteins were designed to contain: N terminus, three hemagglutinin (3HA) tags; MS2 coat protein; protein to be tested; and C terminus. XTUT7 (also known as ZCCHC6) and XTUT4 (also known as ZCCHC11) cDNAs were cloned from both *Xenopus laevis* and *Xenopus tropicalis* stage VI oocytes. cDNAs corresponding to XTUT7-FL (X. laevis), XTUT7-C (X. laevis), and XTUT4-C (X. laevis) were deposited to GenBank™ with accession numbers KC493151, KC493152, and KC493153, respectively. Mutations and deletions in XTUT7 were inserted by site-directed mutagenesis using *Pfu*-Ultra DNA polymerase (Agilent), and mutated/deleted residues are listed in *supplemental Table 1*. Specific amino acids (*i.e.* His-1269) discussed under “Results” and “Discussion” are referenced by their location in XTUT7-FL. Domain predictions of XTUT7 proteins were completed using the InterProScan referenced by their location in XTUT7-FL. Domain predictions of XTUT7 proteins were completed using the InterProScan.

**Multiple Sequence Alignments**—XTUT7 sequence homologs were identified by reciprocal best BLAST (National Center for Biotechnology Information (NCBI)): *Ciona intestinalis* (GI number: 198429679), *Stronglylocentrotus purpuratus* (115933324), *Bos taurus* (329664700), *Canis lupus familiaris* (73946401), *Macaca mulatta* (10912038), *Mus musculus* (259016375), *Rattus norvegicus* (293354419), *Homo sapiens* (221116335), *Monodelphis domestica* (334332807), *Ornithorhynchus anatinus* (345314193), *Danio rerio* (326668285), *H. sapiens* (297307111), *Caenorhabditis elegans* (17554128), and *Amphimedon queenslandica* (340382961). Sequence logos were derived from MUSCLE (39) sequence alignments of the putative XTUT7 orthologs using WebLogo (40).

**Reporter RNA Plasmids**—The pLG-MS2 (firefly luciferase), pLG-MS2+MS2 (polyadenylated firefly luciferase), pSP65-ren (*Renilla* luciferase), pLGMS2-luc (RNA with three MS2-binding sites), pLGMS2+3A9-luc (RNA with three MS2-binding sites and a poly(A)39 tail), pLG-FBE-ACAmut (RNA that lacked MS2 binding sites), and pLG-FBE-ACAmut+3A9 (RNA with a poly(A)39 tail that lacked MS2 binding sites) plasmids have been described (41–44).

**In Vitro Transcriptions**—RNAs were *in vitro* transcribed from restriction digested plasmids using either the Ampliscribe SP6 high yield transcription or T7-Flash transcription kits (Epichem). RNAs encoded in pCS2+3HA:MS2 (NotI, SP6), pSP65-ren (SalI, SP6), pLG-MS2+A39 (BamHI, T7), pLGMS2+A39-luc (BamHI, T7), pLG-MS2 (BglII, T7) and pLGMS2-luc (BglII, T7) plasmids were prepared with the indicated reagents. All reactions included m’G(5’)ppp(5’)G cap analog (New England Biolabs). In some cases, [α-32P]UTP was included to radio-label the RNA.

**Oocyte Injections and RNA Analysis**—Oocyte injections were performed as described (41, 44). Oocytes were collected after overnight incubation (~16 h). Total RNA was extracted from 10 oocytes using TRI reagent (Sigma). Total RNA from three oocytes was separated on denaturing 6% polyacrylamide gels and analyzed by phosphorimaging. Densitometric analyses were completed using ImageQuant software (GE Healthcare).

**Luciferase Assays and Western Blotting**—Dual-Luciferase assays (Promega) and Western blotting were performed as described (35, 43). Student’s two-tailed *t* tests were used to calculate all *p* values.

**RT-PCR Assays**—Total RNA was treated with 4 units of TURBO-DNase (Invitrogen) for 1 h at 37 °C and then purified using the GeneJET RNA purification kit (Fermentas). 1 μg of total RNA was reverse-transcribed using ImPromII reverse transcriptase (Promega) and 1 μm oligo(dA)18, -(dT)18, -(dC)20, or -(dG)20 for the RT primers, as indicated. cDNA was PCR-amplified using a firefly luciferase-specific forward primer (GCGTTAATCGAGGCGAATTAGTG) and the corresponding RT primer. For quantitative RT-PCR assays, 100 ng of total RNA were reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) and a random hexamer primer, and 5% of the cDNA was amplified using the PerfeCTa qPCR FastMix UNG Low ROX kit (Quanta Biosciences). Firefly luciferase levels were compared with *Renilla* luciferase and β-actin mRNA levels.

**Tail Sequencing Assays**—The tail sequencing assay was performed essentially as described (45) with the following modifications. The P1 anchor primer (AATATTCACCTTGTCTGAATCTTG) was 5’ phosphorylated using polynucleotide kinase enzyme (Promega) and 3’-blocked with cordycepin (Sigma-Aldrich) using terminal deoxynucleotidyl transferase enzyme (New England Biolabs). 400 ng of modified P1 primer were pre-annealed with 400 ng of P1’ (GCTTCAATCGAGGCGAATTGGTG) and ligated to 1–2 μg of total RNA using T4 RNA Ligase (Fermentas) at 37 °C for 1 h. Reverse transcription reactions were completed using 1 μM P1’ oligonucleotide and SuperScript III reverse transcriptase (Invitrogen). Two rounds of nested PCR amplification were performed using forward primer 1 (GCGTTAATCGAGGCGAATTGTGG) and forward primer 2 (ACCTCTCTCTCTCTCTCAGGGCTT). 2 μl of the PCR product was sequenced using the P1 primer.
arginine-rich segment of basic amino acids (the basic region or BR). The amino acids that span the C-terminal NTD and PAPD are 84% identical and 95% similar between *Xenopus* and *H. sapiens* TUT7. Overall, the proteins are 57% identical and 77% similar. TUT7 includes the conserved motif characteristic of known nRTase active sites, which contains two of its three putative catalytic aspartates (Fig. 1B) (28).

TUT7 and its orthologs encode two distinct NTDs and PAPDs. XTUT7 orthologs have canonical NTDs in their C-terminal halves and cryptic NTDs (NTD*) in their N-terminal halves. The NTD contains the three canonical catalytic aspartates (Fig. 1C) (28).

In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartates (Fig. 1C). In contrast, the NTD* contains aspartate to asparagine and aspartate to lysine substitutions of aspartases.
two and three, respectively. XTUT7 orthologs also have distinct NRMs encoded in their N- and C-terminal PAPDs. The NRMs encoded in their C-terminal PAPDs contain highly conserved type-2 NRMs characteristic of rNTases, which includes an invariant histidine (Fig. 1D) (28). The NRMs encoded in their cryptic, N-terminal PAPDs (PAPD*) are more divergent among species and contain an arginine in place of the histidine (Fig. 1D).

**XTUT7 Is a Poly(U)-adding Enzyme—**To test whether XTUT7 can catalyze rNTase activity, XTUT7 was tethered to reporter RNA using MS2 coat protein and MS2-binding sites (Fig. 2A). mRNAs encoding portions of XTUT7 fused to three HA tags and MS2 coat protein (3HA/MS2) were microinjected into *X. laevis* oocytes to directly translate the proteins. Radiolabeled RNA substrates that contained three MS2-binding sites were then microinjected and analyzed on polyacrylamide gels. The XTUT7 proteins tested were depicted in Figure 2B. XTUT7-FL and XTUT7-C are derived from *X. tropicalis* and XTUT7-C is derived from *X. laevis*. XTUT7-DADA proteins contain aspartate to alanine substitutions as indicated in panel B. All proteins tested here and in subsequent figures were derived from *X. laevis*, except when noted. The bottom panels indicate protein levels as determined by Western blotting for HA-tagged fusion proteins and actin. D, the indicated 3HA/MS2 fusion proteins were assayed as in panel C. All proteins tested here and in subsequent figures were derived from *X. laevis*, except when noted. The bottom panels indicate protein levels as determined by Western blotting for HA-tagged fusion proteins and actin. E, schematic of the assays used to determine the nucleotide(s) added by XTUT7. F, RT-PCR analysis for the indicated fusion proteins. All samples were analyzed on the same gel but were separated for clarity. RT primers are indicated. The *dT* lane indicates the addition of adenosines, the *dA* lane indicates addition of uridines, and so forth. G, seven representative, independently cloned sequences that illustrate the nucleotides added by XTUT7-C are shown.
XTUT7 is a poly(U)-adding enzyme. Products confirmed that uridines had been added (Fig. 2). Thus, indicated that the enzyme added adenosines to the reporter GLD-2 yielded products only with an oligo(dT) primer, which were present (Fig. 2) (dA) primed reactions and only when the catalytic aspartates were present (Fig. 2F). Therefore, XTUT7 added uridines to the reporter RNA. Conversely, the control poly(A) polymerase F indicated sufficient to add nucleotides to RNAs.

XTUT7 Extends RNAs Independent of MS2 Tethering—Two lines of evidence demonstrate that XTUT7 extends RNAs independent of MS2 tethering. First, XTUT7-FL and XTUT7-C extended an RNA that lacked MS2-binding sites by ∼30–50 nucleotides, and this activity was eliminated by the mutation of catalytic aspartates (Fig. 3A). Second, an XTUT7-C construct that lacked both MS2 coat protein and the 3HA tag extended a reporter RNA that contained three MS2-binding sites by up to 50 nucleotides (Fig. 3B, last lane). Tethered XTUT7-C extended the same RNA by ∼200 nucleotides on average. As expected, both tethered and untethered XTUT7-C added uridines to reporter RNA (Fig. 3C). Therefore, the C-terminal half of XTUT7 uridylates RNAs independent of MS2 tethering, and the activity of XTUT7 is increased when tethered.

The BR and CCHC Zinc Finger Domains Mediate the Tethering-independent Activity of XTUT7—To examine the role of the BR and CCHC zinc finger domains in XTUT7, mutant enzymes were constructed in the context of the C-terminal half of the protein (Fig. 4A), which possesses the same rNtase activities as the full-length protein (Figs. 2 and 3). The mutant enzymes were first assayed on a radiolabeled reporter RNA that contained MS2-binding sites. Deletion of a single zinc finger (ΔZ1), all three zinc fingers (ΔZ123), or the BR (ΔBR) yielded nucleotide tail lengths similar to XTUT7-C (Fig. 4B). The XTUT7 enzyme that lacked both the zinc fingers and the BR (ΔZ123ΔBR) yielded many fewer nucleotides than wild-type XTUT7-C when expressed at comparable levels (Fig. 4D). Consequently, the BR and zinc finger domains likely act redundantly to contribute to the catalytic activity of XTUT7. However, the diminished activity also could result from a population of misfolded enzyme.

To further examine the role of the BR and CCHC zinc finger domains, the XTUT7 mutants were assayed on a reporter RNA that lacked MS2-binding sites. The mutant XTUT7 enzyme that lacked the zinc fingers and BR (ΔZ123ΔBR) was inactive on the RNA without binding sites (Fig. 4C). Mutant XTUT7 enzymes that lacked the BR (ΔBR) or the zinc fingers (ΔZ123) were less active than wild-type XTUT7-C when expressed at a comparable level (Fig. 4D). In addition, tethered XTUT7 mutants retained uridine specificity (Fig. 4E). Thus, the BR, as well as the CCHC zinc fingers, mediates the tethering-independent uridylation activity of XTUT7.

The BR is a conserved domain that may bind nucleic acids. The BR and CCHC zinc finger domains of XTUT7 are con-

FIGURE 3. XTUT7 extends RNAs independent of MS2 tethering. A, the indicated 3HA/MS2/XTUT7 fusion proteins were assayed on a radiolabeled reporter RNA that lacked MS2-binding sites. The size of the unmodified reporter RNA is 146 nucleotides (146 nt). Diagrams of the XTUT7 proteins tested are depicted in Fig. 2B. The bottom panels indicate protein levels as determined by Western blotting for HA-tagged fusion proteins and actin. B, the indicated XTUT7 proteins were assayed on the radiolabeled reporter RNA with three MS2-binding sites. The size of the unmodified reporter RNA is 133 nucleotides (133 nt). The last XTUT7 protein tested lacks both the MS2 fusion protein and the 3HA tag. The bottom panels indicate protein levels as determined by Western blotting for HA-tagged fusion proteins and actin. RT-PCR assays were performed as in Fig. 2F for the indicated proteins. All samples were analyzed on the same gel but were separated for clarity. RT primers are indicated. The dT lane indicates the addition of adenosines, and the dA lane indicates the addition of uridines.
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The BR and CCHC zinc finger domains mediate the tethering-independent activity of XTUT7. A diagram of XTUT7 mutants tested. CCHC zinc fingers (ZF) 1, 2, and 3 are indicated. The red D's denote catalytic aspartates, whereas the red A's denote asparagine to alanine substitutions. B, C, and D indicated 3HA/MS2/XTUT7 fusion proteins were assayed in parallel on radiolabeled reporter RNA with (B) or without (C) three MS2-binding sites as in Fig. 2C. The sizes of the unmodified reporter RNAs are 133 and 146 nucleotides (nt), respectively. All proteins tested in panels B and C are were assayed in parallel and analyzed on the same gel, which was subsequently split into two panels for clarity. D, protein levels for all samples tested in panels B and C as determined by Western blotting for HA fusion proteins and actin. E, RT-PCR assays were performed as in Fig. 2. All samples were analyzed on the same gel but were separated for clarity. RT primers are indicated. The dT lane indicates the addition of adenines, and the dA lane indicates addition of uridines. F, multiple sequence alignment of the BR-containing regions from the indicated XTUT7 orthologs. Critical residues for defining CCHC zinc finger domains are highlighted in orange. The BR was defined by the above deletion experiments. Conserved arginines are highlighted in purple. The . . . in the alignments indicate a break in sequence. G, BRs from XTUT7 orthologs were compared with ARMs from viral RNA-binding proteins and a basic stretch from PAPD5. Purple, arginines; pink, lysines and histidines. The percentage of arginine composition of each region was also calculated as a comparison.

The BR Binds Nucleic Acids—To test whether the BR directly binds nucleic acids, wild-type (BR-WT) and mutant BR (BR-R1–6A) segments were fused to an MBP-His6 tag (Fig. 5A), purified (Fig. 5B), and tested using electrophoretic mobility shift assays. BR-WT bound an RNA that contained three MS2-binding sites in a concentration-dependent manner, with an apparent $K_d$ of 70 ± 5 nM (Fig. 5, D and E). At the highest protein concentrations, the protein-nucleic acid complexes migrated progressively more slowly, which may indicate that multiple copies of BR-WT can bind the same nucleic acid molecule. A mutant BR in which arginines 1–6 had been changed to alanine (BR-R1–6A) bound the RNA and ssDNA substrates poorly, with estimated apparent $K_d$ values of greater than 400 and 350 nM, respectively (Fig. 5E). Protein-RNA complexes were not observed with MBP-His6 backbone alone on either substrate (Fig. 5E). Therefore, the BR directly binds nucleic acids and requires conserved arginines for optimal binding.

XTUT7 Represses a Polyadenylated RNA—To examine the rNtase activity of XTUT7 on polyadenylated RNA, we tethered XTUT7 to an RNA with a poly(A)$_{39}$ tail. XTUT7-FL and XTUT7-C extended the polyadenylated reporter RNA by a distinct number of nucleotides, which on average was 60 ± 10 nucleotides (Fig. 6A). RT-PCR of the polyadenylated RNAs with either substrate (Fig. 5E). Therefore, the BR directly binds nucleic acids and requires conserved arginines for optimal binding.

XTUT7 Represses a Polyadenylated RNA—To examine the rNtase activity of XTUT7 on polyadenylated RNA, we tethered XTUT7 to an RNA with a poly(A)$_{39}$ tail. XTUT7-FL and XTUT7-C extended the polyadenylated reporter RNA by a distinct number of nucleotides, which on average was 60 ± 10 nucleotides (Fig. 6A). RT-PCR of the polyadenylated RNAs
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To assess the effect of XTUT7 on translation, XTUT7 was tethered to a poly(U) tail of discrete length to an RNA substrate with 3′-poly(A)39 adenosines. poly(U) tail of discrete length to an RNA substrate with 3′-poly(A)39 adenosines.

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extended by XTUT7-C yielded products with both oligo(dT) and oligo(dA) primers, whereas the substrate yielded a single band in the oligo(dT) lane (Fig. 6B). XTUT7 therefore added a poly(U) tail of discrete length to an RNA substrate with 39 adenosines.

To assess the effect of XTUT7 on translation, XTUT7 was tethered to a poly(A)39 firefly luciferase mRNA (Fig. 6C). As a control, we co-injected Renilla luciferase mRNA that lacked MS2-binding sites and a poly(A) tail. Tethered XTUT7 specifically reduced firefly luciferase activity nearly 3-fold (p value < 0.001), much like the characteristic translational repressor Xp54 (Fig. 6D, top and middle panels) (54). XTUT7-dependent repression was specific to firefly luciferase as all proteins tested had no significant effect on firefly or Renilla luciferase mRNA levels as determined by quantitative PCR. Thus, XTUT7 represses translation of polyadenylated reporter mRNA by adding uridines to the RNA.

**Histidine 1269 Is Important for the Uridine Specificity and Repression Activity of XTUT7**—To visualize the likely structure of the active site region, the three-dimensional structure of the XTUT7 catalytic core was predicted using the I-TASSER server (55–57). This analysis yielded ligand-free and ligand-bound homology models of XTUT7 (C-score = 1.02, expected r.m.s.d. = 4.3 ± 2.9 Å) (Fig. 7A). In the model, putative catalytic aspartates 1041, 1043, and 1102 are adjacent to the triphosphate moiety of UTP (Fig. 7A). Tyrosine 1154 appears to participate in a stacking interaction with uracil. Histidine 1269, contained in the NRM of XTUT7, is predicted to contact a carbonyl oxygen in UTP. As expected, the predicted XTUT7 structure aligns well to a structure of the S. pombe poly(U)-adding enzyme CID1 (alignment r.m.s.d. = 1.2 Å, sequence identity to XTUT7 is 32%) (Fig. 7B) (30). Intriguingly, a hydrogen bond that is observed in CID1 between His-336 and UTP is predicted in XTUT7 (His-1269).

We reasoned that His-1269 might be important for nucleotide specificity due to its proximity to UTP. Therefore, we substituted His-1269 with leucine in the context of the XTUT7 C-terminal half (XTUT7-H1269L) because this substitution ablates a potential hydrogen bond to UTP (Fig. 7C). Indeed, tethered XTUT7-H1269L added cytosines, as well as uridines, to RNA (Fig. 7D). XTUT7-H1269L-dependent tails were ~20% cytosine as compared with ~3% with the wild-type enzyme (p values < 0.005). Both XTUT7-H1269L and XTUT7-C rarely added guanosines or adenosines to RNA (< 3% and 2%, respectively). Thus, His-1269 is important for the uridine specificity of XTUT7.

To test whether altered uridine specificity affected the catalytic activity of XTUT7, XTUT7-H1269L was tethered to a polyadenylated reporter RNA. XTUT7-H1269L added a heterogeneous length tail to an RNA with a poly(A)39 tail, rather than the discrete ~60-nucleotide tail added by XTUT7-C (Fig. 7E). The tail added by XTUT7-H1269L was between ~50 and 150 nucleotides in length, which was shorter than the tail added by the wild-type enzyme to RNA that lacked a poly(A) tail. Accordingly, incorporation of non-uridine residues by XTUT7 prevents formation of the discrete length tail on the poly(A)39 reporter RNA.

To determine the effect of XTUT7-H1269L on translational repression, XTUT7-H1269L was assayed using poly(A)39 firefly luciferase mRNA. XTUT7-H1269L not only prevented translational repression, but instead activated it ~3-fold (p value < 0.05) (Fig. 7F). This increase in firefly luciferase activity was less than the increase yielded by the poly(A) polymerase GLD2. Firefly and Renilla luciferase mRNA levels were not significantly affected by any protein tested. Thus, the H1269L substitution alleviated XTUT7-dependent translational repression, which could result from either the relaxed nucleotide specificity of the mutant enzyme or its addition of a heterogeneous length tail to poly(A)39 firefly luciferase mRNA.
The C-terminal Half of XTUT7 Is Sufficient for rNTase Activity—Our studies demonstrate that XTUT7 is a poly(U)-adding enzyme and identify key domains and residues important for activity. The NTD and PAPD of XTUT7 were sufficient for activity, and mutations in the NTD of full-length XTUT7 abolished rNTase activity. Therefore, the NTD and PAPD are the core catalytic domains. The BR and CCHC zinc finger domains flanking the catalytic core likely enable efficient XTUT7-dependent uridylation as their removal decreased XTUT7 rNTase activity. In contrast, the conserved NTD* and PAPD* of XTUT7 lacked activity and were dispensable for it in the context of the full-length protein. The analogous domains in XTUT4 were also dispensable for catalytic activity. Thus, the C-terminal half of XTUT7 is sufficient for rNTase activity.

Recent work on the mammalian TUT7 ortholog, TUT4, suggested that its NTD* was necessary for rNTase activity and therefore contrasts with our finding that the analogous domain of XTUT7 was dispensable (16). Although the NTD* and PAPD* of XTUT7 orthologs are dispensable for catalytic activity, they nonetheless may have critical roles in vivo. For example, they may mediate protein-protein interactions, as suggested by LIN28-pre-let-7-TUT4 experiments (16). Indeed, TUT4 segments that contain its NTD* and PAPD* promote cell proliferation independent of catalytic activity (58).

The XTUT7 Basic Region—XTUT7 contains nucleic acid binding-domains, including the arginine-rich BR. XTUT7 possesses tethering-independent rNTase activity redundantly mediated by its BR and CCHC zinc finger domains. These domains are likely required for efficient catalytic activity when XTUT7 is tethered to RNA. Together, these findings suggest that the BR and at least one of the CCHC zinc finger domains bind RNA. Indeed, we show that the BR binds both RNA and ssDNA in vitro. Given the modest preference of the BR for binding RNA and that rNTases lack catalytic activity on DNA substrates (59), we suggest that the BR binds RNA in vivo.

The XTUT7 BR resembles RNA-binding domains present in certain viral proteins, such as the ARM found in HIV Rev (49, 60). ARMs are flexible RNA-binding domains that typically confer specificity for particular RNAs by recognizing RNA sequences and/or structures (61, 62). For example, the ARM in Rev specifically recognizes and binds its RNA target (63–66). Critical arginines in the ARM make base-specific contacts with the RNA and are necessary for binding. Similarly, the XTUT7 BR requires highly conserved arginines for optimal RNA binding activity.

**Nucleic Acid Binding and Repression by XTUT7**

**FIGURE 6.** XTUT7 represses a polyadenylated RNA. A, 3HA/MS2 fusion proteins were assayed as in Fig. 2C, except that the reporter RNA contained poly(A)_{39} on the 3’ end. The bottom panels indicate protein levels as determined by Western blotting for HA-tagged fusion proteins and actin. B, samples were assayed by RT-PCR as in Fig. 2F, except the RNA contained poly(A)_{39} on the 3’ end. All samples were analyzed on the same gel but isolated for clarity. C, schematic of the assay used to determine the effect of XTUT7 on translation. RNAs were injected as in Fig. 2A, except that two nonradiolabeled reporter RNAs were co-injected. The first contained the firefly luciferase open reading frame (FF) upstream of three MS2-binding sites and a poly(A)_{39} tail. The second contained the Renilla luciferase open reading frame (Ren) that lacked MS2-binding sites and a poly(A)_{39} tail. After 16 h, luciferase levels were determined. D, relative luciferase levels in oocytes that express the indicated fusion proteins were determined. Luciferase levels were normalized to reporter alone samples (no fusion protein) in each panel. Error bars represent the S.D. from three experiments. The bottom and middle panels represent the average relative Renilla or firefly luciferase levels, respectively. The top panel represents the average firefly/Renilla luciferase levels. Protein levels from a representative experiment are depicted below the luciferase data, and all bands are from the same Western blot but were separated for clarity.
BRs are present in other nRTases. Human PAPD5, a noncanonical poly(A) polymerase, binds a subset of RNAs likely through a small, lysine-rich stretch of amino acids (52). The basic stretch of PAPD5 is also required for efficient catalytic activity, much like the BR in XTUT7. A search for similar BRs in human nRTases reveals that five of the seven enzymes contain characterized or putative BRs, including TUT7, TUT4, and PAPD5. Thus, BRs appear to be a common feature of nRTases that are likely utilized to bind RNA substrates and/or facilitate catalytic activity.

We speculate that the BR and CCHC zinc fingers facilitate TUT7 binding to particular RNAs in vivo. TUT7 orthologs uridylate pre-let-7 in the absence of LIN28 both in vitro and in vivo, and this activity requires the pre-let-7 stem (16, 17, 67). We therefore propose that the BR of TUT7, likely in cooperation with the zinc fingers, binds the accessible region of the pre-let-7 stem.

**XTUT7 Homology Model and Nucleotide Specificity**—We generated a homology model of the three-dimensional structure of the XTUT7 catalytic core that identified an amino acid important for uridine specificity. Not surprisingly, the predicted structure of XTUT7 is similar to those of other poly(U)-adding enzymes, particularly CID1, and predicted that a histidine would be important for uridine specificity (30). The analogous histidine in CID1 is required for optimal uridine specificity in vitro (30–32). Indeed, substituting His-1269 with leucine broadened the nucleotide specificity of XTUT7 in vivo so that it added both uridines and cytosines. These data suggest that a histidine-UTP contact is a critical determinant for XTUT7 uridine specificity and likely represents a common mechanism of uridine recognition among XTUT7 orthologs. Direct determination of the XTUT7 structure is needed to test this rigorously.

**XTUT7 and Translational Control**—XTUT7 can repress translation and may represent a new class of translational repressor proteins. XTUT7 repressed translation of a polyadenylated reporter mRNA without affecting mRNA stability. We propose that the U-tail added by XTUT7 binds poly(A).

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**FIGURE 7.** Histidine 1269 is important for the uridine specificity and repression activity of XTUT7. **A**, The predicted structure of the XTUT7 core catalytic domains bound to UTP (XTUT7/UTP) as generated by the I-TASSER server (55–57). The XTUT7 homology model had a C-score of 1.02, an expected TM-score of 0.85 ± 0.08, and an expected r.m.s.d. of 4.3 ± 2.9 Å. Asp-1041, Asp-1043, Asp-1102, Tyr-1154, and His-1269 are highlighted in green. The UTP molecule is gray. **B**, Alignment of the predicted XTUT7 structure (blue) with a described S. pombe CID1 structure (gold/orange, Protein Data Bank (PDB) ID 4E8F) (r.m.s.d. = 1.2 Å) (30). Analogous histidines and their position relative to UTP are indicated. **C**, Alignment of the predicted wild-type XTUT7 structure (blue) to the predicted XTUT7-H1269L mutant structure (pink) (r.m.s.d. = 0.96 Å). The XTUT7-H1269L homology model had a C-score of 0.99, an expected TM-score of 0.85 ± 0.08, and an expected r.m.s.d. of 4.4 ± 2.9 Å. The position of the histidine and the H1269L substitution relative to UTP are indicated. **D**, The nucleotides added by XTUT7-H1269L were determined as in Fig. 2G. Six representative, independently cloned sequences that illustrate the nucleotides added by XTUT7-H1269L are shown. The average percentages of nucleotide composition of nine representative cloned tails added by XTUT7-C and XTUT7-H1269L are indicated. **E**, Indicated proteins were assayed using the poly(A)9 radiolabeled reporter RNA as in Fig. 6A. The top panel represents the average firefly/Renilla luciferase levels. Protein levels from a representative experiment are depicted below the luciferase data. All bands are from the same Western blot but were separated for clarity. **F**, Relative luciferase levels were determined as in Fig. 6D. Error bars represent the S.D. from three experiments. The bottom and middle panels represent the average relative Renilla or firefly luciferase levels, respectively. The top panel represents the average firefly/Renilla luciferase levels. Protein levels from a representative experiment are depicted below the luciferase data. All bands are from the same Western blot but were separated for clarity. **FF**, firefly luciferase open reading frame.
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