Multi-domain utilization by TUT4 and TUT7 in control of let-7 biogenesis

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The uridyl transferases TUT4 and TUT7 (collectively called TUT4(7)) switch between two modes of activity, either promoting expression of let-7 microRNA (monoU) or marking it for degradation (oligoU). Link4 modulates the switch via recruitment of TUT4(7) to the precursor pre-let-7 in stem cells and human cancers. We found that TUT4(7) utilize two multidomain functional modules during the switch from monoU to oligoU. The catalytic module (CM) is essential for both activities, while the Link4-interacting module (LIM) is indispensable for oligoU. A TUT7 CM structure trapped in the monoU activity state revealed a duplex-RNA-binding pocket that orients group II pre-let-7 hairpins to favor monoU addition. Conversely, the switch to oligoU requires the ZK domain of Link4 to drive the formation of a stable ternary complex between pre-let-7 and the inactive LIM. Finally, ZK2 of TUT4(7) aids oligoU addition by engaging the growing oligoU tail through uracil-specific interactions.

The microRNA (miRNA) let-7 is broadly expressed in somatic cells and regulates cellular proliferation and differentiation, as well as the repression of several oncogenes and key regulators of mitogenic pathways, including HMG2A, MYC and RAS. Humans have 12 let-7 variants (let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7g, let-7i and let-98), and many human tumors are associated with the coordinated downregulation of multiple let-7 family members. Mature let-7 is produced via the canonical miRNA biogenesis pathway; however, its production levels are tightly controlled post-transcriptionally through the Lin4–let-7 pathway. An elevated amount of Link4 in stem cells and a subset of human cancers triggers the destruction of let-7 precursors (pre-let-7) via recruitment of the redundant terminal uridyltransferases TUT4 (ZCCHC11) and TUT7 (ZCCHC6), referred to collectively as TUT4(7). The oligoU tail added by TUT4(7) is a signal for degradation of pre-let-7 by Dicer.

As cells differentiate, Link4 levels decline, causing TUT4(7) to switch their catalytic mode from processive oligouridylation to distributive monouridylation of group II pre-let-7 RNAs (ref. 18). Group II pre-let-7s acquire a 3'-nt overhang from Drosha processing and must be monouridylation before serving as a substrate for Dicer. Monouridylation by TUT4(7) promotes let-7 biogenesis by supplying the precursor with a proper 2-nt 3'-end overhang.

TUT4(7) are members of the noncanonical poly(A) polymerases of the DNA polymerase β superfamily. Unlike most uridytransferases, TUT4(7) are modular, multidomain enzymes composed of an N-terminal CCHC zinc knuckle domains (ZK1–ZK3; Fig. 1a). Structurally, the TUT4(7) NTDs closely resemble TUTases from trypanosomes and the Schizosaccharomyces pombe CID1 poly(U) polymerase.

However, the domain architecture of TUT4(7) is distinct and more complex, with two tandem NTDs accompanied by ZF and ZK domains, whose function remains to be determined. Curiously, NTD1 is an active nucleotidyl transferase, as it lacks critical catalytic aspartate residues.

Besides pre-let-7, a plethora of mammalian RNAs are targets of TUT4(7) uridylation, including mature miRNAs, other pre-miRNAs, mRNAs and noncoding RNAs. Small-RNA uridylation is also widespread in several animal model organisms, with Argonaute-bound short interfering RNA (siRNA) in flies and worms as examples. In plants, the TUTase HESO1 competes with methylation in an active system of miRNA turnover.

The mechanisms driving the Link4–let-7 pathway are of particular interest in terms of human health, as the principal players in the pathway are linked to numerous diseases. Link4 strongly reduces the tumor-suppressor activity of let-7. As such, Link4 activation is associated with several human primary tumors, and its ectopic expression promotes cellular transformation. Furthermore, Link4 expression is correlated with development of Wilms' tumors and with advanced stages and poor clinical outcomes of ovarian carcinoma, colon adenocarcinoma, germ-cell tumors, hepatocellular carcinoma and chronic myeloid leukemia. Because Link4 recruits TUT4, it is not surprising that TUT4 can promote tumor growth and metastasis and is often overexpressed in human cancers, especially Link4-expressing tumors. Interestingly, TUT4 inhibition blocks tumorigenicity and
invasiveness of breast cancer cells in vivo and in vitro, making the Lin28–let-7 pathway a superb therapeutic target. Finally, genetic deletion of Dis3L2, the effector of the Lin28–let-7 pathway, is the primary cause of Perlman syndrome, a congenital growth defect that often leads to Wilms’ tumors.

In this study, we set out to determine the mechanisms used by TUT4(7) in carrying out two alternative activities that occur in different cellular contexts with opposite outcomes. We used a structural biochemistry approach to show that TUT4(7) are multidomain modular enzymes. TUT4 and TUT7 are each composed of two functional modules: the CM, which is essential for both modes of uridine (U) addition, and the LIM, which is critical for interaction with Lin28 and the switch to processive oligoU addition.

RESULTS

TUT4(7) utilize multiple domains to regulate pre-let-7

To understand the TUT4(7) activity switch on pre-let-7 substrate, we examined the uridylation activities of mouse TUT4 and human TUT7 purified from insect cells. We selected pre-let-7g as a substrate for biochemical characterization because it has been extensively studied and there is a published structure of the Lin28–pre-let-7g pre-element. It has previously been reported that immunopurified and/or bacterially expressed TUT4(7) catalyze monoU addition and Lin28-dependent oligoU addition to pre-let-7 (refs. 13,14,18,46,48). We found that baculovirus-infected insect cells produced higher yields and more stable TUT4(7) proteins for in vitro studies, enabling us to establish assay conditions to monitor both activities. In accordance with previous biochemical data, we found that both TUT4(7) catalyze distributive monoU addition on the group II pre-let-7g substrate (1-nt 3′-end overhang; Supplementary Fig. 1a,b) with a marked preference over group I pre-let-7g (existing 2-nt 3′-end overhang; Supplementary Fig. 1a,c). Also, in the presence of Lin28, TUT4(7) switched to processive oligoU addition for both group I and group II pre-let-7 substrates (Supplementary Fig. 1a).

Our principal aim was to understand the TUT4(7) activity switch. We reasoned that a structure of TUT4(7) in complex with Lin28–pre-let-7g would be invaluable toward this goal. Unfortunately, recombinant TUT4(7) proteins are not stable and there is a published structure of the Lin28–pre-let-7g pre-element. Overall, our data are consistent with previous biochemical data, we found that both TUT4(7) catalyze distributive monoU addition on the group II pre-let-7g substrate (1-nt 3′-end overhang; Supplementary Fig. 1a,b) with a marked preference over group I pre-let-7g (existing 2-nt 3′-end overhang; Supplementary Fig. 1a,c). Also, in the presence of Lin28, TUT4(7) switched to processive oligoU addition for both group I and group II pre-let-7 substrates (Supplementary Fig. 1a).

We identified construct mT5 (composed of NTD2 and three ZK domains) as the minimal domain tested that catalyzed monoU addition (Supplementary Fig. 1f). We refer to mT5 as the catalytic module (CM), as it was also indispensable for Lin28-dependent oligoU activity (Supplementary Fig. 1g). In addition, oligoU activity required the ZF and NTD1 domains, which we therefore term the Lin28-interacting module (LIM). The LIM was inactive on its own, yet together with the CM it facilitated processive oligoU addition (Supplementary Fig. 1g). Deletion up to and including the ZF in the LIM abrogated Lin28-dependent oligoU addition, as observed previously. A construct with ZK2 deleted (mT4) catalyzed processive Lin28-dependent oligoU addition but consistently added shorter oligoU tails (oligoUshort, with up to ten uridines) to pre-let-7 compared with construct mT1 (∼30 uridines), suggesting a role for ZK2 in oligoU extension (Supplementary Fig. 1g). Overall, our data are consistent with a previous domain-mapping study of TUT4(7)46. However, that study reported no activity for ZK2 truncation mutants, in contrast to our oligoUshort phenotype. This discrepancy could be due to a difference in the constructs used or the expression system.

Next, we incubated mT1 with pre-let-7g and analyzed the complex by gel-filtration chromatography (GF). The mT1–pre-let-7g complex was transient, as the two components eluted separately by GF, consistent with distributive monoU addition in the absence of Lin28 (Fig. 1c). We hypothesized that Lin28 would stabilize the interaction between TUT4 and pre-let-7 RNA to switch on processive oligoU activity. Indeed, Lin28 compelled the stable association of TUT4 with pre-let-7g so that all three components eluted as a ternary complex (Fig. 1c). Given that the LIM is essential for oligoU activity, we next asked whether the LIM is in communication with Lin28 during ternary complex formation. Indeed, construct mT2 (Fig. 1a, encompassing only the LIM, while not catalytically active, formed a ternary complex with Lin28–pre-let-7g (Fig. 1d)).

Pre-let-7 family members are highly conserved in the double-stranded stem but slightly more divergent in the terminal loop. However, a conserved GGAG element within the terminal loop of pre-let-7 is essential for the oligoU activity switch of TUT4(7).13 Pre-let-7 family members are highly conserved in the double-stranded stem but slightly more divergent in the terminal loop. However, a conserved GGAG element within the terminal loop of pre-let-7 is essential for the oligoU activity switch of TUT4(7).13
Figure 2 Structure of human TUT7 CM in the monoU-addition state. 
(a) Domain schematic of human TUT7 with LIM and CM indicated. The CCHH zinc finger (pink), inactive NTD1 (light purple), disordered linker, three CCHC zinc knuckles (ZK1–ZK3, purple), and the catalytic NTD2 (green) are labeled. (b) Schematic of the truncated CM2 construct used for crystallization of CM–dsRNA, with the N lobe (light green) and C lobe (gray) labeled. (c) Structure of CM–dsRNA representing the monoU-addition state. The N and C lobes are labeled as in b. The RNA duplex (purple and orange strands) rests against the 5′ anchor (dark green) and the groove loop (blue). The 1-nt U overhang (U15, orange strand) sits in the −1 position and is stacked with the incoming UTP nucleotide (yellow sticks) in the +1 position. During monoU addition, ZK2 is displaced by the 5′ strand (purple) of the RNA duplex.

Figure 3 Structure of human TUT7 CM in the oligoU-addition state. 
(a) Schematic of the truncated CM1 construct used for crystallization of CM–U2, with the N lobe (light green) and C lobe (gray) labeled. (b) Structure of CM–U2 trapped in the oligoU state. ZK2 (purple ribbon) coordinates Zn2⁺ (lime green sphere) and is engaged with U1 in the −2 position. Incoming nucleotide (UMPNPP, yellow sticks) sits in the +1 position and is poised for the next uridylation cycle. (c) Superposition of a single chain of CM–apo (light blue cartoon) onto CM–U2 (purple cartoon) and CM–dsRNA (orange cartoon). The movement of the N lobe in the presence of RNA substrates is highlighted (gray shade). (d) Close-up view of the N-lobe movement induced upon substrate binding. The degree of movement is illustrated by the displacement of Val1104 (sticks) by ~2.7 Å going from the open to the closed conformation.

Structure of the CM in the monoU state

Since the CM is essential for both modes of TUT4(7) activity, we set out to understand the molecular underpinnings of monoU addition and oligoU addition. We reasoned that a structure of the CM in complex with a group II pre-let-7 double-helical stem would effectively represent monoU addition. To this end, we identified two truncated forms of the human TUT7 CM suitable for crystallization (CM1, residues 963–1,365; and CM2, residues 983–1,365). CM2 begins at NTD2 (Fig. 2b), whereas CM1 includes ZK1 (Fig. 3a). Crystals for both constructs diffracted X-rays to comparable resolution limits. Overall, human TUT7 and mouse TUT4 share 48% sequence identity, whereas the CMs are very similar, with 69% sequence identity. We concluded that our structural interpretations of the TUT7 CM would likely be conserved for TUT4. We determined the structure of CM1 by SAD phasing of a selenomethionine derivative to 3.0-Å resolution, followed by molecular replacement (MR) phasing of native data to 2.6-Å (CM–apo; Table 1 and Supplementary Fig. 3a). Next, we co-crystallized CM2 (D1060A active site mutant) in complex with a 14-bp palindromic RNA duplex and UTP nucleotide (CM–dsRNA; Fig. 2) and determined its structure by MR using CM–apo as a search model (Table 1).

Two CMs were observed at each end of the dsRNA, but we considered only one CM, along with the dsRNA duplex, to represent the relevant structure for monoU addition (Supplementary Fig. 3b,c). The dsRNA is a mimic of the duplex stem of group II pre-let-7 (Supplementary Fig. 1d), as it contains a 1-nt 3′-end U overhang. We observed electron density accounting for most of NTD2, but curiously, no density was observed for ZK2 (residues 1337–1365). NTD2 is bilobal; the N-terminal lobe (N lobe; residues 987–1124) consists of a mixed five-stranded β-sheet wrapped tightly by two α-helices, while the C-terminal lobe (C lobe; residues 1125–1336) is made up of six α-helices joined by several long loops (Fig. 2c). The overall fold of NTD2 resembles the determined structures of single-domain TUTases (including S. pombe CID1 (refs. 24–27) and Trypanosoma brucei TUT1 (TUT1, ref. 22), TUT4 (ref. 19), RET2 (ref. 23) and MEAT1 (ref. 20)) and noncanonical poly(A) polymerases (including GLD-2...
entire ZK2 domain (residues 1337–1363; unlike in the CM–dsRNA complex, we observed clear density for the lobes of NTD2, whose conformation is similar to the closed conformation observed in CM–dsRNA. However, when we compared the CM–U2 and CM–dsRNA structures to CM–apo, we noticed a modest conformational change observed in the substrate U nucleotide in the −2 position (Fig. 3b). Both the CM–dsRNA and CM–U2 structures represent the precatalytic state before oligoU addition, with an incoming nucleotide occupying the +1 position (Figs. 2c and 3b).

Structure of the CM in the oligoU state

Next we set out to better understand the role of the CM during Lin28-dependent oligoU addition. We imagined the CM in complex with nucleotide and oligoU substrate would suitably mimic the oligoU-dependent oligoU addition. We determined the structure of CM1 in complex with a 2-nt oligoU RNA substrate and a nonhydrolyzable UTP analog, UMPNP (CM–U2; Table 1, Fig. 3a,b and Supplementary Fig. 5a,b). We did not observe density for ZK1 (residues 963–984); however, unlike in the CM–dsRNA complex, we observed clear density for the entire ZK2 domain (residues 1337–1363; Fig. 3b).

The fold of NTD2 in CM–U2 was identical to that observed in the substrate U nucleotide in the −2 position (Fig. 3b). The U5 sits in the +1 nucleotide-binding site with the remaining nucleotides occupying the −1, −2 and −3 positions. In both the pre- and postcatalytic states, ZK2 remains engaged with the U in the −2 position (Fig. 4a,b and Supplementary Fig. 5e). We hypothesize that ZK2 might aid translocation of the oligoU tail (Fig. 4c). Earlier biochemical studies of TUT4 have shown that ZK2 is essential for catalysis56. However, our activity data showed that ZK2 mutants still maintain monoU and processive oligoU addition activities (Supplementary Figs. 1 and 6), albeit with oligoU_port as product in the presence of Lin28. These data support a role for ZK2 in oligoU extension, perhaps stabilizing the oligoU product or aiding oligoU synthesis.

Table 1 Data collection and refinement statistics

|                   | SeMet | CM–apo (PDB 5WOB) | CM–U2 (5W0N) | CM–U3 (5W0M) | CM–dsRNA (5WDD) |
|-------------------|-------|-------------------|--------------|--------------|-----------------|
| **Data collection** |       |                   |              |              |                 |
| Space group       | P61   | P61               | P61          | P61          | P21,2,2,1       |
| Cell dimensions   | a, b, c (Å) | 135, 135, 179   | 141, 141, 174 | 136, 136, 182 | 135, 135, 179  |
| α, β, γ (°)       | 90, 90, 120 | 90, 90, 120     | 90, 90, 120  | 90, 90, 120   | 90, 90, 90     |
| Resolution (Å)    | 178–3.0 (3.03–3.0) | 100–2.6 (2.62–2.6) | 99–2.5 (2.51–2.5) | 179–2.3 (2.31–2.3) | 90–2.5 (2.51–2.5) |
| Rmerge (%)        | 9.4 (111) | 4.6 (59.7)       | 6.3 (63.7)   | 4.9 (55.6)    | 4.9 (73.4)      |
| Rfre(%)/Rmerge    | 22.8 (2.2) | 32.8 (2.1)       | 15.5 (2.1)   | 22.7 (2.1)    | 19.3 (2.1)      |
| CC1/2             | 0.998 (0.825) | 1.00 (0.828)    | 0.998 (0.872) | 0.999 (0.784) | 0.999 (0.733)  |
| Completeness (%)  | 100 (99.7) | 100 (98.4)      | 99.6 (99.9)  | 99.9 (99.6)   | 99.5 (100)      |
| Refinement        |       |                   |              |              |                 |
| Resolution (Å)    | 100–2.6 | 99–2.5            | 179–2.3      | 91–2.5       |
| No. reflections   | 59,474 | 65,534            | 82,606       | 35,149       |
| Rmerge / Rfree    | 0.232 / 0.255 | 0.187 / 0.215     | 0.188 / 0.229 | 0.205 / 0.256 |
| No. atoms         | Macromolecules | 8,361           | 8,936        | 9,094        | 5,977           |
|                   | Ligands       | 39              | 211          | 26           | 58              |
|                   | Water         | 22              | 218          | 205          | 50              |
| B factors         | Macromolecules | 86.3           | 63.2         | 59.9         | 78.9            |
|                   | Ligands       | 103.7           | 53.8         | 61.4         | 62.5            |
|                   | Water         | 59.9            | 51.0         | 52.3         | 61.8            |
| R.m.s. deviations | Bond lengths (Å) | 0.003          | 0.003        | 0.005        | 0.006           |
|                   | Bond angles (°) | 0.61           | 0.56         | 0.68         | 0.84            |

Values in parentheses are for the highest-resolution shell.
translocation. We also find it plausible that ZK1 and ZK3 could act redundantly with ZK2 to aid oligoU addition to pre-let-7 (refs. 13,14) and perhaps other RNA substrates11.

**ZK2 is displaced by pre-let-7 stem during monoU addition**

We compared the structures representing monoU addition and oligoU addition. In the −1 and −2 positions, the oligoU in CM–U2 tracks along the same path as the 3′ strand in CM–dsRNA, but U2 in the −3 position takes an alternative pathway (Supplementary Fig. 7a). The deviation in the path taken by oligoU compared to the 3′ strand of dsRNA is due to the engagement of ZK2 (Supplementary Fig. 7a,b). When ZK2 is engaged in the oligoU activity state, it occupies the same position as the first −3 nt of the 5′ strand of dsRNA in the monoU activity state, which forces oligoU to diverge at the −3 position (Supplementary Fig. 7a,b). Conversely, in CM–dsRNA, since ZK2 is displaced by the 5′ strand of the RNA duplex (Fig. 4d,e), the nucleotide in the −2 position (C14) is stabilized by a base pair with G1 (double arrow). ZK2 is displaced by the 5′ strand of dsRNA.

**TUT4(7) active site and conserved UTP selectivity**

Next we evaluated the substrate interactions occurring in the oligoU state of the CM (Fig. 5). In CM–U2, the incoming UMPNPP base is wedged between Tyr1171, the uracil base of U2, and Val1104. These interactions help determine substrate specificity in *T. brucei* TUT4 (ref. 19) and likely contribute to specificity for UTP as the incoming nucleotide in the CM as well (Fig. 6). UTP specificity is also facilitated through U-specific interactions with C-lobe residues Asn1130 and His1286, and through a water-mediated interaction with Asp1280. The role of His1286 in UTP selection is conserved in *S. pombe* CID1 (refs. 24–26), but this residue is not conserved in *T. brucei* TUTases (Fig. 6). Otherwise, the UTP binding site (+1 position) and the catalytic site are remarkably conserved between the TUT7 CM and other TUTases (Fig. 6), despite overall low sequence identity (~20%). Although not involved in nucleotide selection, C-lobe (Lys1152, Ser1170) and N-lobe (Ser1047, Ser1057) residues stabilize the γ-phosphate of UMPNPP. The catalytic Mg2+–binding residues Asp1058 and Asp1060 coordinate a single Mg2+, which further stabilizes the β- and γ-phosphates of UMPNPP with the help of a water. Asp1119 completes the active site, where the α-phosphate of incoming UTP is positioned for the uridyl transfer (Figs. 5 and 6).

U specificity is not unique to the +1 position, but is also evident in the substrate RNA binding pocket. The N-lobe residue Asn1124 makes a U-specific interaction with U2. This asparagine is conserved and essential for CID1 activity22 (Fig. 6a). Furthermore, residues His1355 and Lys1352 of ZK2, which engage only in the oligoU state, make U-specific interactions with U1 in the −2 position (Fig. 5). These interactions are not evident in any of the other TUTase structures, which all lack ZK domains (Fig. 5). The TUT4(7) CM is elegantly designed to catalyze oligoU addition, which is exemplified by U-specific interactions with UTP, the 3′-terminal nucleotide and the penultimate nucleotide by ZK2.

**The 5′ anchor orients the pre-let-7 duplex**

UTP binding and selection is accomplished in an identical manner in the monoU-addition structure, CM–dsRNA (Supplementary Fig. 7c,d). However, since ZK2 is displaced and unavailable to stabilize the nucleotide in the −2 position, this role is taken over by the 5′ strand of the duplex (Fig. 4e and Supplementary Fig. 7d). We found that the 5′ anchor is crucial for positioning of the dsRNA hairpin stem that results in
precise placement of U15 in the active site (Fig. 7a). The 5′ anchor residues Leu1097 and Ile1099 create a hydrophobic platform for the first base pair of the duplex, C14–G1, while Val1104 positions the U15 base. The hydrophobic platform is conserved in mammalian TUT4(7) but quite divergent in CID1 and T. brucei TUTs. Additionally, Thr1101 forms a hydrogen bond with C14, and Lys1103 connects the bridging phosphate of C12 and G13 (Fig. 7a). Opposite the 5′ anchor, the groove loop from the C lobe stabilizes the minor groove of the dsRNA duplex primarily through van der Waals forces with Ala1163, Ser1164 and Arg1165 as well as a hydrogen bond with Ser1164 (Supplementary Fig. 7e). The interactions with the duplex stem are non-sequence-specific and are consistent with TUT4(7)′s ability to monouridylate multiple pre-let-7 family members as well as other group II pre-miRNAs18. Interestingly, the 5′ anchor is also involved in oligoU substrate binding, as Val1104 interacts with the −1 nucleotide base regardless of whether the substrate is dsRNA or single-stranded RNA (ssRNA; Fig. 5). In addition, the hydrophobic anchor residue Ile1099 stabilizes the base of the −2 nucleotide for both dsRNA and ssRNA substrates, while L1097 engages only the 5′ strand of the dsRNA duplex (Fig. 7a).

Group II pre-let-7 RNAs must be repaired by TUT4(7) monoU addition before their biogenesis. We asked how TUT4(7) might discriminate between group I (2-nt overhang; Supplementary Fig. 1c) and group II (1-nt overhang; Supplementary Fig. 1b) pre-let-7 substrates. First, TUT4(7) must ensure precise positioning of group II pre-let-7 substrates in the precatalytic state, with UTP in the +1 position so that monoU addition can proceed (Fig. 7b). On the basis of the CM–dsRNA structure, we hypothesized that the CM of TUT4(7) measures the end structure of group II substrates through the hydrophobic platform of the 5′ anchor. To validate the CM–dsRNA structure, we mutated the hydrophobic platform of full-length human TUT7 to make it more bulky (L1097W I1099W) and measured miRNAs are measured by the 5′ anchor and groove loop. From the 5′ anchor, Val1104, Thr1101 and the hydrophobic platform (Leu1097, Ile1099) make direct contact with the dsRNA (green sticks). The C14–G1 base pair of dsRNA is stabilized by the hydrophobic platform. (b) Schematic comparison of the recognition of group II and group I pre-let-7 substrates during monoU addition. (c,d) MonoU addition assay time courses conducted with full-length human TUT7 (hTUT7). WT, wild-type hTUT7 (c). Bulky substitutions within the hydrophobic platform (L1097W I1099W; d) severely disturb monoU addition. Gels shown are representative of three technical replicates. Uncropped gel images are shown in Supplementary Data Set 1.

**DISCUSSION**

Our study is the first to describe a structural model of the two catalytic modes of mammalian TUT4(7) (Fig. 8). We find that group II pre-miRNAs bind TUT4(7) in the precatalytic state, which is favored for monoU addition (Fig. 8a). We propose that the transient interaction between TUT4(7) and pre-let-7 favors the addition and release of the RNA substrate before oligouridylation can occur. In contrast, group I pre-miRNAs bind in the inactive postcatalytic state and are released before uridylation (Fig. 7b). All the while, the double-stranded helical stem of pre-let-7 prevents ZK2 engagement.

Lin28 controls the ‘oligoU switch’ by recruiting the LIM of TUT4(7) to the GGAG motif within the terminal loop of pre-let-7 (Fig. 8b). The stable ternary complex supports processive oligoU addition by the
Notably, Lin28 ZK mutants can bind pre-let-7 but not recruit TUT4(7). Since Lin28 induces a stable long-lived ternary complex, Lin28 dramatically increases the dwell time of TUT4 on pre-let-7 oligoU addition is also supported by single-molecule studies, in which ZKLin28–GGAG complex creates a unique binding surface for the LIM in our experiments) as the interaction partner for the Lin28–binary complex to initiate oligoU addition. Lin28 and TUT4(7) do not interact without pre-let-7. It is tempting to speculate that the LIM in complex with Lin28–pre-let-7 would shed crucial light on these details.

In summary, this study takes the first steps in distinguishing the mechanism of the two catalytic modes of TUT4(7). We expect this approach will provide a blueprint for the design of therapeutic compounds to target TUT4(7) activity, in particular Lin28-dependent oligoU activity in human cancers.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.R.F., J.W. and L.J. designed all experiments. C.R.F. and J.W. conducted all experiments. All authors contributed to data analysis and wrote the paper.

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The authors declare no competing financial interests.

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ONLINE METHODS
Protein expression and purification. Plasmids containing the ORF of mouse TUT4 (mTUT4) and human TUT7 (hTUT7) were kind gifts from Richard J. Gregory and V. Narry Kim, respectively. TUT4(7) constructs were expressed in S09 or High Five insect cells as N-terminal Strep-sumo-TEV fusion proteins from the pFL vector of the MultiBac baculovirus expression system. Insect cells were infected with baculovirus at 27 °C for 60 h of protein expression, after which the cells were centrifuged at 1,200 r.p.m. and suspended in Wash buffer (50 mM Tris pH 8, 100 mM NaCl and 5 mM DTT), flash frozen in liquid nitrogen and stored at −80 °C. Cells were thawed, then the NaCl concentration was increased to 500 mM, and the cells were lysed by sonication. The lysate was treated with 0.2% polyethylene imine (PEI) to precipitate bulk nucleic acid before ultrafiltracritigation at 35,000 r.p.m. at 4 °C for 1 h. The soluble fraction was incubated with 1 mL of Strep-Tactic superflow resin per 10 mL of lysate for 1 h on a rolling shaker. The resin was applied to a gravity flow column and washed extensively with Wash buffer. The protein was eluted with Wash buffer containing 2 mM desthiobiotin. The eluted fraction was treated with TEV protease (1:100 TEV/protein ratio) and incubated overnight at 4 °C. The cleavage efficiency and purity was verified by SDS-PAGE. TEV protease treated protein was diluted with an equal volume of MonoS buffer A (25 mM Hepes pH 7.5, 5 mM DTT) to a final NaCl concentration of 50 mM. TUT4(7) proteins were loaded onto a Poros H5 or HiTrap SP HP cation exchange column equilibrated with 25 mM Hepes pH 7.5, 50 mM NaCl and 5 mM DTT. A linear gradient between 0.05 M and 1 M NaCl was used to elute TUT4(7). Fractions that contained TUT4(7) were analyzed by SDS-PAGE, pooled and concentrated and loaded onto either a HiLoad 16/60 Superdex 200 or Superdex 200 10/300 gel filtration column equilibrated in 20 mM Hepes pH 7.5, 150 mM NaCl, and 5 mM DTT. TUT4(7) was concentrated to 15 mg/mL flash frozen in liquid nitrogen and stored at −80 °C. Selenomethionine (SeMet) substituted human TUT7 constructs were expressed in High-five cells infected with baculovirus in ESF921 media (Expression Systems) for 6 h, followed by media exchange to a methionine-free media. After growth for an additional 4 h, SeMet was added to a final concentration of 198 mg/L. The cells were harvested after 48 h. SeMet-substituted TUT7 was purified in the same manner as the native protein. All mutant proteins (mouse TUT4 and human TUT7) used in this study were expressed and purified in an identical manner as the wild-type proteins, and exhibited similar size exclusion chromatography profiles, indicative of proper folding.

Mouse Lin28 (mLin28) was cloned into the pET28 vector and expressed as a N-terminal His6-SUMO fusion protein. mLin28 was expressed in BL21-Ripl (DE3) cells grown in TB media. Cells were grown to an OD > 1, followed by addition of 100 μM zinc chloride, then expression was induced with 0.1 mM IPTG for 3 h at 37 °C. Cell pellets were collected by centrifugation at 18,000 r.p.m., and suspended in Wash buffer (50 mM Tris pH 8.0, 400 mM NaCl, 20 mM imidazole, and 2 mM β-mercaptoethanol). mLin28 was purified by Ni2+-affinity chromatography, followed by elution with Wash buffer containing 250 mM imidazole. Eluted mLin28 fractions were treated with Ulp1 protease to remove the SUMO tag and diluted into ion exchange buffer A (25 mM Hepes pH 7.5 and 5 mM DTT) to a final NaCl concentration of 200 mM. mLin28 was contaminated with substantial amounts of nucleic acids, which were removed by passage through a HiTrap Q HP column. The flow-through fraction (nucleic acid-free mLin28) was loaded onto a HiTrap SP HP column and eluted with a linear gradient from 0.2 M to 1 M NaCl. mLin28 fractions were loaded onto a HiLoad Superdex 75 16/60 gel filtration column equilibrated in 20 mM Hepes pH 7.5, 150 mM NaCl, and 5 mM DTT, and then flash frozen in liquid nitrogen for storage at −80 °C.

Structure determination of human TUT7 CM complexes. We identified two hTUT7 catalytic module constructs (CM1 and CM2) suitable for crystallization by limited proteolysis with thermolysin. CM1 is composed of zinc knuckle-1 (ZK1), nucleotidyltransferase domain-2 (NTD2), and zinc knuckle-2 (ZK2) (residues 963–1365), while CM2 lacks ZK1 (residues 983–1365). Both CM constructs were crystallized at 18 °C by sitting drop vapor diffusion by mixing protein (12 mg/ml concentration) 1:1 with well solution (0.8 to 1 M lithium sulfate, 75 to 200 mM sodium citrate pH 5–6, and 50 to 200 mM potassium iodide) using a mosquito’ Crystal liquid handling robot (TTP Labtech). Crystals were cryoprotected by increasing the lithium sulfate concentration to 80% to 90% saturation, and flash cooled in liquid nitrogen. A SeMet derivative of CM1 was crystallized and flash cooled in an identical manner. Crystals were also obtained in similar conditions for both wild type and active site mutant CMs (D1060A) by co-crystallization with 2 mM UTP, 2 mM UMPNPP and the dinucleotide U2, and 1 mM U3 RNA. Initial phases were derived from a SAD data set collected on a SeMet substituted crystal at beamline X25 at the NSLS at Brookhaven National Laboratory to 3.0 Å resolution (λ = 0.979 Å). Se site determination, phasing, and automatic model building were performed with the SAD phasing module as implemented in Phenix39. The SAD phased map was of excellent quality, which allowed the AutoBuild42 utility in Phenix to build a near complete atomic model. The partial model was subsequently used for molecular replacement (MR) phasing of a native data set collected at the Zn2+ absorption edge (λ = 1.28 Å) on apo CM crystals to 2.6 Å resolution (CM–apo). The CM–apo structure contained three copies of the CM in the asymmetric unit. We did not observe density for ZK1, due to disorder in the region, but a nearly complete model was built for the NTD2 domain (residues 986–1335) in all three copies. In addition, we modeled ZK2 into ordered density for one copy (chain A, residues 1336–1361), but observed disorder in the remaining copies, so we excluded ZK2 from chains B and C in the final refined model. To confirm the presence and location of the Zn2+ coordinated by the CCHC zinc-binding motif within ZK2, we calculated an anomalous difference map, which clearly identified the presence and location of Zn2+ (Supplementary Fig. 3a).

Next, we examined data sets collected from crystals co-crystallized with nucleotides and/or substrate RNA. Upon inspection of the electron density maps it was evident that sulfate and iodide ions occupied the nucleotide and substrate binding sites in place of the desired nucleotide or RNA. We assumed that high lithium sulfate and iodide concentrations are detrimental to substrate binding. We therefore devised an alternative strategy to lower the ionic strength of the crystal mother liquor before soaking of substrate.

Apo hLin28 CM1 or CM2 crystals were transferred to a stabilizing cryo-solution consisting of 20% PEG 3350, 30–30% glucose, 150 mM sodium citrate pH 6, and 100 mM lithium sulfate. Next, nucleotide and/or RNA substrate was introduced (at concentrations of 5 mM UTP or nonhydrolyzable analog UMPNPP, 1 mM U2 or U3 RNA) to the drop for 1.5 to 24 h before cryo-cooling in liquid nitrogen. Complete X-ray diffraction data sets were collected for CM–U2 (apo CM1 crystals soaked in 2 mM MgCl2, 2 mM UMPNPP, 1 mM U2) at the Advanced Photon Source (Beamline 19ID, λ = 0.979 Å) and CM–U3 (apo CM2, D1060A active site mutant crystals soaked in 1 mM U3) at the Advanced Light Source (Beamline 8.2.2, λ = 1.0 Å). Data sets were processed with XDS50 as implemented in the AutoPROC59 software package. The unit cell, space group, and crystal packing arrangement were identical to that observed for CM–apo. Phases were determined by molecular replacement (MR) using the CM–apo structure as a search model in Phaser43. Model refinement was performed with Phenix and manual correction to the models was performed with Coot44. Difference electron density maps clearly indicated the unambiguous presence of substrate for both CM–U2 and CM–U3. Final refinement statistics are presented in Table 1.

To determine the structure of the CM–dsRNA complex we incubated CM2 (D1060A active site mutant) with a 14 bp palindromic RNA duplex with a 1 nt overhang at a 1:1 molar ratio and 5 mM UTP. The complex crystallized at 10 mg/ml using sitting drop vapor diffusion in a well solution of 200 mM ammonium acetate, 100 mM sodium citrate pH 5.5, and 24% PEG 400. Crystals were harvested and flash-cooled directly from the mother liquor. Diffraction data were collected to 2.50 Å resolution at the Advanced Photon Source (Beamline 19ID). Data were processed with XDS as implemented in the AutoPROC software package and phases determined by MR using the CM–apo structure as a search model in Phaser. Refinement cycles were performed with Refmac56 and Phenix and manual correction to the model was performed with Coot44. The final model consists of two copies of the CM (clear density for residues 987–1068, 1073–1195, 1202–1336) bound to each 1 nt overhang end of the palindromic RNA duplex. Final refinement statistics are presented in Table 1. Throughout this manuscript we describe one CM and the duplex as representative of the monoU state structure. We selected the CM copy that generally displayed more ordered density for further analysis. The geometry of the refined structures reported here were validated with MolProbity45. All structure figures were created with the PyMOL Molecular Graphics System.

Uridylation assay. Pre-let-7g hairpins were in vitro transcribed and purified by denaturing urea PAGE. We used pre-let-7g in all of our biochemical assays to maintain consistency between activity and binding assays. Also, pre-let-7g was previously used to characterize the biochemistry of TUT4(7)46, and the structure...
of the Lin28–pre-let-7g pre-element has been determined47. We have tested other pre-let-7 family members and observed similar results to those reported here, as have others13. Monouridylation time course assays were performed by incubation of purified TUT4(7) constructs (5 mM concentration) with 32P radiolabeled pre-let-7 RNA (100 nM) in U-assay buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM DTT, 3 mM MgCl2) in a final volume of 50 µL. Mixtures were pre-heated to 30 °C followed by addition of 1 mM UTP to trigger the reaction. 5 µL aliquots were removed at the indicated time points and were quenched by dilution into 20 µL of stop buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol) and heated for 10 min at 65 °C. 1 µL of each reaction was resolved by denaturing urea PAGE using 10% sequencing gels. The RNA products were visualized with phosphor imaging.

Oligouridylation time course assays were performed in an identical manner to that described for monouridylation, except that 5′ 32P radiolabeled pre-let-7 RNA (100 nM) was pre-incubated with 150 nM of the indicated Lin28 construct in U-assay buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM DTT, 3 mM MgCl2) in a final volume of 50 µL. Mixtures were pre-heated to 30 °C followed by addition of 5 nM of the indicated TUT4(7) construct. Following an additional 1-min incubation, 1 mM UTP was added to trigger the reaction. Reactions were prepared and resolved in the same manner as described for monouridylation assays.

Gel filtration chromatography assay. To monitor TUT4, Lin28, and pre-let-7 ternary complex formation we resolved the complexes by gel filtration chromatography (GF) on a Superdex 200 10/300 column equilibrated in 25 mM Hepes pH 7.5, 150 mM NaCl, and 5 mM DTT. Combinations of TUT4, Lin28, and pre-let-7 were mixed at concentrations of 20 µM in 100 µL total volume and incubated on ice for 1 h before loading of the entire volume on the column. Control GF experiments were also conducted under identical conditions with Lin28, TUT4 and pre-let-7 alone. All GF experiments were monitored by measuring the absorbance at 280 nm and 260 nm.

Data availability. Coordinates and structure factors for CM–apo (PDB 5W0B), CM–U2 (PDB 5W0N), CM–U2 (PDB 5W0M), and CM–dsRNA (PDB 5W0O) have been deposited in the Protein Data Bank. All other data are available upon reasonable request.

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