Supporting Information

SI Materials and Methods

Protein Purification. Mouse LIN28 protein (residues 16-184) (1), human NTUT7 (202-307), and various TUT4 N-terminal constructs were overexpressed in BL21 (DE3) Rosetta pLysS and purified using Ni-NTA affinity, ion-exchange (SP column) and size exclusion chromatography (Superdex 75), as described previously (1). Full-length mouse TUT4 was cloned in a pFastBac vector and overexpressed in *S*f9 insect cells. Full-length TUT4 was purified from insect cells using Ni-NTA affinity followed by ion-exchange chromatography (SP column). Nanobodies were cloned into a pET26b vector containing an in-frame C-terminal 6xHis tag, overexpressed in BL21 (DE3) cells, and purified using Ni-NTA followed by ion-exchange chromatography (SP column) in Buffer A (20mM Bis-Tris pH 6.0 and 5% glycerol) and Buffer B (20 mM Bis-Tris pH 6.0, 2 M NaCl and 5% glycerol).

Protein Labeling. Purified TUT4 constructs were labeled by biotin or fluoro-488 using EZ-link NHS-PEG4-Biotin (N-hydroxysuccinimide biotin) or Alexa Fluor 488 NHS Ester (N-hydroxysuccinimide ester fluorophores), according to the manufacturer's protocols (ThermoFisher Scientific). The labeled protein was further purified by size exclusion chromatography in a storage buffer (20 mM Bis-Tris pH6.0, 1 M NaCl, 5% Glycerol, 5 mM DTT).

Functional Epitope Nanobody Selection Platform I - MACS Enrichment: Positive Panning to Enrich NTUT$^{282\text{-}759}$ Binders. Nanobodies were selected from a synthetic yeast display nanobody library (2). The first step of selection was to enrich the NTUT$^{282\text{-}759}$ nanobody binders from the yeast display nanobody library (Fig. 2*A*)**.** 10^9 cells of the yeast display nanobody library were incubated with 1 μM biotinylated NTUT$^{282\text{-}759}$ in 5 ml binding buffer (20 mM Bis-Tris PH 7.0, 500 mM NaCl, 5 mM Maltose, 0.1% BSA) for 30 min and then pelleted and washed twice using 5

ml of binding buffer. The bound biotinylated NTUT$^{282-759}$ was further labeled by Alexa-647 conjugated streptavidin for 30 min, followed by pelleting and washing to remove excess streptavidin. Anti-Alexa Fluor 647 MicroBeads (Miltenyi biotec) were then used to pull down the yeast displaying NTUT$^{282-759}$ binders stained by Alexa-647 conjugated streptavidin (R1 pool). 4.9% of the R1 pool was positive for NTUT$^{282-759}$ binding (Fig. 2*B*, Panel 1, UR region). In the second round of MACS enrichment, 100 nM biotinylated NTUT$^{282-759}$ were used to further enrich NTUT$^{282-759}$ binders in the yeast display library (R2 pool). The binders in the R2 pool were significantly improved to ~25% when 100 nM NTUT$^{282-759}$ was added to stain the resulting pool (Fig. 2*B*, Panel 2).

Functional Epitope Nanobody Selection Platform II - FACS Enrichment of High Affinity Nanobodies. The enriched R2 pool was used to further select high affinity binders by sorting with 1 nM of Alexa Fluor 647 labeled NTUT$^{282-759}$ using FACS. In brief, 5 x 10^7 cells from the enriched R2 pool were incubated with 1 nM Fluor 647 labeled NTUT$^{282-759}$ in 1 ml binding buffer at 25 °C. After incubation, cells were washed with ice-cold binding buffer and fluorescently labeled with PE-conjugated anti-HA antibody (Cell Signaling Technology). After 30 minutes of incubation, cells were washed once with binding buffer, suspended in 1 ml of binding buffer, and sorted on a SONY SH800 sorter. Typically 0.03% of the NTUT$^{282-759}$ binding population was gated for collection. Collected cells were grown in SD-CAA media. ~27% of the resulting high affinity pool (R3) was positively stained by 1 nM NTUT$^{282-759}$ (Fig. 2*B*, Panel 3)

Functional Epitope Nanobody Selection Platform III - Functional Epitope Selection. The functional epitope selection was performed to quickly separate nanobodies binding either to the interface (inhibitory binders) or the exposed surfaces (complex binders) of the LIN28:pre-let-7:TUT4 ternary complex. A 3' biotin-labeled pre-let-7 fragment was used to form the LIN28:pre-let-7-biotin binary complex and approximately 200 nM of this binary complex was added to 5 x

10^6 cells from the R3 pool and competed with 200 nM of non-labeled $NTUT^{282-759}$ protein to bind to yeast-displayed nanobodies. Yeast expressing nanobodies that bound to the interface between $NTUT^{282-759}$ and the LIN28:pre-let-7 binary complex were not stained by Alexa Fluor 647 Streptavidin and flowed through the column (inhibitory binders, R5 pool), while yeast displaying nanobodies that bound to the exposed surfaces of the ternary complex containing pre-let-7-biotin were stained by anti-Alexa Fluor 647 magnetic microbeads and retained on the column (complex binders, R4 pool) (Fig. 2*A*). The resultant R5 pool underwent FACS enrichment at a stringency of 10 pM of Alex Fluor 488 labeled $NTUT^{282-759}$ to further identify inhibitory nanobodies with high binding affinity.

Isothermal Titration Calorimetry (ITC). The dissociation constants (K_d) and thermodynamic parameters of nanobody upon binding to the LLI fragment were measured by isothermal titration calorimetry using a MicroCal ITC200 calorimeter at 25°C. Purified nanobodies and protein samples were dialyzed overnight against ITC buffer (300 mM NaCl, 20 mM Bis-Tris pH 6.0, 0.5 mM β-mercaptoethanol) at 4°C overnight. 20 successive injections of 2 µl 200 µM Nb-S2A4 into the sample cell containing 20 µM $NTUT^{282-387}$ were carried out with space of 150 s. The data for Nb-CB11:$NTUT^{282-387}$, Nb-S2A4:$NTUT7^{202-307}$ and Nb-S2A4: $NTUT7^{202-307}$ were collected using the same protocol. Data analysis was performed using the Origin 7.0 software suit.

RNA Electrophoretic Mobility Shift Assay (EMSA). 25 pmol of pre-let-7g RNA was end-labeled with alpha-^{32}P ATP using T4 polynucleotide kinase (New England Biolabs), cleaned up with an Amersham MicroSpin G25 column (GE), and diluted into 100 µl buffer (20 mM Tris pH7.4, 1mM EDTA). 1 µl of ^{32}P labeled RNAs were added into each 20-µl scale reaction. Binding of RNA to the LIN28 protein was carried out in the buffer containing 20 mM Tris pH7.5, 75 mM NaCl, 10 mM DTT, 3 mM $MgCl_2$, 5 µM $ZnCl_2$, 1 mg/ml tRNA, 1 mg/ml BSA, and 10% glycerol at room

temperature for 30 min. Reactions were run on a 10% native polyacrylamide gel in TBE buffer at 100V (4 °C).

***In vitro* TUTase Oligouridylation Assay**. Nanobodies, 0.3 µg full-length TUT4/TUT7 and 1.6 µM LIN28 proteins were incubated with ^{32}P-labeled pre-let-7g at 37 °C in a 15 µl reaction in buffer A (20 mM Tris 7.5, 5% glycerol, 6 mM MgCl2, 6 mM DTT, 50 µM $ZnCl_2$, and 40 mM KCl and 0.14 U/µL RNase inhibitor) for 30 min. 1.2 µl of 10 % SDS and 0.8 µl of 0.5M EDTA were added to stop the reaction and subsequently treated by 1 U protease K (Thermo Scientific) at 50°C for 30 min. Reactions were heated at 100°C for 2 min before electrophoresed on 15% denaturing polyacrylamide gels.

***In vitro* TUTase Pan-uridylation Assay Using Cold RNAs**. Nanobodies and 0.3 µg full-length TUT4/TUT7 were incubated with 10 nM cold pre-let-7g or mRNA and 1 µM α^{32}P-UTP at 37 °C in a 15 µl reaction in buffer A for 30 min. 1.2 µl of 10 % SDS and 0.8 µl of 0.5M EDTA were added to stop the reaction and subsequently treated by 1 U protease K (Thermo Scientific) at 50°C for 30 min. Reactions were heated at 100°C for 2 min before electrophoresed on 12% denaturing polyacrylamide gels. The sequence of SHOC2-A10 is UUUAUUACAGCUCUACCUAGAAAAAAAAAA; the sequence of CALM1-A10 is GCCUUUCAUCUCUAACUGCGAAAAAAAAAA (3).

Cell Lines. HEK-293T and HeLa cells were purchased from ATCC. Cells were transfected with Lipofectamine LTX (Life Technologies). HeLa cells were infected with retrovirus produced from pBABE-puro containing N-FLAG-tagged LIN28B. Levels of mature let-7 were measured by quantitative PCR using miScript microRNA primer assays (QIAGEN).

Reporter Assays. A dual luciferase vector was constructed by cloning a Renilla luciferase-8x-let-7-HSV TK-Firefly luciferase cassette into a modified pLenti CMV GFP Neo (657-2) vector. HEK-293T lines stably expressing pLenti-8x-let-7 dual luciferase were transfected with pCDNA3.1 vectors containing either nanobody Nb-S2A4 or Nb-CB11 for 48 hours. Cells were lysed and dual luciferase activity monitored using Dual Luciferase Assay System (Promega), and results normalized to blank samples.

Quantitative PCR. RNA was isolated from HeLa cell lines transiently transfected with nanobody expression constructs using Trizol reagent (Life Technologies). cDNA was synthesized using the miScript kit (QIAGEN). Levels of nanobody transcripts were measured using the following primers: Nb-BV025 F: AAGAACGCGAACTTGTTGCC; Nb-BV025 R: CAGCCGCGCAATAATACACC; Nb-S2A4 F: AAGAACGCGAACTTGTTGCC; Nb-S2A4 R: CCCAAGCCGCGCAATAATAC.

Cell Cycle Analysis. Cells were trypsinized to a single cell suspension, and fixed and permeabilized with ice-cold ethanol, and incubated on ice for 30 minutes. Cells were then washed twice with PBS and resuspended in propidium iodide/RNase solution (BD Biotechnologies) prior to analysis on an LSRFortessa flow cytometer (BD Biotechnologies).

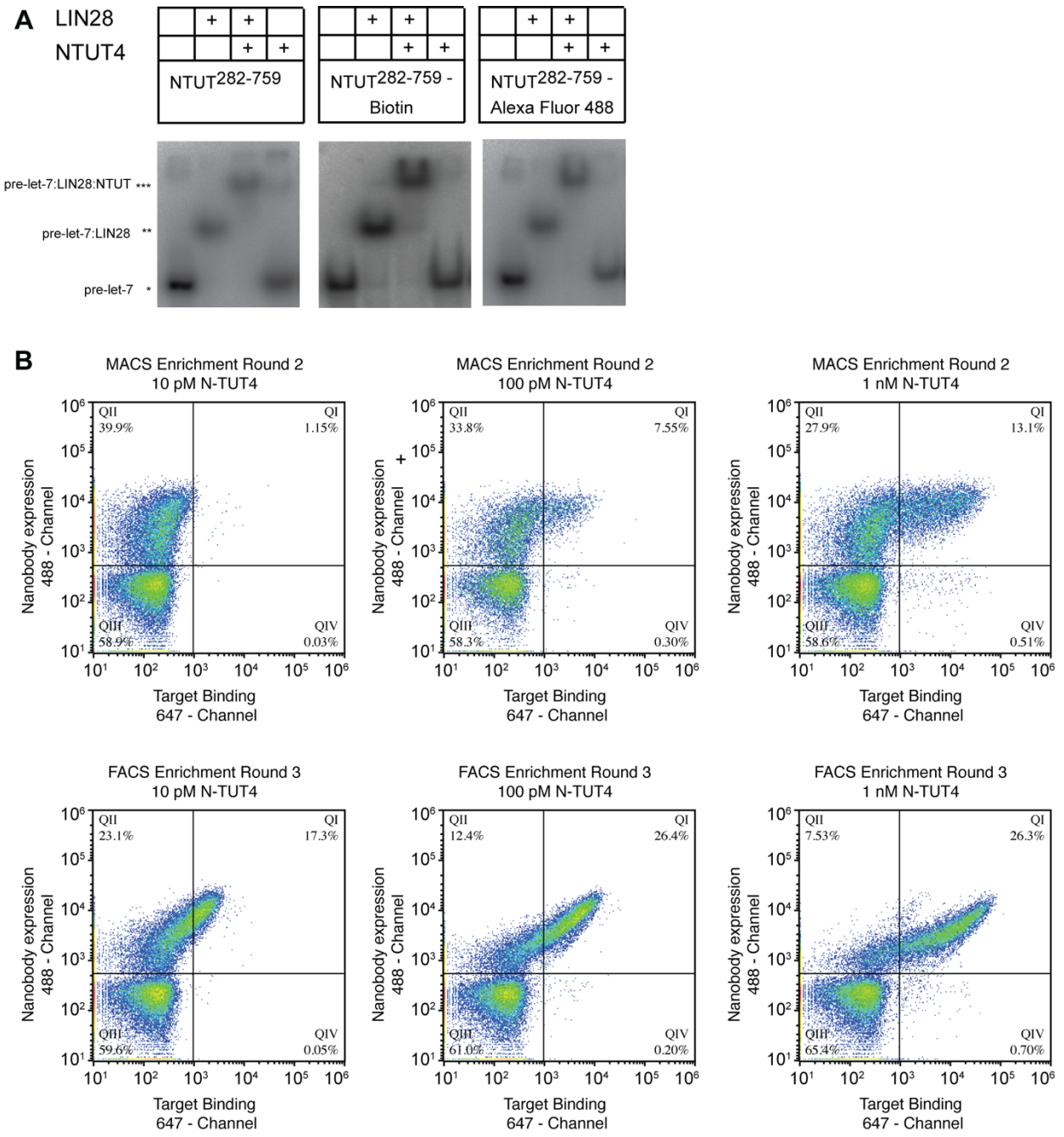

Fig. S1. Functional test of the labeled NTUT$^{282-759}$ and comparison of NTUT$^{282-759}$ binding affinities between R2 and R3 pool. (*A*) Biotin- or Alexa Fluor 488- labeled NTUT$^{282-759}$ retained complete ternary complex formation activity determined by RNA EMSAs. LIN28 (1.25 μM) and NTUT$^{282-759}$ (2 μM), NTUT$^{282-759}$-biotin (2 μM) or NTUT$^{282-759}$-Alexa Fluor 488 (2 μM), were incubated with P^{32} labeled pre-let-7g, respectively. *, free pre-let-7g; **, LIN28:pre-let-7g binary complex; ***, LIN28:pre-let-7g:NTUT4 ternary complex. (*B*) Comparison of NTUT$^{282-759}$ binding affinities between R2 and R3 pool. Nanobodies expressed on the yeast cells were labeled by Alexa Fluor 488 conjugated anti-HA antibodies and biotinylated NTUT$^{282-759}$ bound to the yeast displayed nanobodies was subsequently stained by Alexa Fluor 647 conjugated streptavidin.

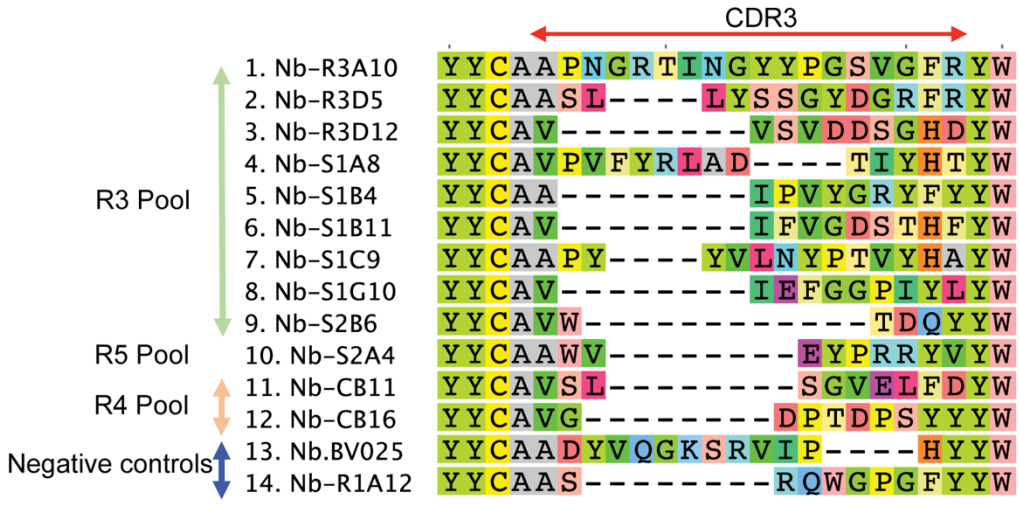

Fig. S2. Alignment of the highly diverse CDR3 regions of nanobodies from the high affinity R3 Pool, the complex binder R4 Pool, the inhibitory binder R5 Pool as well as the negative control Nb.BV.025 and Nb-R1A12.

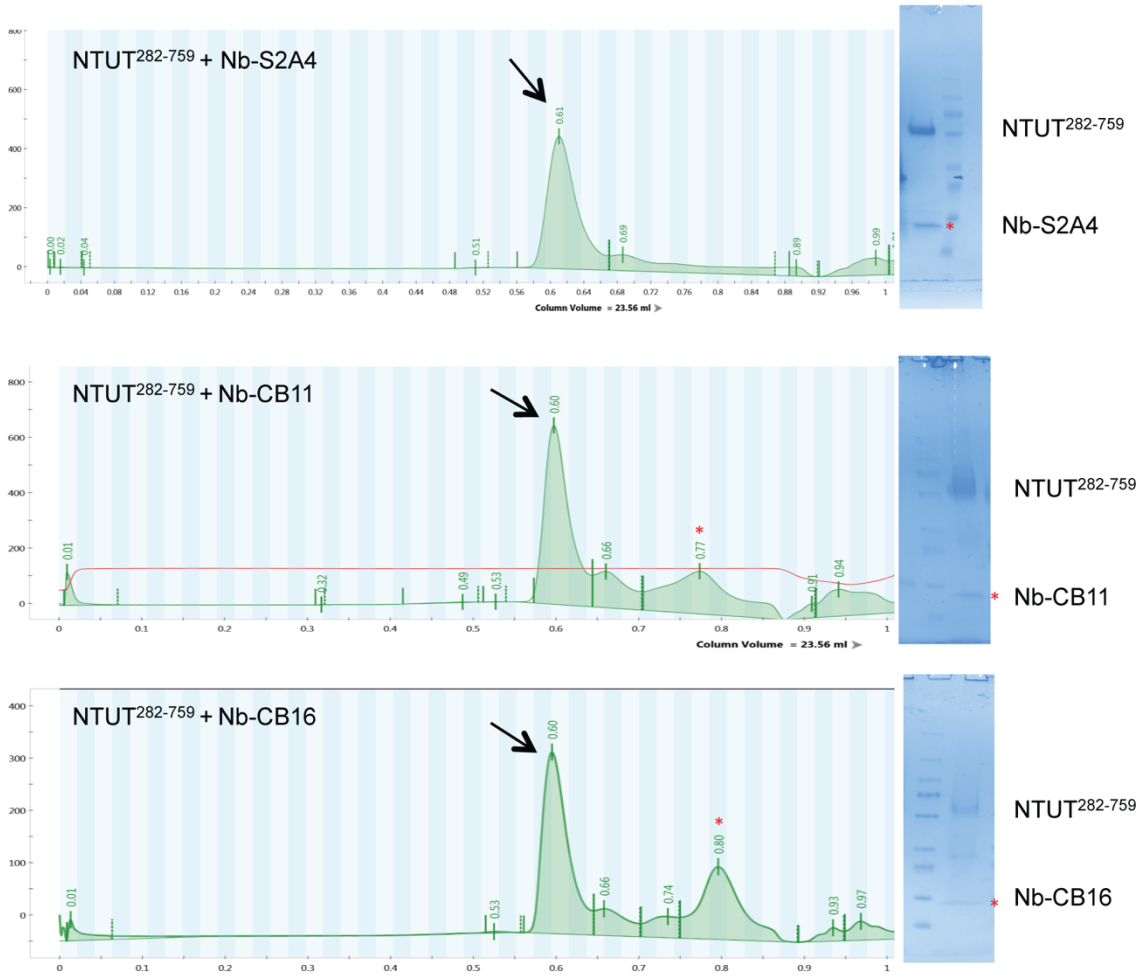

Fig. S3. Nanobodies bound to NTUT$^{282-759}$ and co-eluted from size exclusion chromatogram. Arrows indicated the peak representing the Nanobody:NTUT$^{282-759}$ complex. NTUT$^{282-759}$ and * indicated the nanobody.

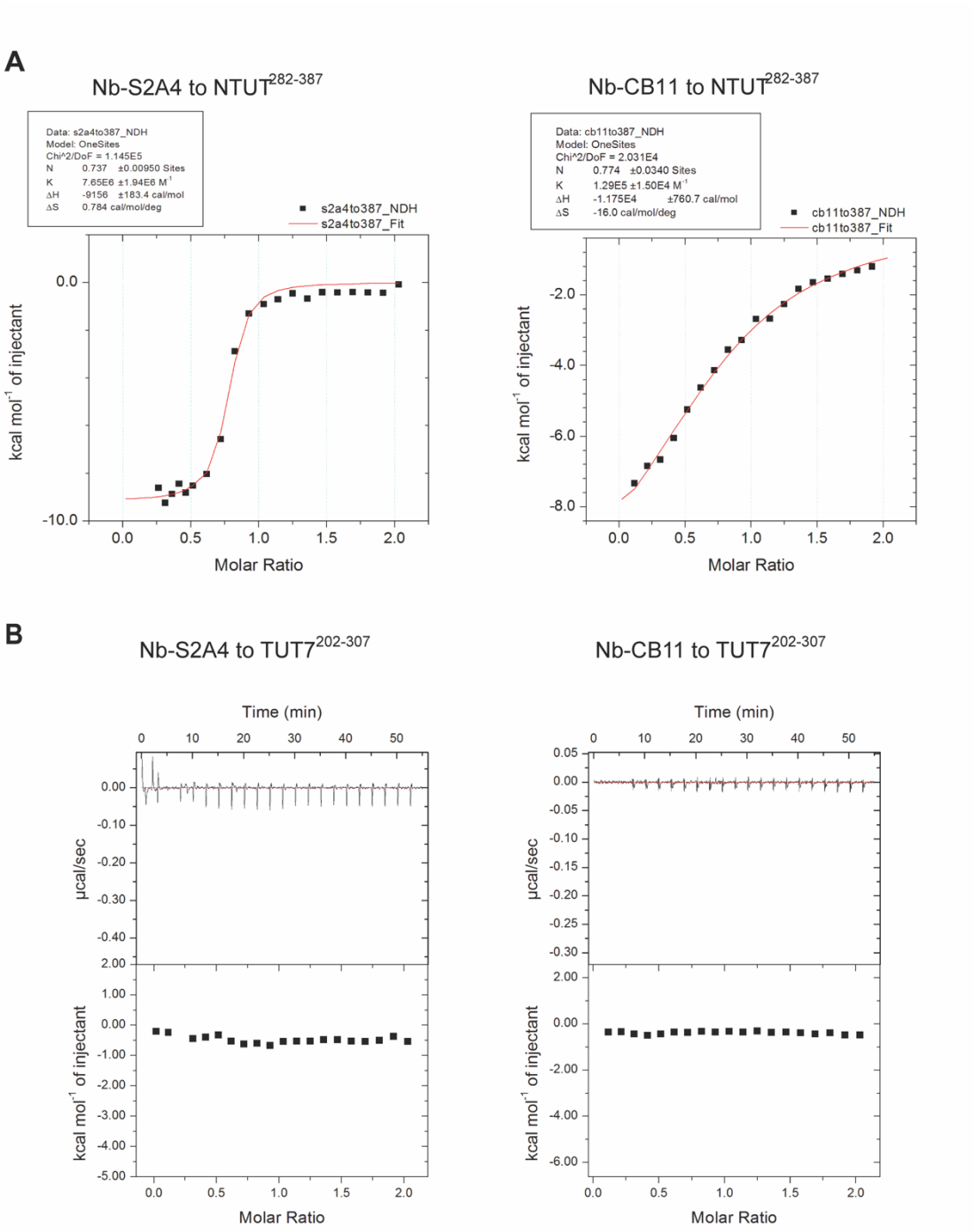

Fig. S4. Isothermal titration calorimetry (ITC) analysis of nanobodies. (*A*) The Fit of ITC data of nanobodies binding to NTUT$^{282-387}$. (*B*) ITC analysis of nanobodies binding to NTUT7$^{202-307}$. Neither Nb-S2A4 or Nb-CB11 binds to NTUT7$^{202-307}$ in this assay.

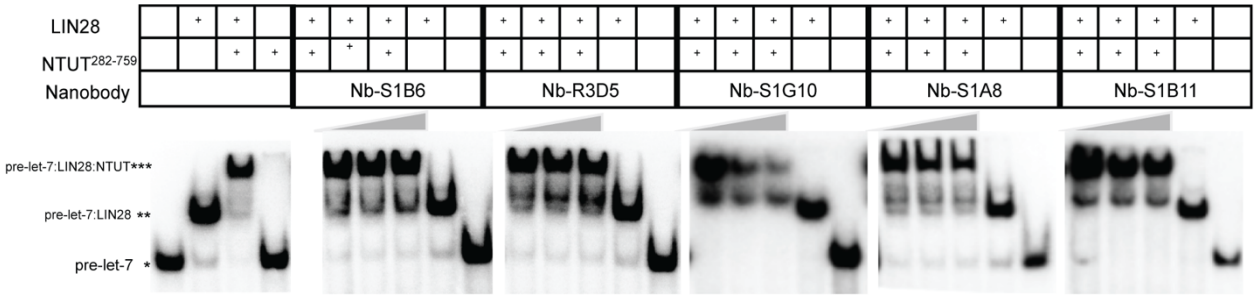

Fig. S5. Inhibitory abilities of nanobodies from R3 pool on blocking the formation of the LIN28:pre-let-7:TUT4 ternary complex determined by RNA EMSA. LIN28 (1.25 μM) and NTUT$^{282-759}$ (2 μM) were incubated with ^{32}P labeled pre-let-7g. Nanobodies were added at 1.5-fold, 3.75-fold, and 7.5-fold molar excess to NTUT$^{282-759}$. *, free pre-let-7g; **, LIN28:pre-let-7g complex; ***, LIN28:pre-let-7g:NTUT4 ternary complex. Nb-R3D5 and Nb-S1G10 partially inhibited the TUT4 recruitment to the LIN28:pre-let-7 binary complex. Nb-S1B6, Nb-S1A8 and Nb-S1B11 did not affect ternary complex formation.

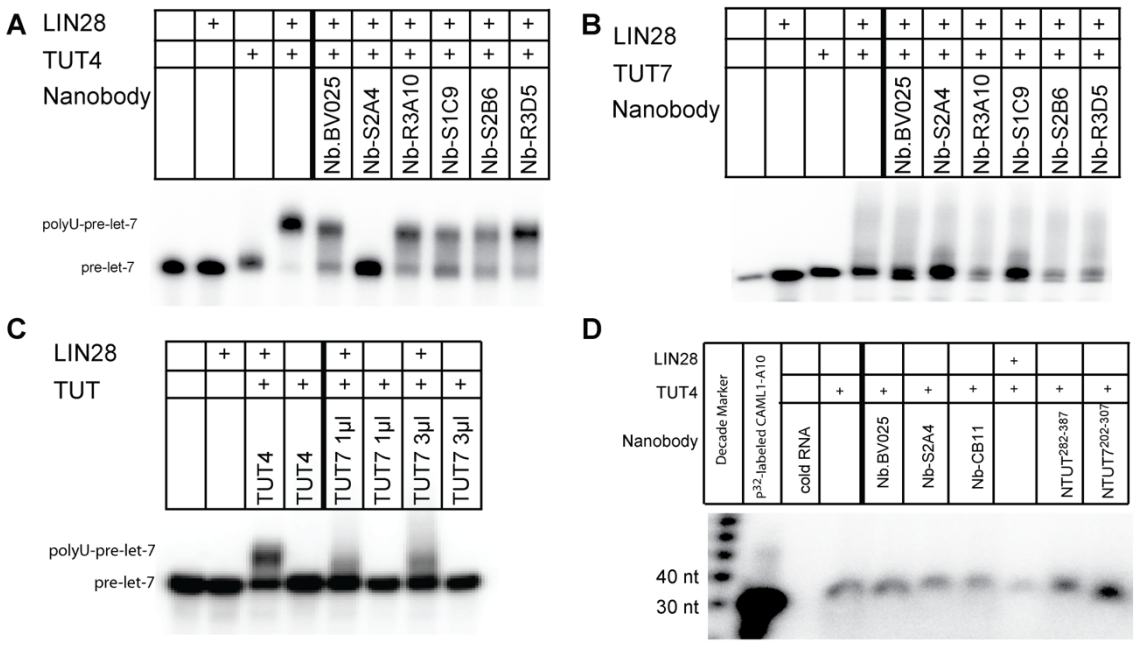

Fig. S6. *In-vitro* TUTase assay carried out by full length TUT4 or TUT7. (*A*) *In-vitro* TUTase assay carried out by TUT4 with ^{32}P labeled pre-let-7g, 1.6 µM LIN28 and 13.3 µM nanobodies. *, oligouridylated pre-let-7g. (*B*) In-vitro TUTase assay carried out by TUT7 with ^{32}P labeled pre-let-7g, 1.6 µM LIN28 and 13.3 µM nanobodies. TUT7 displayed weaker oligouridylation activity (represented as a smear of poly-U tailed RNA) compared to TUT4 did. (*C*) In-vitro monouridylation assay carried out by TUT4 with P^{32} labeled UTP, cold mRNA sample CAML1-A10, 1.6 µM LIN28 and 13.3 µM nanobodies. None of the nanobodies inhibited monouridylation of mRNA SHOCK2-A10 and CAML1-A10. (*D*) *In-vitro* TUTase assay carried out by full length TUT4 and TUT7 with ^{32}P labeled pre-let-7g and 1.6 µM LIN28. *, oligouridylated pre-let-7g. TUT7 displayed weaker oligouridylation activity (represented as a smear of poly-U tailed RNA) compared to TUT4 did. Oligouridylation of pre-let-7g by TUT7 is LIN28 dependent.

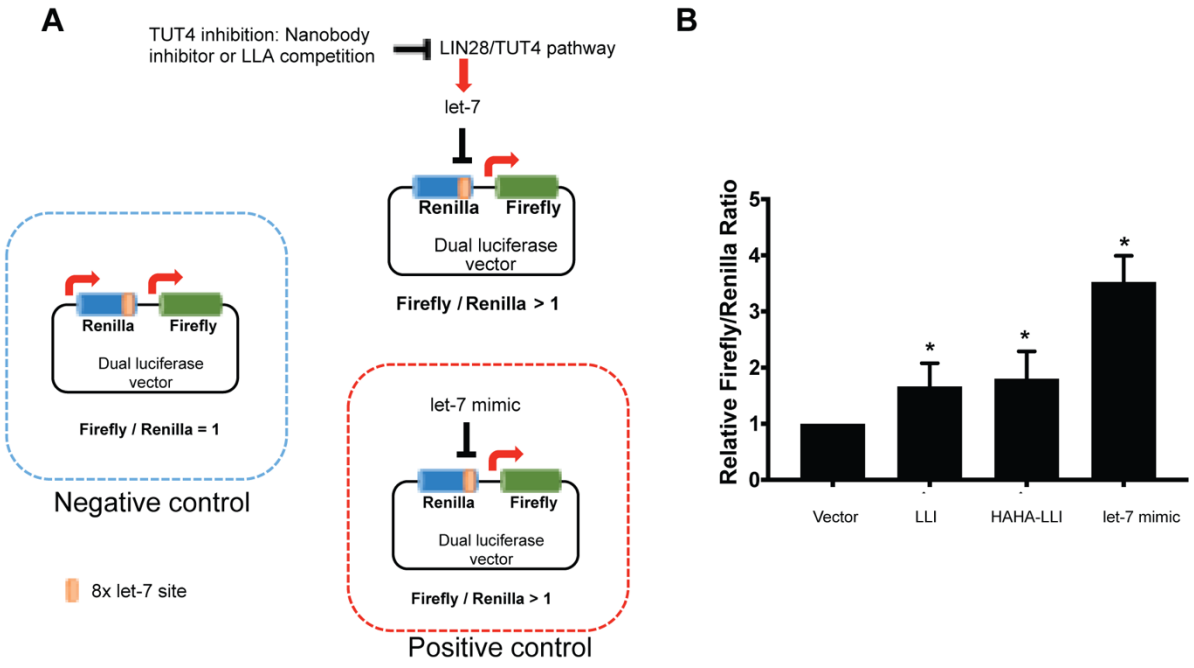

Fig. S7. The LLI fragment ($NTUT^{282-387}$) outcompeted TUT4 activity in cells. (*A*) Schematic of the Dual Luciferase assay. (*B*) HEK-293 cells were transfected with the indicated nanobody constructs, or the LLI fragment, or a control RNA duplex or a let-7a mimic, along with the dual luciferase reporter construct. 48 hours later, Renilla and Firefly luciferase activities were quantified. * indicated statistically significant compared to the vector control.

References:

1. Y. Nam, C. Chen, R. I. Gregory, J. J. Chou, P. Sliz, Molecular basis for interaction of let-7 microRNAs with Lin28. *Cell* **147**, 1080-1091 (2011).
2. C. McMahon *et al.*, Yeast surface display platform for rapid discovery of conformationally selective nanobodies. *Nat Struct Mol Biol* **25**, 289-296 (2018).
3. J. Lim *et al.*, Uridylation by TUT4 and TUT7 marks mRNA for degradation. *Cell* **159**, 1365-1376 (2014).