Supporting Information

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SI Materials and Methods

Cell Transfections. Before transfection, RNA/DNA complexes were preincubated in serum-free medium (Opti-MEM medium; Invitrogen) for 20 min at room temperature. U2OS cells (1×10^5 per well) or NSC34 cells (1.5×10^5 per well) were plated in 24-well plates for 24 h and then transfected with 750 nM synthetic tiRNAs/tiDNAs using 2.5 μL of Lipofectamine 2000 (Invitrogen). In cotransfection experiments (Fig. 3*D*), U2OS cells were transfected as described above, but equal amounts of competitor oligos were added (final oligo concentration of 1.5 μM, 1:1 G4/competitor RNA), and cells were transfected using 5 μL of Lipofectamine 2000. In the case of cotransfection of extended repeats [C9ORF72 (23×) and AACCCC (17×)], 2 μg and 2.7 μg of in vitro-transcribed C9ORF72 (23×) or AACCCC (17×) RNAs were added to the indicated RNA oligos, respectively (to keep the total number of GGGGCC or AACCCC repeats in the transfection reaction comparable).

NMM Fluorescence. Fluorescence assays were performed in 30 μL of 10 mM sodium phosphate buffer (pH 6.4), 100 mM KCl, 4 mM $MgCl_2$, and 5 μM NMM. The ODN concentration ranged from 0 to 50 μM. All fluorescence experiments were performed using a FlexStation III (Molecular Devices) plate reader with excitation and emission wavelengths of 399 nm and 614 nm, respectively. Fluorescence measurements were repeated three times for each sample, and the intensities were averaged and corrected by running a buffer control without RNA before each series of experiments. Fluorescence intensities were normalized to the maximum intensity of the c-MYC G-quartet. Results shown are the average of three to five independent replicates. Error bars represent the SD between experiments.

Reagents. Goat polyclonal anti-eIF3b, goat polyclonal anti-eIF4A, rabbit polyclonal anti-eIF4G, mouse monoclonal anti–YB-1, mouse monoclonal anti-eIF4E, and mouse monoclonal anti-Vigilin antibodies were purchased from Santa Cruz Biotechnology. Mouse polyclonal anti-G3BP was purchased from Biomedical Biosciences. Anti-mouse, anti-goat, and anti-rabbit secondary antibodies conjugated with HRP were purchased from GE Healthcare. Cy2-, Cy3-, and Cy5-HRP–conjugated secondary antibodies were purchased from Jackson Immunoresearch Labs.

The 3′-end biotinylated oligos (control DNA/RNAs or tiRNA/tiDNAs) were obtained from Integrated DNA Technology. Streptavidin agarose precipitations were as described [Ivanov et al. (1)]. Recombinant YB-1 (a gift from Lev Ovchinnikov, Institute of Protein Research, Russian Academy of Sciences, Pushchino, Russia) and/or its GST-tagged derivatives were added to the biotinylated RNA/streptavidin bead complexes, incubated for 2 h at 4 °C with rotation, and washed three times with wash buffer [15 mM Tris·HCl (pH 7.2), 0.5 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40]. Proteins were eluted using 60 μL of 1× SDS/PAGE loading buffer.

All non–sU-containing RNA and DNA ODNs used in this study were synthesized and purified by Integrated DNA Technology. DNA oligos are analogous to their RNA counterparts. The sU-containing RNA ODNs were synthesized and purified by Thermo Scientific. All ODNs are at least 95% homogeneous. Sequences are reported below.

ODN Sequences.

Nonbiotinylated oligos.

Control RNA1: 5′-UGA AGG GUU UUU UGU GUC UCU AUU UCC UUC-3′ (Piwi-interacting RNA piR006650)

Control RNA2: 5′-Phospho-UGU GAG UCA CGU GAG GGC AGA AUC UGC UC-3′ (piR58620)

Control RNA3: 5′-Phospho-GCA UUC ACU UGG AUA GUA AAU CCA AGC UGA A-3′ (random)

5′-tiRNA^{Ala}: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG AGC GCG UGC-3′

U4G: 5′-Phospho-UGG GGU GUA GCU CAG UGG UAG AGC GCG UGC-3′

UU3G: 5′-Phospho-UUG GGU GUA GCU CAG UGG UAG AGC GCG UGC-3′

5′-tiRNA$^{Ala/Cys}$: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG AGC AUU UGA-3′

5′-tiRNA$^{Cys/Ala}$-bio: 5′-Phospho-GGG GGU AUA GCU CAG UGG UAG AGC GCG UGC-3′

Helix-mis: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG UCC GCG UGC-3′

UU3G-helix-mis: 5′-Phospho-UUG GGU GUA GCU CAG UGG UAG UCC GCG UGC-3′

5′-tiRNA^{Cys}: 5′-Phospho-GGG GGU AUA GCU CAG UGG UAG AGC AUU UGA-3′

5′-tiRNA^{Val}: 5′Phospho-GUU UCC GUA GUG UAG UGG UUA UCA CGU UCG CC-3′

5′-tiRNA^{Pro}: 5′-Phospho-GGC UCG UUG GUC UAG GGG UAU GAU UCU CGG-3′

5′-tiRNA^{Met}: 5′-Phospho-GCC UCG UUA GCG CAG UAG GUA ACG CGU CAG U-3′

C9ORF72 (4×): 5′-GGG GCC GGG GCC GGG GCC GGG GCC-3′

AS1411: 5′-GGT GGT GGT TGT GGT GGT GGT GG-3′

C-myc: 5′-GGG GAG GGT GGG GAG GGT GGG G-3′

AACCCC (4×): 5′-AAC CCC AAC CCC AAC CCC AAC CCC-3′

Biotinylated oligos.

Control RNA1-bio: 5′-UGA AGG GUU UUU UGU GUC UCU AUU UCC UUC-3′-/biotin/

Control RNA2-bio: 5′-Phospho-UGU GAG UCA CGU GAG GGC AGA AUC UGC UC-3′-/biotin/

Control RNA3-bio: 5′-Phospho-GCA UUC ACU UGG AUA GUA AAU CCA AGC UGA A-3′-/biotin/

5′-tiRNA^{Ala}-bio: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG AGC GCG UGC-3′-/biotin/

U4G-bio: 5′-Phospho-UGG GGU GUA GCU CAG UGG UAG AGC GCG UGC-3′-/biotin/

UU3G-bio: 5′-Phospho-UUG GGU GUA GCU CAG UGG UAG AGC GCG UGC-3′-/biotin/

24mer-bio: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG AGC-3′-/biotin/

5′-tiRNA$^{Ala/Cys}$-bio: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG AGC AUU UGA-3′-/biotin/

5′-tiRNA$^{Cys/Ala}$-bio: 5′-Phospho-GGG GGU AUA GCU CAG UGG UAG AGC GCG UGC-3′-/biotin/

Helix-mis-bio: 5′-Phospho-GGG GGU GUA GCU CAG UGG UAG UCC GCG UGC-3′-/biotin/

UU3G-helix-mis-bio: 5′-Phospho-UUG GGU GUA GCU CAG UGG UAG UCC GCG UGC-3′-/biotin/

AS1411-bio: 5′-GGT GGT GGT TGT GGT GGT GGT GG-3′-/biotin/

C-myc-bio: 5′-GGG GAG GGT GGG GAG GGT GGG G-3′-/biotin/

M3Q: 5′-GAG GGA GGG AGG GAG AGG GA-3′-/biotin/

M3Q-Mut: 5′-GAG ATA GTG AGT GAG AGA GA-3′-/biotin/

sU-containing RNA oligos.

5′-Ala-sU RNA: 5′-Phospho-GGG GG 4-S-U GUA GCU CAG 4-S-UGG UAG AGC GCG UGC-3′-/biotin/

5′-Met-sU RNA: 5′-Phospho-GCC UCG 4-S-U UA GCG CAG 4-S-U AG GUA GCG CGU CAG U-3′-/biotin/

Control-sU RNA: 5′-Phospho-GGC UCG U 4-S-U G GUC UAG GGG 4-S-U AU GAU UCU CGG-3′-/biotin/

1. Ivanov P, Emara MM, Villen J, Gygi SP, Anderson P (2011) Angiogenin-induced tRNA fragments inhibit translation initiation. *Mol Cell* 43(4):613–623.

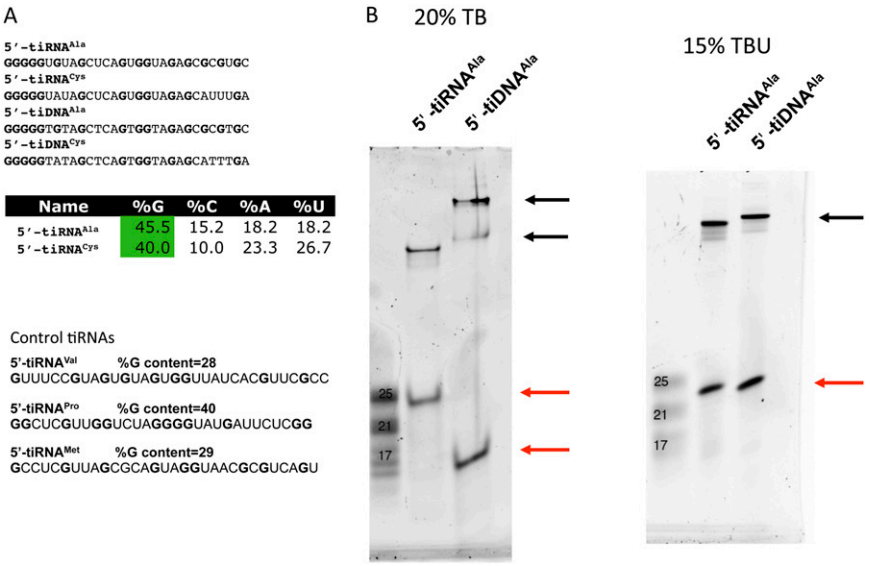

Fig. S1. G-rich 5′-tiRNA^{Ala} and 5′-tiRNA^{Cys} assemble polymorphic structures. (*A*) Translationally active 5′-tiRNA^{Ala} and 5′-tiRNA^{Cys} are highly G-rich in composition. (*Upper*) Sequences of 5′-tiRNA^{Ala}, 5′-tiRNA^{Cys}, and their DNA analogs. Guanine is shown in bold black. (*Lower*) Nucleotide content of 5′-tiRNA^{Ala} and 5′-tiRNA^{Cys} is highlighted. Sequence and Guanine content of other tiRNAs used in this study are shown. (*B*) Translationally active 5′-tiRNA^{Ala} assembles monomeric and multimeric structures. 5′-tiR/DNA^{Ala} forms compact and multimeric structures. PAGE analysis in denatured (15% TBU, *Right*) and native (20% TB, *Left*) conditions reveals stable, fast-migrating RNA/DNA species with compact shapes (25 nt and 17 nt, respectively; red arrows), as well as stable oligomers/multimers migrating slowly and resistant to denaturation (black arrows).

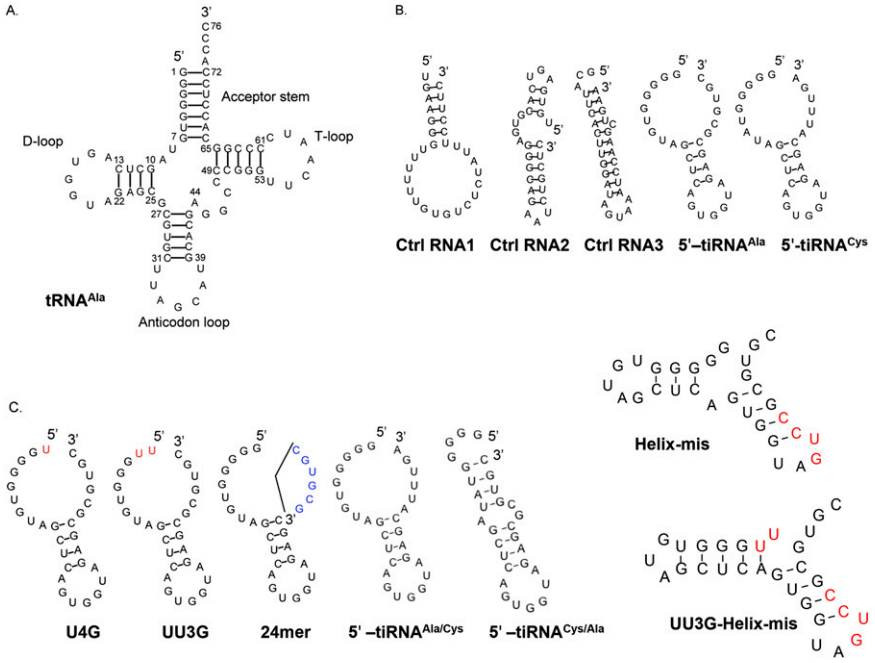

Fig. S2. Predicted secondary structures of selected 5′-tiRNAs and their mutants. (*A*) Secondary structure of $tRNA^{Ala}$. (*B*) Predicted secondary structures of control (Ctrl) RNAs and 5′-$tiRNA^{Ala}$ and 5′′-$tiRNA^{Cys}$. (*C*) Mutants of 5′-$tiRNA^{Ala}$ and 5′-$tiRNA^{Cys}$. Substituted nucleotides are shown in red, and deleted nucleotides are shown within wedges in blue. Prediction of secondary structures was done online at the RNAFold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

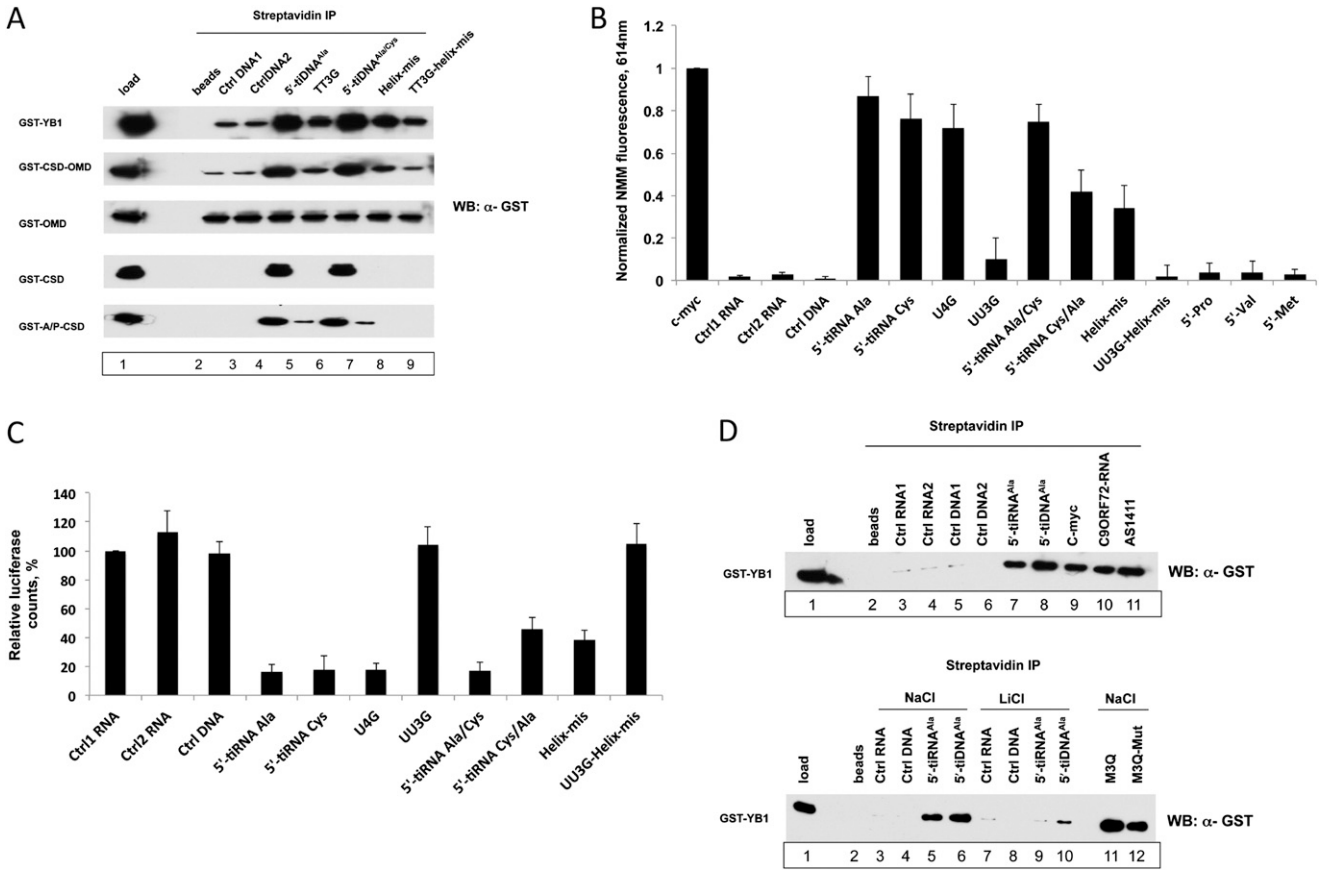

Fig. S3. YB-1 specifically interacts with G4 oligos in vitro. (*A*) Recombinant GST–YB-1 and its truncation mutants were incubated with selected biotinylated DNA oligos. Streptavidin beads were used for coimmunoprecipitation (co-IP) of biotinylated RNA/protein complexes as described in Fig. 2*D*. The precipitated GST–YB-1 variants were visualized by standard Western blotting (WB) using anti-GST antibodies. (*B*) Translationally active tiRNAs assemble G4s. NMM fluorescence analysis was used as described in Fig. 1*A*, with exception that the concentration of RNA/DNA oligos was kept constant (25 μM). Means and SDs are from two independent experiments [Student *t* test ($n = 2$)]. (*C*) G4 variants of 5′-tiDNA^{Ala} inhibit translation of mRNA reporters in vitro. Uncapped *Firefly* luciferase mRNA (Promega) was translated in RRLs in an in vitro translation system in the presence of control RNAs (Ctrl1 and Ctrl2 RNA; derived from piwi-interacting RNA (Piwi-interacting RNA, piRNA) or random sequences) or control DNA (Ctrl DNA1; sequence analogs of control RNAs), 5′-tiRNA^{Ala} or its mutants, and 5′-tiRNA^{Cys}. Luciferase expression is relative to luciferase expression in the absence of any RNA or DNA (no RNA/DNA oligo = 100%). Means and SDs are from two independent experiments [Student *t* test ($n = 2$)]. (*D*) 5′-tiDNA^{Ala} and 5′-tiRNA^{Ala} interact with G4 oligos in vitro. (*Upper*) Streptavidin co-IP with biotinylated RNA/DNA oligos without G4 structures (Ctrl DNA/RNA oligos, lanes 3–6); 5′-tiDNA^{Ala} and 5′-tiRNA^{Ala} (lanes 7–8); and known G4s c-myc, C9ORF72, and AS1411 (lanes 9–10) was performed with recombinant GST–YB-1. (*Lower*) Streptavidin co-IP with GST–YB-1 and biotinylated Ctrl RNA/DNA oligos or 5′-tiDNA^{Ala}/5′-tiRNA^{Ala} was done as above (*Upper*) in the presence of sodium ions (NaCl, supporting G4 formation) or lithium ions (LiCl, not supporting G4 formation), or with biotinylated M3Q oligo (supporting G4 formation) or its mutant (M3Q-Mut, not supporting G4 formation). Note the decrease in the binding of GST–YB-1 to 5′-tiDNA^{Ala} and 5′-tiRNA^{Ala} in the presence of lithium cations and to the mutant form of M3Q.

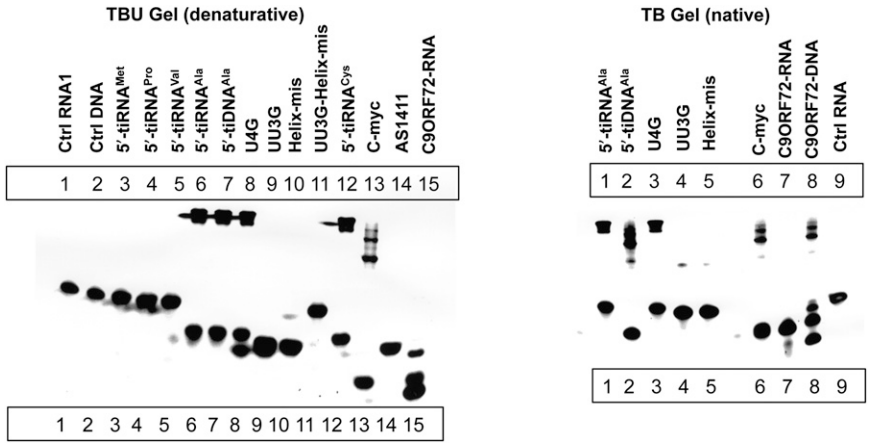

Fig. S4. In-gel analysis of G4 oligos. 5′-tiR/DNA$^{Ala/Cys}$ forms compact and multimeric structures. PAGE analysis in denatured (15% TBU, *Left*) and native (20% TB, *Right*) conditions reveals stable, fast-migrating RNA/DNA species with compact shapes, as well as stable oligomers/multimers migrating slowly and resistant to denaturation. U4G mutant, but not UU3G or Helix-mis/UU3G-helix-mis mutant, of 5′-tiRNA^{Ala} assembles stable oligomers/multimers. Other tiRNAs (tiRNAs$^{Met/Pro/Val}$) and control oligos (Ctrl RNA1 and Ctrl DNA) move according to the predicted length. Validated G4 oligos (AS1411, C9ORF72, and c-myc) demonstrate a broad spectrum of anomalous mobility in both denatured and native conditions.

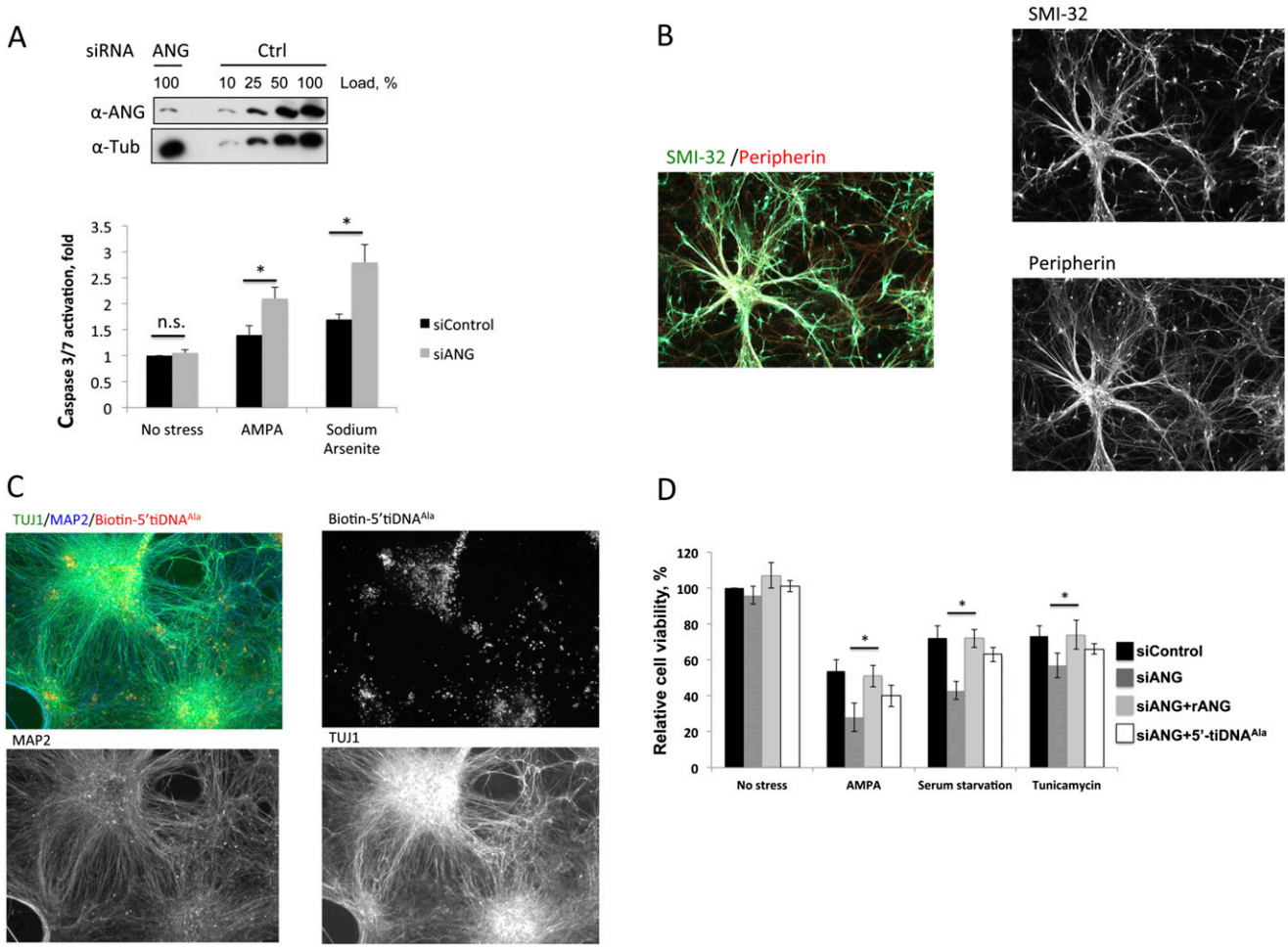

Fig. S5. Neuroprotective properties of ANG and tiDNAs. (*A*) ANG-depleted hMNs are sensitive to stress. The hMNs were treated twice with control siRNA (siCtrl) or ANG-specific siRNA (siANG) on days 17 and 20 of differentiation. ANG depletion has been verified by Western blotting using ANG-specific antibody. A series of dilution of protein lysates from control siRNA-treated cells (from 10–100%) is compared with the protein lysate from ANG-depleted cells (siANG, 100%). Tubulin (Tub) is used as a loading control. (*Upper*) Efficiency of ANG knockdown is 80–90%. On day 21, siRNA-treated cells were challenged with no stress, AMPA, serum withdrawal, or tunicamycin. (*Lower*) CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to determine the relative viability of motor neurons. Means and SDs are from three independent experiments [$*P < 0.05$, comparison of control siRNA-treated and ANG-depleted cells under selected stress stimuli, Student *t* test ($n = 3$)]. (*B* and *C*) Cultures of hMNs and delivery of 5′-tiDNA^{Ala}. Neuronal cultures are positive for several neuronal markers, such as SMI-32 (neurofilament heavy polypeptide that visualizes neuronal cell bodies, dendrites, and thick axons; green) and peripherin (type III Intermediate filament protein that stains thin and thick axons; red), TUJ1 (major neuron-specific class III β-tubulin that visualizes dendrites and axons) (*B*), and MAP2 (neuronal microtubule-associated protein 2 predominantly found in neuronal dendrites) (*C*). The images are shown both as combined colored and separate gray. (Magnification 10×.) (*C*) 5′-tiDNA^{Ala} spontaneously enters hMNs. The hMNs were differentiated from hMN progenitors (Lonza) for 21 d according to the manufacturer's protocol. On day 21, 3′-end biotinylated 5′-tiDNA^{Ala} oligo (IDT Tech) was applied to the medium for 72 h. Neurofilament staining using TUJ1 antibody (green), MAP2 antibody (blue), and streptavidin-Cy3 visualizing biotinylated 5′-tiDNA^{Ala} oligo (red) was used. (*D*) ANG and tiDNAs rescue motor neurons from stress-induced injuries. For the rescue experiment, ANG-depleted hMNs were pretreated with recombinant WT ANG (0.5 μg/mL, 24 h) or 5′-tiDNA^{Ala} oligo (750 nM, 72 h) before challenge with selected stresses. Means and SDs are from three independent experiments [$*P < 0.05$, comparison of control siRNA-treated and ANG-depleted cells under selected stress stimuli, Student *t* test ($n = 3$)].

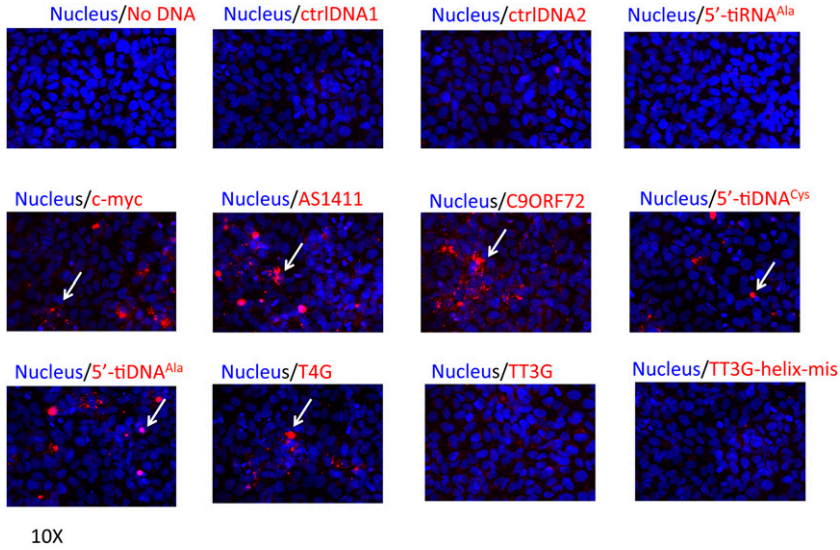

Fig. S6. 5′-tiDNA^{Ala} is spontaneously taken up by osteosarcoma U2OS cells. The 3′-end biotinylated control DNAs (ctrlDNA1 and ctrlDNA2) and 5′-tiRNA^{Ala} (first row, all negative controls), AS1411 (validated G4 oligo, positive control for uptake) and G4 DNA oligos (C9ORF72, c-myc, and 5′-tiDNA^{Cys}) (second row), or 5′-tiDNA^{Ala} and its mutant variants (T4G, TT3G, and TT3G-helix-mis) (third row) oligos were added to U2OS cells for 72 h. Streptavidin-Cy3 (red) and Hoechst (blue) were used to visualize biotinylated DNA oligos and nuclei, respectively. AS1411, c-myc, C9ORF72, 5′-tiDNA^{Cys}, and 5′-tiDNA^{Ala} (and its bioactive T4G variant) oligos (internalized oligos are shown with white arrows), but not control DNAs (ctrlDNA1 and ctrlDNA2) or 5′-tiDNA^{Ala} translationally inactive (TT3G and TT3G-helix-mis) oligos, spontaneously enter U2OS cells. 5′-tiRNA^{Ala} oligo is degraded in the medium within minutes.