Supplementary Information for:

Targeting non-coding RNA family members with artificial endonuclease XNAzymes

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Supplementary Figure 1. Further characterisation of XNAzymes targeted to microRNAs.

Urea-PAGE gels showing (a,b) single-turnover reactions between (0.25 μM) microRNA substrates and 1.25 μM XNAzyme (a) Fz_miR_17 or (b) Fz_miR_20a, for 8h under quasi-physiological conditions (37 °C, 1 mM Mg²⁺, pH 7.4). (c,d) multiple-turnover reactions between (50 μM) (c) miR-17 or (d) miR-20a RNA substrates and (0.5 μM) XNAzyme (c) Fz_miR_17 or (d) Fz_miR_20a, under quasi-physiological conditions (37 °C, 1 mM Mg²⁺, pH 7.4). (e,f) single-turnover reactions between (0.5 μM) (e) miR-17 or (f) miR-20a and (2.5 μM) (e) Fz_miR_17 or (f) Fz_miR_20a, in DMEM tissue culture media (4 h), Mg-free cell wash buffer (5 h) or 90% HEK293 cell lysate (5 h).
Supplementary Figure 2. Engineering XNAzymes targeted to miR-106a and miR-21.

(a) Schematic and urea-PAGE gel showing putative secondary structure and (lack of) activity of a variant of the FR6_1 XNAzyme, “Fz_miR_106a”, engineered to cleave human miR-106a. No detectable product was observed after 15 h with (1 µM) miR-106a RNA substrate and (5 µM) XNAzyme Fz_miR_106a. Dashed red line indicates possible complementarity responsible for destabilising secondary structure in the catalytic core. (b) Schematic and urea-PAGE gel showing putative secondary structure and activity of a variant of the FR6_1 XNAzyme, “Fz_miR_21”, engineered to cleave human miR-21. Black arrows indicate site of substrate cleavage. (1 µM) miR-21 RNA substrate and (5 µM) XNAzyme Fz_miR_21 were incubated for 15 h. Red circles indicate positions mutated in variants of Fz_miR_21 and (c) screened for improved activity; (1 µM) miR-21 RNA substrate and (5 µM) each XNAzyme Fz_miR_21 variant were incubated for 2 h. (d) Urea-PAGE gel showing multiple-turnover reaction between (50 µM) miR-21 RNA substrate and (0.5 µM) XNAzyme “Fz_miR_21B” [Fz_miR_21: fA8fG, fC10fA, fC19fU, fG25fU]. All reactions shown were performed under quasi-physiological conditions (37 °C, 1 mM Mg²⁺, pH 7.4).
**Supplementary Figure 3. Characterisation of DNAzymes targeted to miR-21.**

(a-c) Schematics showing DNAzymes targeting miR-21 based on (a) the 8-17 DNAzyme, “miR21dz1”\(^1\), and (b,c) the 10-23 DNAzyme, “RVN541”\(^2\), graphs showing their RNA-cleavage activity in pseudo first-order single-turnover reactions between (0.5 \(\mu\)M) miR-21 RNA substrate and (2.5 \(\mu\)M) DNAzyme, in ‘high magnesium’ conditions (37 °C, 10 mM Mg\(^{2+}\), pH 7.4), or quasi-physiological conditions (37 °C, 1 mM Mg\(^{2+}\), pH 7.4) (Solid red lines indicate DNAzyme data. Dashed grey lines indicate data for analogous reactions with FANA XNazy Fz_miR_21B, and Urea-PAGE gels showing their degradation in 50% human serum at 37 °C (with data for FANA XNazy Fz_miR_21B shown for comparison). Black arrows indicate site of substrate cleavage. DNA residues are indicated in cyan. Substrate RNA residues are indicated in orange.

DNAzyme “RVN541” (b) is the sequence as published by Larcher et al.\(^2\), but as this sequence had little activity in our hands, a variant (c) “RVN541B” was also synthesised with an inserted dC (indicated in red) to restore canonical binding arm pairing with the miR-21 RNA substrate.
Supplementary Figure 4. Characterisation of ribozymes targeted to miR-21.

(a,b) Schematics showing ribozymes targeting miR-21 based on the extended hammerhead ribozyme, “miR21rz1_TLR” 1, graphs showing their RNA-cleavage activity in pseudo first-order single-turnover reactions between (0.5 µM) miR-21 RNA substrate and (2.5 µM) ribozyme, in ‘high magnesium’ conditions (37 °C, 10 mM Mg²⁺, pH 7.4), or quasi-physiological conditions (37 °C, 1 mM Mg²⁺, pH 7.4) (Solid red lines indicate DNAzyme and ribozyme data. Dashed grey lines indicate data for analogous reactions with FANA XNAzyme Fz_miR21B), and Urea-PAGE gels showing their degradation in 50 % human serum at 37 °C. Black arrows indicate site of substrate cleavage. Catalyst RNA residues are indicated in green. Substrate RNA residues are indicated in orange.

Ribozyme “miR21rz1_TLR” (a) is the sequence as published by Belter et al.1 but as this sequence was found to be inactive in our hands, a variant (b) “miR21rz1_TLRB” with three mutations (indicated in red) was synthesised that restores the canonical hammerhead ribozyme core sequence.
Supplementary Figure 5. Screening XNAzymes targeted to non-coding Y RNA hY5.

(a) Schematic showing the secondary structure of human hY5 RNA indicating potential cleavage site motifs targeted by a series of variants of the FR6_1 XNAzyme, “Fz_hY5_[1-5]”. (b) Urea-PAGE gels showing activity of XNAzymes Fz_hY5_[1-5] in pseudo first-order single-turnover reactions between (0.5 µM) hY5 RNA substrate and (2.5 µM) XNAzymes under quasi-physiological conditions (37°C, 1 mM Mg²⁺, pH 7.4) for 15 hrs. Bands with PAGE mobility corresponding to the expected molecular weight of cleavage products could be observed in reactions with (c) XNAzymes “Fz_hY5_4” and “Fz_hY5_5”. Black arrows indicate site of substrate cleavage.
Supplementary Figure 6. Electrophoretic mobility of TFz_miR₃ tetrahedron components in native and denaturing PAGE.

(a) Native PAGE gel showing annealed individual FANA component strands of the TFz_miR₃ tetrahedron. For each component, bands indicated by dotted line boxes were extracted and re-analysed by (b) Denaturing Urea-PAGE. Mwt = NEB low molecular weight ladder (dsDNA).
| Name             | Sequence (5' – 3')                                                                 | Description                                                                                   |
|------------------|-----------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| drP2_Ebo         | [6FAM] AATCTACCACATCGCTCATTTG                                                    | Primer for synthesis of XNAzymes (3' RNA residue allows primer removal after synthesis)      |
| Fz_miR_17temp    | AAAGTGCCCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_17                                                |
| Fz_miR_20atemp   | AAAGTGCCCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_20a                                              |
| Fz_miR_106atemp  | CAAGATGGGCGATGCAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]           | Template for synthesis of Fz_miR_106a                                                       |
| Fz_miR_21temp    | GCTTTACCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_21                                               |
| Fz_miR_21[A8G]temp | GCTTTACCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_21 with A8G mutation                               |
| Fz_miR_21[C10A]temp | GCTTTACCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_21 with C10A mutation                              |
| Fz_miR_21[C19U]temp | GCTTTACCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_21 with C19U mutation                             |
| Fz_miR_21[G25U]temp | GCTTTACCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_21 with G25U mutation                             |
| Fz_miR_21Btemp   | GCTTTACCGGAAGTCAAAACCGGTAGTCAGCACAA TGAGCGATGTGGTAGATT [BiotinTEG]               | Template for synthesis of FANAzyme Fz_miR_21B (Fz_miR_21: A8G, C10A, C19U & G25U)         |
| miR21dz1         | CATCAGTTCCGAGCCGAGAGACAGAAGC                                                    | 8-17 DNAzyme targeting miR-21 described by Belter et al.                                   |
| RNV541           | TCAACAGGCTAGCTACAAGAAGTCTGATAAGCTA                                              | 10-23 DNAzyme targeting miR-21 described by Larcher et al.                                 |
| RNV541B          | TCAACAGGCTAGCTACAAGAAGTCTGATAAGCTA                                              | 10-23 DNAzyme targeting miR-21 described by Larcher et al., with insertion C22 (underlined) |
| miR21rz1_TLR     | CUAAGGGCCCAAGCUAUGGGCAGUCAGAUGC GGCCGAAAGGCGUAUAAGC                            | Extended hammerhead ribozyme targeting miR-21 described by Belter et al.                  |
| miR21rz1_TLRB    | CUAAGGGCCCAAGCUAUGGGCAGUCAGAUGC GGCCGAAAGGCGUAUAAGC                            | Extended hammerhead ribozyme targeting miR-21 described by Belter et al., with mutations A27U, C32A, U46A (underlined) |
| Fz_hY5_1temp     | GTTGTGTGGGCGGAAGTCAAAACCGGGTTTGTAA GTTCAATGACCGATGTGGTAGATT [BiotinTEG]          | Template for synthesis of Fz_hY5_1                                                         |
| Fz_hY5_2temp     | TGGGTTATCGGAAGTTCAAAACCGGGTTGATTTGAT TTCAATGACCGATGTGGTAGATT [BiotinTEG]          | Template for synthesis of Fz_hY5_2                                                         |
| Fz_hY5_3temp     | TTAAGTTGATCGGAAGTCAAAACCGGTACATTGT CTCAAATGACCGATGTGGTAGATT [BiotinTEG]          | Template for synthesis of Fz_hY5_3                                                         |
| Fz_hY5_4temp     | GTCTCCCCCCCCGGGAAGTCAAAACCGGTACCGGC TGCAATGAGCGATGTGGTAGATT [BiotinTEG]          | Template for synthesis of FANAzyme Fz_hY5_4                                               |
| Fz_hY5_5temp     | CGGCCGTTGACGGAAGTCAAAACCGGGTGCTGTGAT TTCAATGACCGATGTGGTAGATT [BiotinTEG]          | Template for synthesis of FANAzyme Fz_hY5_5                                               |
| Fz_hY5_4Btemp    | GTCTCCCCCCCCGGGAATCAAAACCGGTACCGGC GC TGCAATGAGCGATGTGGTAGATT [BiotinTEG]          | Template for synthesis of FANAzyme Fz_hY5_4B                                              |
| Oligonucleotide | Sequence | Notes |
|-----------------|----------|-------|
| TFz_miR_17temp | CGTGGGAAGACACATTCTAAGTCTGAAGTAAAGTG CCGGAAAGTCACACCAACGGGCTAGTGCAAAAACG ACCTATGAGGATGTGCTGAAGT [BiotinTEG] | Template for synthesis of FANAzyme Fz_miR_17 with sequences for tetrahedron assembly [analogous to 'S3' in ref 3] |
| TFz_miR_21Btemp | CCATAGTAGATTCCCTCTCTTCTGAGTTATCCTGAGTTCTTCAGA CAATGAGCGATGTGGTAGATT | Template for synthesis of FANAzyme Fz_miR_20B with sequences for tetrahedron assembly [analogous to 'S5' in ref 3] |
| TFz_miR_20atemp | AGGAATGTGTTACTATGGCGGCTCTTCTCAAAGT GCCGGAAGTCAAACCGGGTGAGTGGCAAGTACCAATGAGCGATGTGGTAGATT | Template for synthesis of FANAzyme Fz_miR_20a with sequences for tetrahedron assembly [analogous to 'S4' in ref 3] |
| miR-17 | [6FAM] CAAAGUGCUUACAGUGCAGGUAGU | Mature miR-17 substrate |
| miR-20a | [6FAM] UAAAGUGCUUAUGUGACGAGUAGU | Mature miR-20a substrate |
| miR-20b | [6FAM] CAAAGUGCUCAUAGUGACGAGUAGU | Mature miR-20b substrate |
| miR-21 | [6FAM] UAGGCUAAUCAGACUGAGUGAGA | Mature miR-21 substrate |
| miR-93 | [6FAM] CAAAGUGCUGUUCGUGACAGUAGU | Mature miR-93 substrate |
| miR-106a | [6FAM] CAAAGUGCUACAGUGACGAGUAGU | Mature miR-106a substrate |
| miR-106b | [6FAM] UAAAGUGCUAGACGAGUAGU | Mature miR-106b substrate |
| hY1tempFwd | ATTTAGGTGACATCTAGGTGGTCTGAGGCTAGTAGTGACACTATAGGCTGGTCCGAAGGTAGTGAGTTATCTCAATTGATTGTTCACAGTCAGTTACCACTACTGACTCTCACTCTTCCCCCCTTCT CACTACTGACCTAGACTGCTTTT | Template for synthesis of hY1 RNA substrate using SP6 |
| hY1tempRev | AAAAGACTGTCAAGTCTGAGTTGAGAAGGGG GGAAGAGTAGAACAAGGAGGTCTGCGATCTGTAACCTGACTGTGAACATACCACTACCTACCT TGGGACCAGGCCTATAGGTGCACCTAAAT | Template for synthesis of hY1 RNA substrate using SP6 |
| hY3tempFwd | ATTTAGGTGACATCTAGGTGGTCTGAGGCTAGTAGTGACACTATAGGCTGGTCCGAAGGTAGTGAGTTATCTCAATTGATTGTTCACAGTCAGTTACCACTACTGACTCTCACTCTTCCCCCCTTCT CACTACTGACCTAGACTGCTTTT | Template for synthesis of hY3 RNA substrate using SP6 |
| hY3tempRev | AAAAGGCTAGTCAGTCTGAGTTGAGAAGGGG GGAAGAGTAGAACAAGGAGGTCTGCGATCTGTAACCTGACTGTGAACATACCACTACCTACCT TGGGACCAGGCCTATAGGTGCACCTAAAT | Template for synthesis of hY3 RNA substrate using SP6 |
| hY4tempFwd | ATTTAGGTGACATCTAGGTGGTCTGAGGCTAGTAGTGACACTATAGGCTGGTCCGAAGGTAGTGAGTTATCTCAATTGATTGTTCACAGTCAGTTACCACTACTGACTCTCACTCTTCCCCCCTTCT CACTACTGACCTAGACTGCTTTT | Template for synthesis of hY4 RNA substrate using SP6 |
| hY4tempRev | AAAAGGCAAGCTCAAAATTTAGCAGTGAGGGGTTGT ATACAACTTTTAGTCAGCTAATAGTCTAATTAAACAGTGATCTACGACTACTACCACTGACCAGGCCACACCC TATAGTCACCTAAAT | Template for synthesis of hY4 RNA substrate using SP6 |
| hY5tempFwd | ATTTAGGTGACATCTAGGTGGTCTGAGGCTAGTAGTGACACTATAGGCTGGTCCGAAGGTAGTGAGTTATCTCAATTGATTGTTCACAGTCAGTTACCACTACTGACTCTCACTCTTCCCCCCTTCT CACTACTGACCTAGACTGCTTTT | Template for synthesis of hY5 RNA substrate using SP6 |
| hY5tempRev | AAAACGCAAGCTCAAAATTTAGCAGTGAGGGGTTGT ATACAACTTTTAGTCAGCTAATAGTCTAATTAAACAGTGATCTACGACTACTACCACTGACCAGGCCACACCC TATAGTCACCTAAAT | Template for synthesis of hY5 RNA substrate using SP6 |

**Supplementary Table 1. Oligonucleotide sequences used in the study.**

Black and red text indicates DNA and RNA sequences respectively.
Supplementary References

1. Belter, A. et al. Inhibition of miR-21 in glioma cells using catalytic nucleic acids. *Scientific Reports* **6**, 24516, doi:10.1038/srep24516 (2016).

2. Larcher, L. M., Wang, T. & Veedu, R. N. Development of Novel antimiRzymes for Targeted Inhibition of miR-21 Expression in Solid Cancer Cells. *Molecules* **24**, doi:10.3390/molecules24132489 (2019).

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