Supplemental Information

Modulation of TNFα Activity
by the microRNA Let-7
Coordinates Zebrafish Heart Regeneration

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SUPPLEMENTARY MATERIALS

TRANSPARENT METHODS

Zebrafish heart resection. All animal studies were performed under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at MDI Biological Laboratory. Briefly, wildtype Ekkwill (EK) and transgenic adult zebrafish of 7-11 months of age were anesthetized with immersion in a 1:1000 dilution of 2-phenoxyethanol, and ~20% of the ventricular apex was resected with iridectomy scissors. Animals were allowed to recover in a centralized, recirculating system, and hearts were extracted at defined stages for RNA expression and histology studies.

Anti-miR and pharmacological microinjections. Antisense scrambled and let-7 locked-nucleic-acid (LNA) oligonucleotides were designed and synthesized with phosphorothioate backbone (denoted with *) by Exiqon (www.exiqon.com) and administered into adult animals via intraperitoneal (IP) microinjections at 10ug/g body weight using previously described procedures (25, 58-60). A cocktail of 3 LNA let-7 oligonucleotides, C*A*A*Y*Y*T*A*C*T*A*C*C*T*C, A*C*A*A*H*C*W*A*C*T*A*C*C*T*C and T*G*A*G*G*T*A*G*T*A*G*T*T*T*G* were administered in 1:1:1 ratio for all functional studies on let-7 family. Similarly, in co-treatment studies, the CAY10500 pharmacological inhibitor of TNFα (www.scbt.com) was administered IP immediately after LNA let-7 injection at a dose of 15ug/g body weight.

Histological methods. Zebrafish hearts were extracted and fixed in 4% Paraformaldehyde (PFA) for 1-hr at room temperature, embedded in tissue freezing medium (TFM) (Fisher) and sectioned at 10µm with a Leica CM1860 cryostat. Proliferating CMs (CMs) were identified as double labeled cells for Mef2 (rabbit; Santa Cruz Biotechnology #SC-313; 1:75) and PCNA (mouse; Sigma #P8825; 1:400). CM proliferation indices were defined as the total number of Mef2+PCNA+ cells represented as a percentage of the total Mef2+ population. Three sections containing the largest injury area were quantified for each heart. Alternatively, tissue sections were stained with hematoxylin and eosin as previously described(49). Images were captured on an Olympus BX53 compound microscope at 20x magnification. Electron microscopy images were captured at 15000x magnification on a JEOL 1230 Transmission Electron Microscope, at 90nm thickness, at the Jackson Laboratory (Bar Harbor, ME).
Quantification of fluorescent reporter expression. To quantify tcf21:Dsred and gata4:GFP expression, 3 sections from each heart were analyzed with Photoshop. For tcf21:Dsred expression, Dsred pixel intensity within the resection zone was represented as a percentage of the total area. Similarly, for gata4:GFP quantification, a 1.5 x 5 inch rectangle was drawn at the injury and ventricle lateral wall opposite of the atrium. Pixel intensity of gata4:GFP was expressed as a percentage of the total area.

RNA collection and qPCR relative analysis. Ventricles were extracted directly into Trizol and total RNA was isolated with Zymo Direct-zol RNA microprep kit, as suggested by the manufacturer (Zymo Research Corp., Irvine, CA). cDNA synthesis was performed using either NEB ProtoScript II First Strand cDNA kit or Quanta qScript microRNA cDNA synthesis kit (www.VWR.com). Real-time qPCR expression studies were performed with SYBR Green (Agilent) and transcript specific oligo pairs (Table S1). Relative expression was determined using ΔCT method as previously described(61).

RNA sequencing. Total RNA samples were extracted from triplicate tissue samples from antisense-scrambled and let-7 injected zebrafish (see above) using the Zymo Direct-zol RNA microprep kit (Zymo Research Corp., Irvine, CA). For each biological replicate sample, indexed strand-specific polyA+ selected mRNA libraries were prepared at the HudsonAlpha Institute for Biotechnology (Huntsville, AL) using NEBNext library kits (New England BioLabs, Ipswich, MA). Strand-specific polyA+ selected mRNA libraries were sequenced using paired-end 75bp reads. All sequencing was performed on an Illumina HiSeq2500 at the HudsonAlpha Institute for Biotechnology following the manufacturer’s protocols. Sequence data are available in the Gene Expression Omnibus (GSE125987).

RNA sequencing data analysis. Following sequence read quality control diagnostic analyses using FastQC version 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), paired-end reads were trimmed using Trimmomatic version 0.32.90(62). Trimmed paired-end reads were aligned to the Ensembl-annotated zebrafish transcriptome (version 76)(63) using RSEM version 1.2.25(64) and Bowtie 1.1.2.93(65). Read counts expressed as transcripts per million were analyzed using
R/edgeR(66). Pathway analysis was performed using Ingenuity Pathways Analysis (QIAGEN, Redwood City, CA).

**Fluorescence-activated Cell Sorting and Gene Expression.** Uninjured and amputated transgenic adult zebrafish ventricles were extracted and dissociated in accordance to previously described methods(55). Isolation of fluorescently marked cells from Tg(tcf21:Dsred), Tg(cmlc2:GFP), and Tg(fli1a:GFP) was performed on a FACSArria II instrument (BD Biosciences) at the Jackson Laboratory (Bar Harbor, ME). Cells were sorted directly into Trizol LS (www.thermofisher.com) and total RNA was isolated using Zymo Direct-zol RNA microprep kit, as suggested by the manufacturer (Zymo Research Corp., Irvine, CA). cDNA was synthesized using NEB ProtoScript II First Strand cDNA kit, in accordance to manufacturer’s protocol (NEB, Ipswich, MA). Real-time qPCR expression studies were performed with SYBR Green (Agilent) detection and specific primers for each gene (Supplementary Table S1).

**Engineering Transgenic animals.** To engineer the Tg(hs:lin28a) strain, the lin28a cDNA was amplified from uninjured adult heart RNA using primers described in Table S1. The 600-bp let-7i amplicon extends ~250-bp upstream and downstream of the mature precursor sequence and was amplified with embryonic gDNA. Each amplicon was cloned downstream of the hsp70 promoter in the pHS-2mi vector(29). Each transgene was microinjected into 1-cell stage of zebrafish Ekkwill embryos and at least 2 independent insertions of each transgene was analyzed for expression activation. F2 and later generation animals were used for all studies.

**Heat-treatment Regimen.** Transgenic and Tg(-) clutchmates were injured and subjected to daily heat-treatment whereby the water temperature was elevated from 28°C to 38°C, over a 3 hour period. Water temperature is maintained at 38°C for 45 minutes per 24 hour interval.

**Statistical analysis.** All statistics were performed using Student’s t-test with Welch’s correction. A p-value< 0.05 was deemed statistically significant.

**Supplementary Figure S1.** Sequence alignment of mature let-7 family members reveal 100% identity within the seed sequence, Related to Figure 1.
Supplementary Figure S2. LNA-let-7 dose response, Related to Figure 2. Adult zebrafish hearts were resected and administered either vehicle or LNA-let-7 at 5ug/g, 10ug/g and 20ug/g via IP microinjection. Total RNA was extracted for cDNA synthesis and qPCR evaluation of let-7i expression levels. (A) In comparison to vehicle, hearts treated with 10ug/g and 20ug/g LNA-let-7 resulted in significant repression of 75% and 85% in let-7i levels. (n=5). (B) Treatment with LNA-let-7 10ug/g showed a specific reduction of let-7i levels without affecting the levels of other cardiac miRNAs. Values are means ± S.E. *P<0.05 compared to control heart ventricles.

Supplementary Figure S3. LNA-let-7i induces promiscuous repression of family members, Related to Figure 2. Real-time qPCR studies show treatment with a single LNA antisense oligonucleotide directed against let-7i suppresses additional let-7 family members. Values are means ± S.E. *P<0.05.

Supplementary Figure S4. Activation of Tg(hs:lin28a) suppresses heart regeneration by decreasing let-7i expression levels, Related to Figures 2, 3. (A) Lin28a mRNA expression decreases at the onset of heart resection when compared with uninjured ventricles. Heat-treatment of Tg(hs:lin28a) lines 9, 25 and 30 show increased expression levels of lin28a mRNA (B) and decreases in let-7i levels (C) as revealed by qPCR studies. Expression levels were normalized to either uninjured hearts (A) or heat-treated Tg (-) clutchmates in (C, D). (D) Representative images of heat-treated control and Tg(hs:lin28a) hearts stained with Mef2 and PCNA to identify proliferating CMs. Arrowheads indicate Mef2+PCNA+ cells. (n=12) (E) Quantification of CM proliferation indices reveals suppression of CM proliferation indices from 9.1% in heat-treated control to 3.8% in Tg(hs:lin28a) hearts. Values are means ± S.E. *P<0.05.

Supplementary Figure S5. Delayed LNA-let-7 treatment suppresses cardiomyocyte proliferation, Related to Figure 3. (A) Treatment protocol for delayed LNA-let-7 treatment. (B) Representative images of Tg(tcf21:Dsred) at 14 dpa (B, top panels), and 21 dpa hearts stained with antibodies to detect Mef2 and PCNA (bottom panels). (C) Quantification of cardiomyocyte proliferation indices on vehicle and delayed LNA-let-7 treatment show a decrease from 1.7% to 0.8% from control to delayed LNA-let-7 hearts, respectively. (n=8). Values are means ± S.E. *P<0.05.
Supplementary Figure S6. Overexpression of let-7i enhances heart regeneration, Related to Figure 3. (A) Schematic outline of experimental induction of Tg(hs:let-7i-pre). (B) Heat treatment of Tg(hs:let-7ipre) lines 30 and 5 increases the mature levels of let-7i expression, as reflected in qPCR studies. (C) Representative images of 3 dpa control and Tg(hs:let-7ipre)30 hearts treated with Mef2 and PCNA antibodies. (n=12). Arrowheads mark Mef2+PCNA+ cells. (D) Cardiomyocyte proliferation indices show an increased in cardiomyocyte proliferation from 7.2 in Tg (-) to 10.7% in let-7i overexpression. (E) Representative images of heat treated Tg(tcf21:Dsred) control and Tg(tcf21:Dsred);(hs:let-7ipre)30 double transgenic hearts show no significant differences in rate of wound closure. (n=16). Brackets mark approximate resection plane. (F) Quantification of tcf21:Dsred expression within the resected zone show no significant differences between control and Tg(hs:let-7ipre)30 hearts. Values are means ± S.E. *P<0.05.

Supplementary Figure S7. Heat-treatment of Tg(hs:let-7i)30 induces suppression of TNFα associated transcripts, Related to Figures 5, 6. qPCR studies of the 9 predicted direct target genes of let-7 show significant downregulation of stf3, jund, rrm2 and mcm5. Conversely, fosl2 was upregulated by ~2-fold.

Supplementary Figure S8. CAY10500 mediated change in expression of TNF-α associated genes, Related to Figures 6, 7. Following treatment with vehicle or CAY10500 at 5ug/g and 15ug/g hearts were extracted for total RNA collection and cDNA synthesis. Real-time qPCR studies reveal significant downregulation of most let-7 predicted target genes at 15ug/g. Treatment with 5ug/g did not show differences when compared to PBS control. (n=5). Values are means ± S.E. *P<0.05 compared to control heart ventricles.
**dre-let-7 family**

Let-7a: 5' uagguacuagguuguauaguu 3'
Let-7b: 5' uagguacuagguuguugugguu 3'
Let-7c: 5' uagguacuagguuguauagguu 3'
Let-7d: 5' uagguacuugguuguaugguu 3'
Let-7e: 5' uagguacuagauuggaaauaguu 3'
Let-7f: 5' uagguacuagauuggauaguu 3'
Let-7g: 5' uagguacuagauuguguguuuuuu 3'
Let-7h: 5' uagguacuaguuguauaguu 3'
Let-7i: 5' uagguacuaguuugugcuguu 3'
Let-7j: 5' uagguacuaguuuuguacaguuu 3'
A. wt timecourse

- Lin-28 qPCR fold-change:
  - Uninjured
  - 3 dpa
  - 5 dpa

B. Tg(hs:lin28a)

- Lin-28 qPCR fold-change:
  - Line 9
  - Line 25
  - Line 30

C. Tg(hs:lin28a)

- Let-7i qPCR fold-change:
  - Line 9
  - Line 25
  - Line 30

D. Mef2 PCNA

- Control
- Tg(hs:lin28a)30

E. CM proliferation indices (%)

- Control
- Line 30
### Table S1. Gene expression primer sequences, Related to Figures 1, 2, 5.

| Gene     | Forward Primer (5'-3') | Reverse Primer (5'-3')                     |
|----------|------------------------|-------------------------------------------|
| Let-7a   | CCGAGCTGAGGTAGTTGTATA  | PerfeCTa Universal PCR Primer             |
|          |                        | (Quanta Biosciences)                      |
| Let-7b   | CGTTCTGAGGTAGTTGTAGT   | PerfeCTa Universal PCR Primer             |
|          |                        | (Quanta Biosciences)                      |
| Let-7c   | CCGAGCTGAGGTAGTTGTAGT  | PerfeCTa Universal PCR Primer             |
|          |                        | (Quanta Biosciences)                      |
| Let-7d   | Custom LNA oligo by Exiqon | Custom LNA oligo by Exiqon               |
| Let-7e   | Custom LNA oligo by Exiqon | Custom LNA oligo by Exiqon               |
| Let-7f   | CGGTGGGTGGTAGTAGTAGTA  | PerfeCTa Universal PCR Primer             |
|          |                        | (Quanta Biosciences)                      |
| Let-7g   | CCGAGCTGAGGTAGTTGTAC   | PerfeCTa Universal PCR Primer             |
|          |                        | (Quanta Biosciences)                      |
| Let-7h   | Custom LNA oligo by Exiqon | Custom LNA oligo by Exiqon               |
| Let-7i   | CGTTCTGAGGTAGTTGTCT    | PerfeCTa Universal PCR Primer             |
|          |                        | (Quanta Biosciences)                      |
| Let-7j   | Custom LNA oligo by Exiqon | Custom LNA oligo by Exiqon               |
| U6       | GGAACGATACAGAGAAATTAG  | TGGAAACGTTCACGAAT TT GCG                 |
| Lin28a   | ATGCCCGCGAATCCGACATCTC | CTAATCTGTGCCCTGCGAGTAA                    |
| Let-7i pre| CCCCAGTAGGAGTTAGCTATCA  | GACAGAAAAGCTTTAGCGACGCAGTT               |
| Cm1c2    | TCCAAAGAGGGTGAGGAGAAGA | GCACACTAGGGAGCGTAGA                      |
| Tcf21    | CGACAGATACCTGCGCAATGA  | GTTTGCCAGCCACCATAA A                     |
| Fli1a    | GGGCTGTGCAAAACAT       | GGGCTGTGCAAAACAT                        |
| Atf3     | TTGTCTGCTTCTTCTTAGAT   | GATGTCTTGTTTGTTGTGTTT                    |
| Cxcr4b   | CACTGCTGTCTCAACCCCATC  | GTGACCTGTACTCCTGCTATG                    |
| Fosl2    | CTAGCATACTAGCTGGCTCTAA | GTCATCCAGTAACCTA AAA                    |
| JunD     | CATCAAAGCCGAGGAGAAGAA  | GCTTCTAGGGACCCACTTTT                     |
| Klf6a    | TGTTCGGCTAGCTACTAAAT   | ACCAGCTTATAACAAGGACTAC                   |
| Mcm5     | ACACAGCTACCTACAGACTCT  | CAGTCCCTCTCTCCAAATAC                    |
| Mfsd2ab  | CCGAGATTTTGCTGTCTTACT  | GATATGTCCTCACTCTGGCGTCTTTT               |
| Plaua    | ACAGTCCAGACCTTGCTCATC  | TTGTCACCTGGACCTGCTCATTC                 |
| Rrm2     | TTTCGGCGCTCCTCATTTA    | TCGGACTCTGCTGCTATTC                     |
| Rpl13a   | TCTGGAGCCTAGCTAAGGATGATG | AGACGCAAATCTTGAGAGCGAG                  |

Custom LNA oligo: oligo sequence and location of LNA nucleotides are considered proprietary by Exiqon and were not provided.
Table S3. Predicted binding sites between TNFα related factors 3-UTRs and let-7i, Related to Figure 5.

**Performing Scan: dre-let-7i vs ENSDARG00000035909_ ENSDARG00000009511_tnfa**

| Forward | Score: 124.000000 | Q:2 to 21 | R:30 to 52 | Align Len (20) | (70.00%) | (75.00%) |
|---------|-------------------|-----------|-----------|---------------|---------|---------|
| let-7i: | 3' ttGTCGTGTTTGATGAGt | 5'        |           |               |         |         |
|         | ||| | :||| | | |||||       |
| tnfa:   | 5' aaCAGAAGAACCAATGACCCTc | 3'       |           |               |         |         |

Energy: -15.570000 kCal/Mol

**Performing Scan: dre-let-7i vs ENSDARG00000029072_ENSDART00000033494_klf6a_Kruppel-like**

| Forward | Score: 136.000000 | Q:2 to 19 | R:1503 to 1522 | Align Len (17) | (64.71%) | (76.47%) |
|---------|-------------------|-----------|---------------|---------------|---------|---------|
| let-7i: | 3' ttgtCGTGTTTGATGAGt | 5'        |               |               |         |         |
|         | || | : ||||:||| |       |
| klf6a:  | 5' ccttGC-CTTG-TACTGCCTc | 3'       |               |               |         |         |

Energy: -13.860000 kCal/Mol

| Forward | Score: 145.000000 | Q:3 to 18 | R:1152 to 1173 | Align Len (15) | (73.33%) | (93.33%) |
|---------|-------------------|-----------|---------------|---------------|---------|---------|
| let-7i: | 3' ttgtcGTGTTTGATGAGgt | 5'       |               |               |         |         |
|         | :| | :||:|||:||| |       |
| klf6a:  | 5' tttctTAAGATTACTACCTga | 3'     |               |               |         |         |

Energy: -16.860001 kCal/Mol

**Performing Scan: dre-let-7i vs ENSDARG00000007823_ENSDART00000022060_atf3_activating**

| Forward | Score: 135.000000 | Q:2 to 21 | R:868 to 888 | Align Len (19) | (68.42%) | (78.95%) |
|---------|-------------------|-----------|-------------|---------------|---------|---------|
| let-7i: | 3' ttGTCGTGTTTGATGAGt | 5'        |             |               |         |         |
|         | :| | :||:|||:||| |         |
| atf3:   | 5' ttGGCCAC-TGCTACCACCTc | 3'       |             |               |         |         |

Energy: -20.740000 kCal/Mol
### Performing Scan: dre-let-7i vs ENSDARG00000075265_ENSDART00000110064_plaua_plasminogen

| Forward | Score: 156.000000 Q:2 to 21 R:81 to 105 Align Len (22) (59.09%) (77.27%) |
|---------|--------------------------------------------------------------------------|
| let-7i: | 3' ttGTCGTGT-T-T-GATGATGGAGt 5'                                             |
|         | || :|| | : | :||||| | |
| plaua:  | 5' cgCATTATATATGGCAGCTACCTCt 3'                                           |
| Energy: | -15.360000 kCal/Mol                                                       |

### Performing Scan: dre-let-7i vs ENSDARG00000067850_ENSDART00000097755_jund

| Forward | Score: 143.000000 Q:2 to 21 R:2681 to 2704 Align Len (22) (72.73%) (77.27%) |
|---------|--------------------------------------------------------------------------|
| let-7i: | 3' ttGTCGT-GTTTG-ATGATG-GAGt 5'                                           |
|         | |||| || || || || || || :|| |
| jund:   | 5' caCAGCAGC-AACAAACTACATTCc 3'                                           |
| Energy: | -21.840000 kCal/Mol                                                       |

### Performing Scan: dre-let-7i vs ENSDARG00000040623_ENSDART00000059480_fosl2_fos-like

| Forward | Score: 142.000000 Q:2 to 21 R:739 to 759 Align Len (20) (55.00%) (85.00%) |
|---------|--------------------------------------------------------------------------|
| let-7i: | 3' ttGTCGT-GTTTGATGATGGAGt 5'                                             |
|         | || :| ::::|| | :|||||
| fosl2:  | 5' gcCA-TATTGGGCT-CTGCCTCc 3'                                            |
| Energy: | -14.560000 kCal/Mol                                                       |

| Forward | Score: 138.000000 Q:2 to 21 R:630 to 651 Align Len (21) (71.43%) (76.19%) |
|---------|--------------------------------------------------------------------------|
| let-7i: | 3' ttGTCGT-GTTTGATGATGG-AGt 5'                                           |
|         | || || |||| | ||||: || |
| fosl2:  | 5' agCATCAGCAAA-T-CTACTGTCa 3'                                           |
| Energy: | -17.389999 kCal/Mol                                                       |
Performing Scan: dre-let-7i vs ENSDARG00000041959_ENSDART00000061499_cxcr4b_chemokine

Forward: Score: 115.000000  Q:2 to 21  R:209 to 232 Align Len (22) (59.09%) (72.73%)

let-7i: 3' ttGTC-GTGTTTGATGA-T-GGAGt 5'

|| | :||| :|| | ::

cxcr4b: 5' gcCAGACCTAAA-GGCTGAGCCTTg 3'

Energy: -12.220000 kCal/Mol

Performing Scan: dre-let-7i vs ENSDARG00000019507_ENSDART00000024316_mcm5

Forward: Score: 131.000000  Q:2 to 21  R:177 to 199 Align Len (21) (61.90%) (76.19%)

let-7i: 3' ttGTCGT-GTTTGATGGAG-AGt 5'

|| | :: ::|| || ||::

mcm5: 5' taCAACAGTATGCT-CTACTGTCt 3'

Energy: -20.150000 kCal/Mol

Performing Scan: dre-let-7i vs ENSDARG00000035909_ENSDART00000047461_mfsd2ab_major

Forward: Score: 143.000000  Q:2 to 21  R:309 to 333 Align Len (23) (56.52%) (78.26%)

let-7i: 3' ttG-TCGTG-TTTG-AT-GATGGAGt 5'

| | | :: ::|| || ||::

mfsd2a: 5' gaCGAG-GTGGGACATATCTACCTTt 3'

Energy: -15.890000 kCal/Mol