High content image analysis reveals function of miR-124 upstream of Vimentin in regulating motor neuron mitochondria

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microRNAs (miRNAs) are critical for neuronal function and their dysregulation is repeatedly observed in neurodegenerative diseases. Here, we implemented high content image analysis for investigating the impact of several miRNAs in mouse primary motor neurons. This survey directed our attention to the neuron-specific miR-124, which controls axonal morphology. By performing next generation sequencing analysis and molecular studies, we characterized novel roles for miR-124 in control of mitochondria localization and function. We further demonstrated that the intermediate filament Vimentin is a key target of miR-124 in this system. Our data establishes a new pathway for control of mitochondria function in motor neurons, revealing the value of a neuron-specific miRNA gene as a mechanism for the re-shaping of otherwise ubiquitously-expressed intermediate filament network, upstream of mitochondria activity and cellular metabolism.
control of axonal transport. Our study reveals that Vim functions as a regulator of mitochondrial activity in motor neurons, downstream of miR-124.

Results
Here, we tested the impact of miRNAs on motor neuron morphology and function, which led us to discover a new pathway for regulation of mitochondrial activity, downstream of miR-124.

We first calibrated a transfection system for miRNA in primary mouse motor neurons. We isolated motor neurons from embryonic spinal cords and transfected a hematopoietic miRNA, miR-142, or a scrambled dsRNA sequence at a concentration of 0.1 ng/µl or 0.5 ng/µl. We then measured miRNA levels in cell lysates by quantitative real time PCR (qPCR), 72 hours post transfection. Transfected miR-142 repressed the expression of a known target, Cofilin 2 (Cfl2) by 70% after 72 hrs., relative to untransfected or scrambled mimic controls. Thus, an exogenous, transfected, miRNA mimic functionally silences endogenous targets in primary mouse motor neurons.

Then we selected nine different miRNA candidates for investigation, including miR-9, miR-29, miR-135, miR-138, miR-30e, miR-124, miR-218, miR-10a and miR-206. A qPCR study revealed that the synthetic mimics upregulated miRNA expression, 72 hrs after transfection. None of the nine miRNAs tested influenced cell numbers. miR-124 was the only miRNA to reduce mean axonal outgrowth per cell and mean number of branches per cell. 500 Tuj1+ neurons quantified per field, 2 fields/well and 6 wells per treatment in five independent experimental repeats. Data collected from >30,000 Tuj1+ neurons per treatment. Averages ± SEM, Student’s t-test. *P-value < 0.05.

Figure 1. High content image analysis reveals the impact of miR-124 on primary motor neuron morphology. (a) Values for nine individual miRNAs, all transfected at 0.5 ng/µl, to mouse primary motor neurons. miRNA expression levels displayed on the Y-axis as 40 minus qPCR cycle threshold (40-Ct), on a Log2 scale. All miRNAs were significantly overexpressed. (b) A diagram describing the method: Spinal motor neurons were isolated from E13.5 mouse embryos and seeded on a 384-multwell plate. Culture was transfected with different miRNA mimics using Bravo automated liquid handling robot. 72 hrs later, cells were fixed, stained with anti Tuj1 antibody and DAPI. Two fluorescent micrographs were captured per well (ImageXpress Micro and MetaXpress software, Molecular Devices). (c) Cell numbers (Cell), neurite outgrowth per cell (outgrowth) and number of branches per cell (branches), were quantified with serial doses of the stress-inducing agent, Sodium Arsenite (15, 30 and 60 µM, for 60 minutes). See Methods and Sup. Figure 2. (d) None of the nine miRNAs tested influenced cell numbers. miR-124 was the only miRNA to reduce mean axonal outgrowth per cell and mean number of branches per cell. 500 Tuj1+ neurons quantified per field, 2 fields/well and 6 wells per treatment in five independent experimental repeats. Data collected from >30,000 Tuj1+ neurons per treatment. Averages ± SEM, Student’s t-test. *P-value < 0.05.
To uncover the underlying molecular mechanism responsible for miR-124 activity, we performed transcriptome profiling, using next generation sequencing (NGS). Total RNA was extracted from primary motor neurons and 3′ cDNA libraries were constructed and sequenced. Hierarchical clustering analysis of mRNA expression depicted a unique expression profile for neurons that overexpressed miR-124, which was distinguishable from cells transfected with scrambled control oligos (dendrogram of Pearson correlation coefficient, Fig. 2a).

Sylamer analysis of 6500 expressed mRNAs, from neurons expressing scrambled control mimics or miR-124, uncovered two enriched motifs, which matched the miR-124 ‘seed’ sequence. However, such enrichment was not evident for any other miRNA gene (Fig. 2b). We conclude that miR-124 overexpression had a widespread and specific impact on motor neuron mRNA expression profile.

Gene ontology (GO) analysis was carried out for approximately 1100 mRNAs that were significantly up- or downregulated, following miR-124 overexpression (corrected P-value < 0.05), using database for annotation, visualization and integrated discovery (DAVID) software. This analysis revealed an enrichment for mitochondrial-related genes and is further described in Table 1.

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To characterize the targets and pathways that are regulated by miR-124, we intersected the list of mRNAs that were repressed more than twofold by miR-124 overexpression in the NGS data, with the list of predicted miR-124 targets (TargetScan). Three genes that harbor conserved miR-124 binding sites, were also downregulated more
than twofold by miR-124 overexpression, namely, Poly pyrimidine Tract Binding Protein 1 (Ptbp1, MGI:97791),
Mid kine (Mdk, MGI:96949) and Vimentin (Vim, MGI:98932; Fig. 4a). qPCR study validated that miR-124 over-
expression inhibited Vim to ~1/3 its levels compared with control cells that were treated with scrambled oligos.
Ptbp1, an established miR-124 target49, and Mdk were downregulated to ~1/2 their expression level (Fig. 4b).
Interestingly, Vim is a known regulator of mitochondria localization and activity 31,32, with miR-124 binding
sites that are conserved across several vertebrate species (Fig. 4c). Furthermore, we identified molecular evidence
for direct interactions of miR-124 with the target Vim, in Argonaute CLIP studies 50,51 and a Vim 3′UTR reporter
was inhibited by miR-124 mimics in hepatocellular carcinoma cells 52.

We sought to inhibit Vim independently of miR-124, and test its effect on cell morphology and mitochondria
activity. Lentiviral transduction of primary motor neurons was very efficient (Sup. Figure 5) and allowed us
to effectively knockdown Vim by shRNA53. Vim shRNA reduced Vim mRNA and protein levels (Fig. 4d,e, Sup.
Figure 6a). Accordingly, high content image analysis of Vim knockdown depicted reduction in neurite outgrowth
and branching (Fig. 4f), recapitulating miR-124 activity. In addition, Vim knockdown resulted in inhibition of
mitochondria activity in axons, but not in the soma (Fig. 4g–i). Differences in the effect of miR-124 on soma may
be the result of transfection efficiency. Intriguingly, miR-124 is primarily peri-nuclear 54. Therefore, the soma
compartment may be less amendable to manipulation, whereas, in the axon, where the miRNA is expressed at
lower levels, the effects of miR-124 overexpression were consistent across all observations. In conclusion, Vim
pheno-copies miR-124 functions, further suggesting that both genes are engaged in the same pathway.

Based on the above observations, we hypothesized that Vim is a novel effector of miR-124 in a pathway that
regulates mitochondria function. Therefore, we tested whether upregulating Vim levels is sufficient for recover-
ing mitochondrial activity. Doxycycline-induced expression of Vim, from a lentiviral vector that does not har-
bor the 3′UTR and hence is not inhibited by miR-124, upregulated Vim mRNA and protein levels (Fig. 5a,b,
Sup. Figure 6b). Exogenous Vim was also sufficient to alleviate miR-124-dependent inhibition of mitochondrial

| Gene Ontology term | genes count | P-value |
|--------------------|-------------|---------|
| Organelle envelope  | 27          | 1.80E-05|
| Envelope           | 27          | 1.90E-05|
| Mitochondrial part | 24          | 2.34E-04|
| Organelle inner membrane | 23 | 1.80E-07|
| Mitochondrial membrane | 22  | 1.00E-05|
| Mitochondrial envelope | 22   | 2.50E-05|
| Mitochondrial inner membrane | 21  | 1.30E-06|
| Inorganic cation transmembrane transporter activity | 16 | 8.40E-09|
| Monovalent inorganic cation transmembrane transporter activity | 14 | 4.70E-09|
| Hydrogen ion transmembrane transporter activity | 13 | 2.40E-08|
| Redox-active center | 8           | 4.60E-06|
| Cell redox homeostasis | 8  | 1.00E-04|
| Thioredoxin-like    | 6           | 3.40E-04|
| Thioredoxin-like    | 6           | 3.40E-04|
| Disulphide isomerase | 5   | 1.20E-06|
| Domain:Thioredoxin 2 | 5     | 9.30E-06|
| Domain:Thioredoxin 1 | 5      | 9.30E-06|
| Thioredoxin-like subdomain | 5 | 2.50E-05|
| Thioredoxin, conserved site | 5 | 7.80E-04|
| Thioredoxin domain | 5           | 9.10E-04|
| Oxidoreductase activity, acting on heme group of donors, oxygen as acceptor | 5 | 6.90E-04|
| Oxidoreductase activity, acting on heme group of donors | 5 | 6.90E-04|
| Cytochrome-c oxidase activity | 5 | 6.90E-04|
| Heme-copper terminal oxidase activity | 5 | 6.90E-04|
| PIRSFO01487:protein disulfide-isomerase | 4 | 1.90E-04|
| Protein disulfide isomerase activity | 4 | 3.00E-04|
| Intramolecular oxidoreductase activity, transposing S-S bonds | 4 | 3.00E-04|
| Intramolecular oxidoreductase activity, interconverting keto- and enol-groups | 4 | 4.50E-04|
| Intramolecular oxidoreductase activity | 4 | 3.10E-02|
| Protein disulphide isomerase | 3 | 1.70E-03|

Table 1. Gene ontology (GO) terms for changes in motor neuron mRNA expression after miR-124 overexpression.
Top 30 Gene ontology (GO) term analysis of up- or downregulated mRNAs after miR-124 overexpression (corrected
P-value < 0.05). Table columns include DAVID46 term names, number of genes counted in each category and
p-value for the statistical significance of the category. There is noticeable overrepresentation of gene ontology terms
related to mitochondria structure or function in response to miR-124.
function, relative to miR-124 alone (Fig. 5c–e). Furthermore, when counting the numbers of mitochondria in axons, we observed rescue of axonal mitochondria occupancy by exogenous Vim (Fig. 5f–h; We also noted some effect without Dox that is probably due to leaky Vim expression).

Finally, we used live imaging microscopy to test if the new miR-124-Vim axis regulates mitochondria motility in axons. We identified two subtypes of motile mitochondria: mitochondria that were continuously running in an uninterrupted manner along the axon over a distance of >10 μm at average speed >0.2 μm/sec., or mitochondria that displayed intermittent pauses in the same location for ≥3 frames in succession. Intriguingly, miR-124 caused mitochondria to pause more frequently on anterograde route, than in control samples, but did not affect retrograde transport. This may explain the re-distribution of mitochondria after overexpression of miR-124 and relative axonal depletion. Exogenous Vim expression rescued this phenotype and normalized mitochondria running/pausing dynamics (Fig. 6a–c). Furthermore, measurements of pause duration depicted asymmetry in anterograde vs. retrograde (Fig. 6d,e). We noted some effect without Dox that is probably due to leaky Vim expression. Parameters of run length and mean speed, were not changed by miR-124 and Vim (Sup. Figure 7). Therefore, anterograde mitochondria transport is influenced by the number of mitochondria pausing and the typical time interval spent resting during their run. We conclude that the mechanism for control of mitochondria running/pausing propensities in motor neurons involves Vim and miR-124 in a fashion affecting anterograde but not retrograde transport.

Taken together, we characterized a novel pathway in motor neurons downstream of miR-124 in regulation of mitochondria dynamics, distribution and activity. In this pathway, Vim, functions as an important effector of miR-124, revealing a surprising mechanism for controlling energy metabolism in motor neurons by neuronal miRNAs and intermediate filaments.

Discussion
In the current study, we employed high content image analysis, next generation sequencing and molecular approaches for discovery of a new pathway that is affecting motor neuron axon morphology and mitochondria homeostasis. An initial screen led us to focus on miR-124, which was the only miRNA that exhibited abnormal axonal morphology, out of nine miRNAs that were tested. miR-124 is one of the most abundant miRNA in many neuronal subtypes and is conserved from insects to mammals. miR-124 drives neuronal differentiation, promoting neuroblasts cell-cycle exit and neuron-specific alternative splicing and chromatin remodeling via silencing of Ptp11 and Actin like 6 A/BAF53a, respectively. Furthermore, miR-124 levels remain high in postmitotic neurons, suggesting that it plays a role also in maintenance of the differentiated state of neurons. However, miR-124 roles in motor neuron were not thoroughly investigated.
We demonstrated that miR-124 regulates mitochondrial activity and localization. The cluster of mitochondrial genes that responded to miR-124 overexpression was not particularly enriched in direct targets of miR-124, suggesting indirect regulation. The intermediate filament Vim is a key effector of miR-124 upstream of mitochondria function and localization. Vim knockdown by other means, pheno-copied miR-124 overexpression and an exogenous Vim that does not harbor miR-124 binding sites, rescued the mitochondrial phenotype.

Precise mitochondria localization is critical for maintaining energy and calcium homeostasis in neurons. Appropriate mitochondria localization is essential for neurite outgrowth. Minin et al., have shown that Vim regulates mitochondrial activity and motility in other cell types, which is consistent with the discovery of a new miR-124 - Vim axis for unidirectional control of mitochondria transport in axons. miR-124 and Vim

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**Figure 4.** Vimentin regulates neuron morphology and mitochondria function (a) Venn diagram of predicted miR-124 targets (TargetScan) and transcripts that were experimentally repressed >2 fold by miR-124 overexpression in primary motor neurons, relative to control conditions. (b) qPCR analysis of the three mRNAs that obeyed both criterions: Vim, Ptbp1 and Mdk, in primary motor neurons transfected with miR-124 oligos (N = 3), relative to controls (N = 3). (c) miR-124 recognition element at Vim 3′UTR is conserved in vertebrate species. Knockdown of Vim by shRNA lentiviruses downregulated (d) Vim mRNA and (e) Vim protein expression, relative to non-targeting shRNA controls. A full view of the Western blot is in Sup. Figure 6. (f) High content image analysis of cell numbers, mean axonal outgrowth per cell and mean number of branches per cell. 100 Tuj1+ neurons quantified per field, 8 fields/well, 6 replicates per treatment. Data collected from >24,000 Tuj1+ neurons per treatment. (g) Confocal fluorescent micrographs of primary motor neurons, 72 hrs. post transduction with scrambled-shRNA or Vim-shRNA, stained with TMRE (red) and nuclei (blue, Hoechst). Transduction efficacy >80%. Scale bars, 20 μm. Bar graphs quantification of fluorescent signal intensity in (h) axons and (i) soma. Quantification of 3 random positions per compartment; 9 neurons per condition. Data from three replicates and the study performed in three independent experimental repeats. All graphs present averages ± SEM, Student’s t-test. *P-value < 0.05, **p < 0.01, ***p < 0.001.
Figure 5. miR-124 regulates mitochondria activity via Vimentin. Primary motor neurons of control, miR-124 overexpression alone or transduced in addition with lentiviral vector for Doxycyclin-dependent expression of Vim (>80% transduction efficacy, transduction 24 hrs. before transfection of miRNA mimics), without or with the chemical inducer (Dox). (a) Vim mRNA and (b) VIM protein expression without or with Dox. Three experimental repeats, Averages ± SEM, Student’s t-test. A full view of the Western blot is in Sup. Figure 6. (c) Confocal fluorescent micrographs of primary motor neurons, stained with MitoTracker (green) and TMRE (red), merged signal (yellow) and nuclei (blue, Hoechst). Scale bars, 20 μm. Bar graphs quantification of fluorescent signal intensity in (d) axons and (e) soma. Quantification of three random positions per compartment; 9 neurons per condition; Data from three replicates and the study performed in three independent experimental repeats. Averages ± SEM, ANOVA + Duncan’s new multiple range test (MRT) *P-value < 0.05, **p < 0.01, ***p < 0.001. (f) Snapshot from mitochondria live imaging along axons of primary motor neurons. Bar graphs quantification of (g) axonal mitochondria density/μm and (h) mean TMRE intensity in control axons (n = 48), miR-124 overexpression alone (n = 52), miR-124 and Vim without Dox (n = 34) or with Dox (n = 34). Averages ± SEM, Student’s t-test, P-value *p < 0.05, **p < 0.01.
asymmetric action is further evocative of kinesins and dynein motor proteins that reciprocally serve anterograde and retrograde transport. However, a direct molecular link to the classic kinesin/dynein system is still missing.

miRNA dysregulation and mitochondrial impairments are repeatedly observed in ALS. Our analysis reveals that miR-124 overexpression may be disadvantageous to primary motor neurons, in accordance with reported miR-124 upregulation in late ALS stages in mouse brains and with injurious miR-124 overexpression in adult hippocampus/prefrontal cortex. In summary, we propose that miR-124 expression levels should be...
tightly kept within defined margins and that a novel miR-124 - Vim pathway reveals a mechanism, by which miRNAs regulate of axonal mitochondria transport.

Materials and Methods

Primary motor neuron culture. All experiments were performed in accordance with relevant guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at Weizmann Institute of Science. Primary motor neurons were isolated and cultured as described34 with the following modification: spinal cords were dissected from ICR mouse embryos at embryonic day 13.5 (E13.5). Motor neurons were dissociated with papain (2 mg/ml, Sigma), separated through Optiprep gradient (Sigma) and seeded either on 13 mm coverslips (200,000 cells/cover slip, Thermo scientific) or on 384 multiwell plates (7500 cells/well, Greiner bio-one, cat# 781091) using a liquid handling device (GNF Systems), pre-coated with poly-ornithine (3 μg/ml) or poly-L-Lysine (Sigma P4707, 0.002% in Borate buffer 0.1 M pH 8.5, Sigma) and then Laminin (3 μg/ml, Gibco | Thermo Fisher Scientific). Motor neurons were cultured with Neurobasal (Gibco | Thermo Fisher Scientific)/B27 (Gibco | Thermo Fisher Scientific) medium supplemented with 2% horse serum (Sigma), and 1 ng/ml CNTF, 1 ng/ml GDNF (Peprotech) at 37°C. For live imaging motor neurons were isolated as in67,68.

miRNA mimics transfection. miRNA mimics, were dsRNA oligonucleotides (Integrated DNA Technologies, Inc.), as described in Table 2. dsRNA encapsulated in Neuro9™ nanoparticles (Precision NanoSystems, Inc.)33. Mimics (0.5 ng/μl) were transfected 24 hrs after seeding of primary motor neurons manually or by using Bravo automated liquid handling robot.

Lentiviruses. Vimentin shRNA lentiviruses were described in33. Cells were transduced simultaneously with both versions of sh-Vim lentiviruses, 24 hrs post seeding, and downstream analyses performed at 72 hrs. For overexpression, human Vimentin coding region, was subcloned into FuW-TetO lentiviral vector downstream of tetracyclin response element. A mix of two lentiviruses, one for the expression of Vimentin and the other with FUW-m2rtTA (reverse tetracycline-controlled transactivator), were transduced at MOI = 5, one hour after primary motor neuron seeding. Doxycycline (1 μg/ml, Sigma) was added at 24 hrs, 72 hrs post infection, and downstream analyses performed at 96 hrs.

Mitochondria assays. The following chemicals were added, when mentioned: MitoTracker® Deep Red 633 (M22426, Molecular Probes, 50 nM) tetramethylrhodamine, ethyl ester (TMRE, 200 nM) Hoechst (Sigma, 1 μg/ml) 30 min 37 °C. Oligomycin A (Sigma, 1 μM) for 15 min, in 37 °C and analyzed 24 hrs. afterwards.

Immunostaining and Western Blot analysis. Cells were rinsed with phosphate buffered saline (PBS) 3 time manually or using Biotek EL406 washer/dispenser for automated 384-well microplates, fixed with 4% formaldehyde (ChemCruz®) for 15 min, permeabilized and blocked in 0.1% Triton X – 100 (Sigma), 2% BSA for 20/60 min for 384/24 well plate setup, respectively. Incubation with anti-neuronal Class III beta Tubulin (Tuji) (1:1000, MRB-435P, Covance, 1 hr) and/or anti-ATPSA, (1:500,ab14748, Abcam) or anti-Microtubule-associated protein 2 (MAP2) (1:500, sc-20172) was followed by anti Cy2- conjugated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch, 1 hr), nuclear staining with 4'6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/ml, Sigma, 5 min.) and 3 cycles of PBS rinsing. For Western blot studies, 30 μg protein extracts were denatured by boiling in × 5 sample buffer (60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 3-mercaptoethanol, 0.1% bromophenol blue) for 5 min., resolved by 8% SDS-PAGE, 100–120 V , 70 min. and transferred to nitrocellulose membrane (Whatmann, 1040383) at 250 mA, 70 min. Membranes were stained with Ponceau (Sigma-Aldrich, P7170), blocked for 60 min. at Room Temperature (RT) with 5% milk, 0.05% TWEEN-20 and incubated, rock with primary antibodies O.N. at 4 °C, namely, monoclonal mouse anti-Vim supernatant (a gift from Benny Geiger, Weizmann Institute of Science) or monoclonal mouse anti-alpha-Tubulin, (DM1A Sigma T9026 1:2000 in 5% Bovine Serum Albumin, 0.02% sodium azide, 5 drops of phenol red in 0.05% PBST). Membranes were washed 3 × 5 min., RT with 0.05% PBST and incubated 60 min. with horseredish peroxidase (HRP)- conjugated anti-mouse secondary antibodies. Membranes were washed 3 × 5 min., processed with EZ-ECL Chemiluminescence detection kit for HRP (Biological Industries, 20-500-120) and visualized by ImageQuant™ LAS 4000 (GE Healthcare Life Sciences). Vim densitometry values normalized to Tubulin in the same lane. Representative data in main Figs 4c and 5b are depicted on blots, shown in full in Sup. Figure 6.

Imaging. For high content image analysis, eight/two micrographs taken in 24 /384 well plate setups, respectively, using automated fluorescence microscope (ImageXpress Micro and MetaXpress software, Molecular Devices). Motor neuron were defined by positive to Tuji1 staining (Alexa488, FITC channel) and nuclear stain (DAPI). Phenotypic parameters were quantified with relevant MetaXpress High-Content Image Acquisition modules (Neurite Outgrowth, MWCS). Confocal microscopy performed on Carl-Zeiss 710. Mitochondria Live imaging was performed on Nikon Eclipse Ti Spinning disc confocal with Yokogawa CSU-X-1, 60X oil-immersion lens. Andor iXON3 EMCCD camera under controlled environment (37 °C, 5% CO2). Axonal transport analysis was carried by analysis of time-lapse images using imageJ or MATLAB, as in67,68. For transmission electron microscopy, motor neuron were prepared, following66 and electron micrographs were captured with a FEI Tecnai SPIRIT transmission electron microscope (FEI, Eindhoven, Netherlands), operated at 120 kV and equipped with an EAGLE CCD Camera.

RNA analysis and Next Generation Sequencing. Total RNA was isolated with miRNasy micro kit (Qiagen), assessed with Nano Drop ND-1000 Spectrophotometer (Peqlab) and reverse transcribed to cDNA. qPCR, performed with SYBR Green (Thermo Fisher Scientific or Qiagen). miRNA/mRNA levels were normalized to U6/hypoxanthine phosphoribosyltransferase 1 (Hprt), respectively. Primer sequences are described in Table 2.
miRNA mimics, designed as dsRNA oligonucleotides r- RNA bases; r- * - Phosphorothioated RNA base; m- 2' O-methyl RNA base

| miRNA mimics         | Anti- sense mimic | Sense mimic                  |
|----------------------|-------------------|------------------------------|
| miR-142-3p           | rU*rG*rG*rG*rG*rU | TCTGCGTTGGCAGCAGGCA          |
| miR-9-5p             | rU*rC*rU*rG*rG*rU | TCTGCGTTGGCAGCAGGCA          |
| miR-10a-5p           | rU*rA*rG*rG*rU   | TCTGCGTTGGCAGCAGGCA          |
| miR-29a-3p           | rG*rC*rG*rG*rU   | TCTGCGTTGGCAGCAGGCA          |
| miR-30c-5p           | rU*rA*rG*rG*rG*rU | TCTGCGTTGGCAGCAGGCA          |
| miR-218-5p           | rU*rG*rC*rG*rG*rU | TCTGCGTTGGCAGCAGGCA          |
| miR-206-3p           | rU*rG*rC*rG*rG*rU | TCTGCGTTGGCAGCAGGCA          |
| miR-138-5p           | rA                  | TCTGCGTTGGCAGCAGGCA          |
| miR-135a-5p          | rU                  | TCTGCGTTGGCAGCAGGCA          |
| miR-124-3p           | rA                  | TCTGCGTTGGCAGCAGGCA          |
| miR-142-3p           | rA                  | TCTGCGTTGGCAGCAGGCA          |

Library prep for NGS, following [46] and sequencing performed on Illumina 2500 at 50 bp single read. Fasta files created (Illumina CASAVA 1.8.2 software) and mapped (TopHat2 version v2.0.10) against the mouse genome, build mm9. Approximately, 85–90% mapping rate was observed. Only uniquely mapped reads determined the number of reads per gene (HTSeq-count script 0.6.1p1). Differentially expressed genes, were determined by padj < 0.05 and an absolute fold change > 2 (DESeq 2 package v1.4.5) and hierarchical clustering using Pearson dissimilarity and complete linkage was performed to explore gene expression patterns (Matlab 8.0.0.783).

Statistical analysis. Analysis was performed manually or with GraphPad Prism 6 for Student’s t-test, test of proportion or one way ANOVA with post-hoc Newman-Keuls or one way ANOVA with Duncan’s new multiple range test (MRT), as indicated. Results are given as mean ± standard error of the mean (s.e.m). The null hypothesis was rejected at the 0.05 level (*), 0.01 (*) or 0.001 (***) Non-significant values on statistical test are not mentioned in the figures. Gene Ontology analysis was performed using DAVID[9].

Table 2 List of synthetic DNA and RNA oligos used in the study.

| Gene | Forward | Reverse |
|------|---------|---------|
| CR2  | TCTCGTCGCCAGTGCCAGCA | ACTCCAGATGGCATAGTCGCCAGCA |
| Hprt | TCTGGTATGCTATACGGCCAAA | TGGCGGCTATCCCAACTCTGCGAGA |
| Vim  | AAATGCGTCGCCCTCTGGT | AGAAATCTGCTCTCCTGCC |
| Ptbp1| ATTCGCTGTTCGTCCAGACA | GTCACTGGAGAAGTCAGCG |
| Mk2  | CCAGGAGACCATCCGCC | TCCCTTCTTCTTCTTG |
| miR-142-3p | TGATGTGTTCTCTACTTGA | Universal primer (Qiagen) |
| miR-9-5p    | TCTTTGTATCTACGTATGA | Universal primer (Qiagen) |
| miR-10a-5p  | TACCCGTGATATGCGAATTGTG | Universal primer (Qiagen) |
| miR-29a-3p  | TAGACACATGTGAAATCTGGTA | Universal primer (Qiagen) |
| miR-30c-5p  | TGTAAACATCTTACACTTCCGC | Universal primer (Qiagen) |
| miR-124-3p  | TTAGGACCGCCTGTAATTGCC | Universal primer (Qiagen) |
| miR-135a-5p | TATGGCATTATTTACCTATGTGA | Universal primer (Qiagen) |
| miR-138-5p  | AGGTGCTTTATGTAATTGAGCCGCG | Universal primer (Qiagen) |
| miR-206-3p  | TGGAAAGTGGATAGTGGT | Universal primer (Qiagen) |
| miR-218-5p  | TGGTCTCTGTACATTACCTTG | Universal primer (Qiagen) |
| RNU6B(U6) | GATGACACGGCAATTCGTGAA | Universal primer (Qiagen) |

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T.Y., R.F., and E.H. Designed research and wrote the paper. T.Y., R.F., Y.J., T.G.P., N.K., I.R., E.Y., S.N., H.W.T., M.E.B., T.S., A.P., E.P., E.H. performed research or/and analyzed data. H.M.B., contributed analytic tools.
Additional Information

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