# ELAVL1 modulates transcriptome-wide miRNA binding in murine macrophages

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## SUPPLEMENTAL EXPERIMENTAL PROCEDURES.

### BMDM culture and PAR-CLIP

Animals were sacrificed using carbon dioxide and bone marrow cells from mouse femur and tibia were collected by flushing through PBS with a 23-guage needle. Bone marrow cells were cultured in one 150mm Petri dishes with complete DMEM and 20% of L929 cell culture medium for 6 days (Weischenfeldt and Porse, 2008).

BMDM for PAR-CLIP experiment were cultured with 100 μM 4-thiouridine (Sigma) for 16 hours, washed with PBS and UV-crosslinked. BMDM were placed on ice and radiated uncovered with 0.15 J/cm$^2$ total energy of 365 nm UV light in a Stratalinker (Invitrogen). BMDM were then harvested by incubation at 37 $^o$C for 10 min with 0.2 mM EDTA in PBS, washed twice with PBS and frozen at -80 $^o$C. The details of the PAR-CLIP protocol were described previously (Hafner et al., 2010a, b) with several minor modifications as follows. We used mouse monoclonal anti mouse AGO2 antibody (WAKO) for Ago2 IP. The expected radioactive labeled RNA-protein complex, which is around 100 kD (Hafner et al., 2010b), was observed by autoradiography (Supplemental figure 1A). ELAVL1 loss did not influence Ago2 expression (Supplemental figure 1B). The cross-linked miRNA or mRNA fragments were isolated for cDNA library preparation and deep sequencing. We used 3’ adaptors, 5’ adaptors,

RT primers, PCR primers from Illumina TruSeq Small RNA Sample preparation Kit. PAR-CLIP small RNA libraries from WT and *Elavl1* KO samples were sequenced for 45 cycles on Illumina HiSeq 2000 platform (Illumina). For LPS treatment, differentiated BMDM were harvested and seeded on to 100-mm petri dishes for experiments. Cells were subjected to serum starvation with RPMI 1640 medium containing 1% FBS for 16 h before stimulation with 0.1 mg/ml LPS (from *E. Coli* 0111:B4; Sigma-Aldrich, L3024) for the times indicated.

**PARalyzer annotation and miRNA target sites predication**

For WT and *Elavl1* KO PAR-CLIP library, PARalyzer was used to identify binding sites as described previously (Corcoran et al., 2011). Briefly, reads that aligned to a mouse MM9 unique genomic location, after correction of T to C mismatches and overlapped by at least one nucleotide were grouped together. Read groups were analyzed for T to C conversions and nucleotide strings containing a greater likelihood of converted T to Cs than non-converted Ts were extracted as clusters. AGO2 PAR-CLIP clusters are defined as having at least 5 reads, exclude genomic repeat regions, and meeting the T to C conversion criteria. We only considered groups of overlapping sequence reads that (1) contain at least one T to C conversion location per group, (2) show more than 5 T to C conversion reads in combined WT and *ELAVL1* KO samples, and (3) exceed a threshold of ≥ 25% T to C conversion frequency (except miRNAs). To compare WT and *Elavl1* KO Ago2 PAR-CLIP dataset, we merged the PAR-CLIP clusters as follows. The overlapping cluster should be least 18 nt in size and the overlapping sequence between samples should have more than 15 nt. If there are multiple sites in adjacent areas in one sample and the sites can not be distinguished from one another, the reads of each cluster will be combined and represented as the total read number for this integrated large cluster sequence.

With this method, we combined 18,940 clusters from WT and 13,945 clusters from Elavl1 KO sample and got 25,476 clusters in total. Each cluster contains a unique sequence with read numbers from WT and *Elavl1* KO. We select the sites with more than 100 reads in combined samples (>98% overlapping) for further miRNA prediction. Clusters that overlapped with predicted miRNA binding site from Target Scan database (TargetScan 6.2, mouse non-conserved and conserved predictions) were identified using custom scripts. To estimate how many identified PAR-CLIP sites contain miRNA binding sites predicted by TargetScan, we sorted the PAR-CLIP sites with combined reads from high to low. Next, the number of PAR-CLIP sites predicted by TargetScan in the interval of every 500 sites was calculated (Signal). The random sequence background was calculated using a random 26 nt sequence from 3'UTRs in the mouse genome that contains a miRNA seed sequence as predicted by TargetScan (Noise). Since Target Scan uses canonical seed match sites (≥7mer1A, i.e. nt 2-7 match with an A across from position one of the mature miRNA), we also used the PITA algorithm to search for miRNA target sites allowing either 1 G:U wobble or 1 mismatch in the 7-8 nt seed site (Segal Lab of Computational Biology) when no TargetScan predictions were found for a given cluster.

**Total RNA isolation, miRNA isolation and high-throughput sequencing for mRNA or miRNA**

Total RNA and small RNAs were isolated as detailed in the manufacturer's protocols (Clonetech) and RNA quality was checked by an Agilent 2100 bioanalyzer (Agilent technologies). Small RNA and mRNA libraries preparation were followed as manufacturer's protocols (Illumina Small RNA v1.5 Sample Preparation Kit and Illumina mRNA sequencing Sample Preparation Kit, Illumina). All libraries were sequenced for single-reads, 42 cycles on the

Illumina Genome Analyzer IIx (Illumina). Data analysis for mRNAseq was performed with guidance and help from Dr. Fabien Campagne (Institute for Computational Biomedicine, Weill Cornell Medical College (WCMC)). Sequencing data were uploaded into the GobyWeb application (Campagne lab) for alignment to mouse MM9 genome. MicroRNA sequencing results were trimmed and mapped to miRBase 855 mouse stem-loop sequences (http://www.mirbase.org/) (version 19) using the Bowtie alignment (http://bowtie-bio.sourceforge.net/index.shtml) program (version 0.12.7) without any mismatch. The alignment reads were normalized as proportion of total miRNA reads and the normalized reads were analyzed for differentially expressed miRNAs using the Fisher's exact test.

**RNA-binding protein Immunoprecipitation (RIP) assay**

The binding of Ago2 with target mRNA was determined by immunoprecipitation followed by qRT-PCR as described (Lopez de Silanes et al., 2004). BMDM ($3x10^7$) isolated from each mouse were lysed with polysome lysis buffer (PLB) supplemented with RNAse inhibitors and protease inhibitors. The supernatant was incubated overnight at 4 °C with control IgG or Ago2 monoclonal antibody (Wako) and then incubated with protein G Dynabeads (40 μl) for 4 hrs at 4 °C. After washing five times, the pellet was treated with 10 units DNAse I (Promega) in 100μl buffer for 10 min at 37 °C and then treated with 5 μg proteinase K (Roche) in 100μl buffer for 30 min at 55 °C. The supernatant is collected and RNA was extracted using acid phenol chloroform (Ambion). Extracted RNA was subject to qRT-PCR analysis as described above.

**Luciferase miRNA target reporter assay**

PCR products of *Zfp36* 3'UTR fragment (entire 762 bp) generated using the forward primer 5'GAGCTCCAAGTGCCTACCTACCCAGTATG and the reverse primer 5'CAGTTCACACTCAAGATTTTATTTAAA were cloned into the SacI and XhoI sites of the multiple cloning site of pmirGLO vector (Promega), a dual luciferase reporter (Firefly and Renilla). Mutations were made in the seed region of miR-27b on *Zfp36* 3'UTR (408-416). Primers containing mutated target sequences are 5'CTGGAATCTTAAGTGCTGCGCAGAGCCGGCTCCCTAC and 5'GTAGGGAGCCGGCTCTGCGCAGCACTTAAGATTCCAG. Mutation for ARE1 binding site was introduced at the poly T region with GC rich sequence. Primers used to generate this mutation are 5' CCCACAATACTATCCTAATGCGTACTAGACCCTGAAGTTC and 5'GAACTTCAGGGTCTAGTACGCATTAGGATAGTATTGTGGG. Mutation for ARE2 binding area was introduced at the TTATTTATT region with 3 G base. Primers used to generate this mutation are 5' CCTTTATTTATGACGACTGTAGTGATTGTATTAAGATTTTATAG and 5' CTATAAAATCTTAATACAATCACTACAGTCGTCATAAATAAAGG.

HEK293T cells were co-transfected with 50ng/ml pmirGLO vector containing *Zfp36* 3'UTR or mutant *Zfp36* 3'UTR with different dose of (0 nM to 40 nM) miR-27b mimics (Thermo Scientific) using Lipofectamine-2000 (Invitrogen). After 40 hr, the cells were lysed and luciferase activity was measured using a luminometer. Firefly Luciferase values were normalized with Renilla luciferase values.

**SUPPLEMENTAL TABLES.**

**Table S1.** Bowtie alignment results shows 211 miRNAs were detected from the miRNAseq data of 2 WT and 2 Elavl1 KO BMDM, relate to figure 2A.

**Table S2.** Goby analysis result shows a list of mRNAs was expressed in BMDM (average RPKM > 0.1), relate to figure 2B. Input mRNAseq data were from 4 WT and 4 Elavl1 KO BMDM.

**Table S3.** The list of predicted miRNA/mRNA pairs for PAR-CLIP sites. Relate to figure 2B.

**Table S4.** PAR-CLIP analysis result shows a list of angiogenic related genes that are regulated by miRNAs. Relate to figure 5.

**SUPPLEMENTAL REFERENCES:**


Corcoran, D.L., Georgiev, S., Mukherjee, N., Gottwein, E., Skalsky, R.L., Keene, J.D., and Ohler, U. (2011). PARalyzer: definition of RNA binding sites from PAR-CLIP short-read sequence data. Genome biology *12*, R79.

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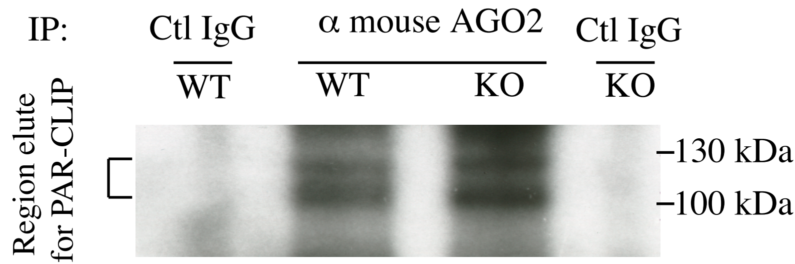
Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, M., Jungkamp, A.C., Munschauer, M.*, et al.* (2010b). PAR-CliP--a method to identify transcriptome-wide the binding sites of RNA binding proteins. J Vis Exp.

Lopez de Silanes, I., Zhan, M., Lal, A., Yang, X., and Gorospe, M. (2004). Identification of a target RNA motif for RNA-binding protein HuR. Proc Natl Acad Sci U S A *101*, 2987-2992.

Weischenfeldt, J., and Porse, B. (2008). Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. CSH Protoc *2008*, pdb prot5080.


**SUPPLEMENTAL FIGURES.**

A.

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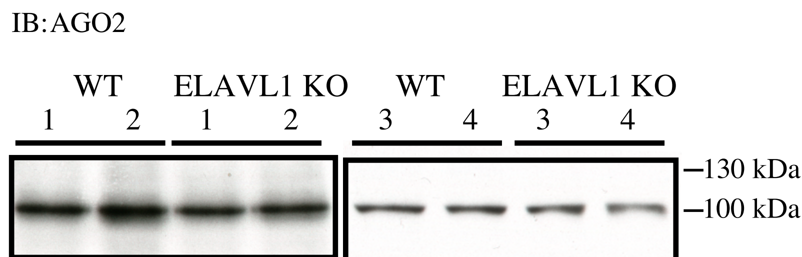


**Figure S1. Mouse AGO2 protein was immuno-precipitated by AGO2 antibody in PAR-CLIP procedure. Related to Figure 1A.**
(A) Autoradiogram image of an SDS-gel resolving radio-labeled RNA cross-linked to Ago2 IPs. Autoradiogram of SDS PAGE gels of RISC complexes are 32P-labeled, cross-linked and immunoprecipitated (IP) with Ago2 IgG but not by non-specific mouse IgG. Protein-RNA complexes on SDS gels were cut from 100 kDa to130 kDa range for library preparation and sequencing. (B) AGO2 expression in Elavl1 KO BMDM. Western blot analysis of Ago2 protein in lysates of BMDM used in the PAR-CLIP experiments.

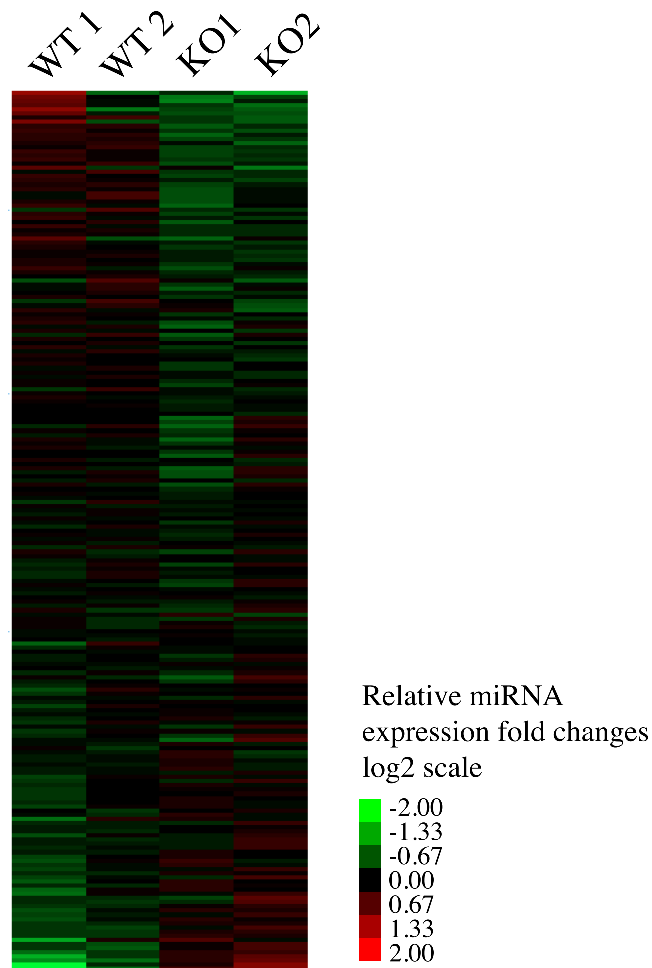


**Figure S2. miRNAseq results from BMDM. Related to Figure 2A.**
miRNA profiles from 2 WT and 2 Elavl1 KO miRNA-seq data. The color code represents log2 scale of relative frequencies fold changes determined by sequencing. Only 5 out of 211 highly expressed mature miRNAs (Reads comprise > 0.001% of total aligned miRNA reads) have more than 2 fold change in expression level (4 increase and 1 decrease after Elavl1 KO).

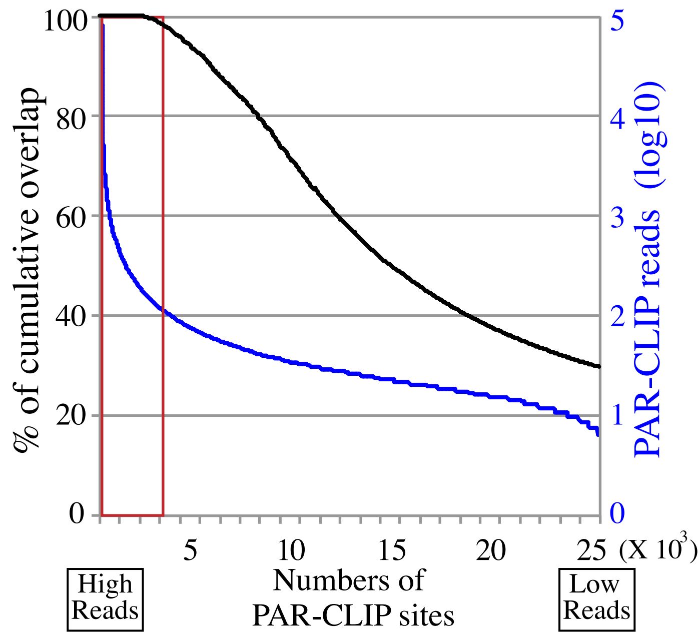


**Figure S3. Identification of high confidence Ago2 PAR-CLIP sites from BMDM. Related to Figure 3A.** Total number of Ago2 PAR-CLIP sites from WT and Elavl1 KO BMDM were sorted based on the number of reads (Right Y axis; blue line). The left Y axis represents % overlap in PAR-CLIP sites between WT and Elavl1 KO samples (black line). Note that high read Ago2 PAR-CLIP sites have high degree of overlap, which is an index of reproducibility between independent replicates. Selected sites that have > 100 combined reads (3,033 sites) were marked with a red box for further analysis.

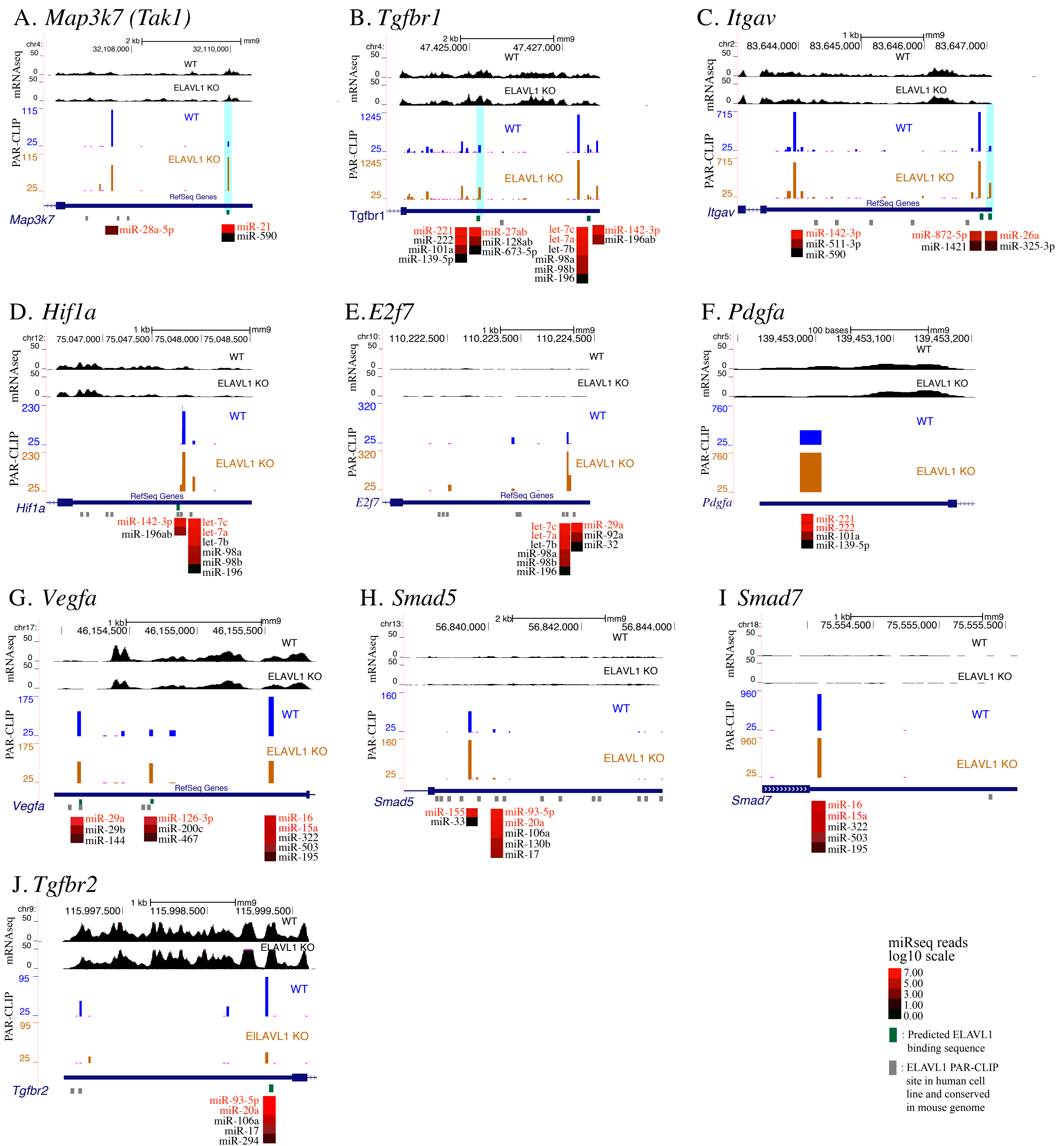


**Figure S4. miRNAs binding is regulated by Elavl1. Related to Figure 5.**
UCSC genome browser views PAR-CLIP data and mRNA-seq data on cell surface receptor genes Tgfbr1 (B), Itgav (C) and Tgfbr2 (J), secreted angiogenic factor genes Pdgfa (F) and Vegfa (G), transcription factor genes Hif1a (D) and E2f7(E), and intracellular signal transducing protein genes Map3k7(A), Smad5 (H) and Smad7 (I). Listed miRNAs below PAR-CLIP signals indicate predicted miRNAs. The color code represents log10 scale of average miRNA reads from miRNA-seq data. Green bars represents potential Elavl1 binding sequences that contain UUUNUUU or less than 2 A base in 7 poly U region (ex. UUAUUAU or UAUUUAU). Red-labeled miRNAs are the most-likely candidates for a given binding site.

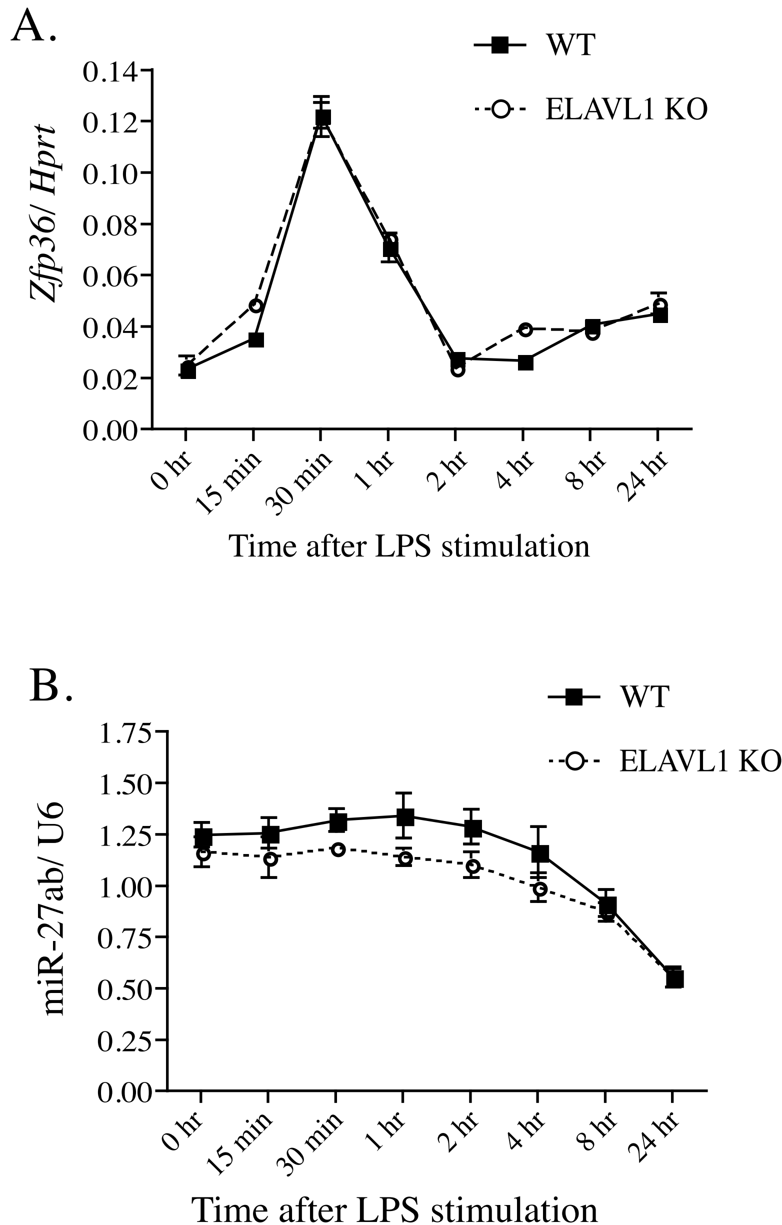


**Figure S5. *Zfp36* gene expression is not significantly affected in Elavl1 KO BMDM. Related to Figure 7E.** (A)Quantitative RT-PCR result of Zfp36 mRNA levels in BMDM after LPS stimulation. Experiments were repeated 3 times in different sets of WT and Elavl1 KO BMDM. (B) Mir-27a/b expression level is not significantly affected in Elavl1 KO BMDM. Quantitative RT-PCR of miR-27b expression level in BMDM after LPS stimulation. (N=2). MiR-27a and miR-27b expression was amplified by Qunata qScript™ microRNA Quantification System and normalized by U6 small nuclear RNA. There is only one nucleotide difference between miR-27a and miR-27b (C to U at 19 nt) and hard to completely distinguish by qRT-PCR results. We used combined miR-27a and miR-27b to represent the overall miR-27 expression level in BMDM.

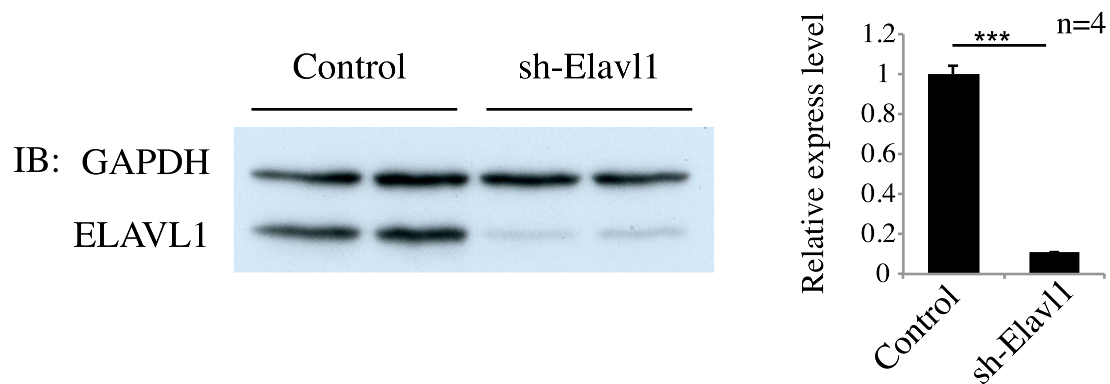


**Figure S6. Elavl1 shRNA suppressed ~90% of Elavl1 polypeptide. Related to Figure 7H.**
Western blot analysis detected the Elavl1 expression level in HEK293T cell transfected with control or Elavl1 shRNA. All error bars indicate the standard deviation. P value were determined by Student's t-test. ***$P < 0.001$.