Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Materials and Methods

References

Materials and methods

Preparation of oligonucleotides

The DNA and RNA oligonucleotides used were received from Genewiz (China), Integrated DNA Technologies (USA), and GenePharma (China), and the plasmids were synthesized by Genewiz (China). The sequence information for all samples can be found in Table S2. Additionally, N-Methyl-mesoporphyrin IX (NMM) and Thioflavin T (ThT) had been synthesized and sold by Frontier Scientific and Solarbio Life Sciences.

Comparative sequence analysis of microRNA-638

miR-638 sequences from different species were obtained from miRbase (https://www.mirbase.org) and miR-638 sequences were further confirmed in National Library of Medicine (https://www.ncbi.nlm.nih.gov/gene/693223).

G4 prediction of microRNA-638

The G4 prediction of miR-638 was obtained by G4RNA screener v.0.2(http://scottgroup.med.usherbrooke.ca/ G4RNA screener/), and the threshold was set to be the default in the corresponding prediction software, i.e., Consecutive G over consecutive C ratio (cGcC)>4.5[1], G4Hunter (G4H) >0.9[2] and G4 Neural Network (G4NN) >0.5[3].

Circular dichroism (CD) spectroscopy

Firstly, a solution of 5 μM miR-638/miR-638mut was prepared by dissolving it in 10 mM LiCac (pH 7.0) and 150 mM KCl/LiCl. After 95 °C denaturing for 5 min, the reaction was cooled to 25 °C for 15 minutes to facilitate renaturation. Next, each sample was loaded into a 1 cm path length cuvette (Hellma Analytics, Germany), and we examined the spectra every 1 nm with a 2 s nm^{-1} response time by a Jasco J-1500 CD spectrophotometer (USA). The wavelength was monitored from 220 to 310 nm, and each sample was scanned twice to attain an average data set[4]. The CD spectra data were normalized to molar residue ellipticity and analyzed using Spectra Manager Suite (Jasco Software) and Microsoft Excel.

UV melting

To prepare the reaction, we dissolved 5 μM miR-638/miR-638mut in 2 mL of 10 mM LiCac buffer with 150 mM KCl and loaded it into a 1 cm path length cuvette (Hellma Analytics, Germany). We sealed the cuvette with three layers of Teflon tape. We denatured each sample at 95 °C for 5 minutes and renatured it 15 minutes at 25 °C. Before scanning, we flushed the Agilent Cary 100 UV-Vis Spectrophotometer with N_2 gas for 5 minutes. We then scanned the sample from 20 °C to 95 °C at 295 nm with the temperature increased 0.5 °C per minute, followed by a reverse scan. To ensure accurate results, we smoothed the data over 10 data points. To calculate the final melting temperature, we determined the average of both forward and reverse scans.

Ligand-enhanced fluorescence

A 100 μl sample with 0.5 μM miR-638/miR-638mut, 150 mM LiCl/KCl, 10 mM LiCac buffer (pH 7.0) was heated at 95 °C to denature and then renatured to 25 °C for 15 min. 0.5 μM ligand (NMM/ThT) was added before transferring to a 1 cm path-length quartz cuvette (Wuxi Jinghe Optical Instrument Co., China). For NMM, the excitation wavelength was at 395 nm, while the emission spectra were scanned from 550 to 750 nm. For ThT, the excitation wavelength was at 425 nm, while the emission spectra were scanned from 440 to 700 nm. Spectra were detected by HORIBA FluoroMax-4 every 2 nm with 5 nm entrance and exit slit widths. Control experiments were performed with no-ligand conditions, and data were normalized with controls and then smoothed over 5 data points in Microsoft Excel. [4]

NMM and SYBR Gold gel staining

The reaction of 20 μl mixtures containing 5 μM of miR-638/miR-638mut, 10 mM Tris-HCl of pH 7.5 and 150 mM KCl was denatured at 95 ˚C for 5 minutes and cooled down to room temperature for 15 mins. 40% sucrose was put in after the sample was incubated for an hour at 37°C. After loading 20 μl sample in each well, the 10% native polyacrylamide gel was run at 100 V, 4 ˚C for 40 min. For NMM staining gel, we stained the gel with 1.72 μM NMM for 5 minutes in 50 mL of milli-Q water, and the gel was scanned under the Alexa 488 mode. For the SYBR gold staining gel, we stained the gel with 1X SYBR Gold for 5 minutes in 50 ml of Mili-Q water, and the gel was scanned with SYBR Gold mode. The gels were scanned with ChemiDoc Touch Imaging System (Biorad, USA).

Electrophoretic mobility shift assay (EMSA)

HEX labeled miR-638/miR-638mut was first dissolved in buffer containing 150 mM LiCl/KCl, 1 mM $MgCl_2$, 25 mM Tris-HCl of pH 7.5. Both miR-638/miR-638mut and *MEF2C* mRNA were heated at 75°C for 3min and renatured on ice for 10 min. A 10 μl mixture containing 2 nM miR-638/miR-638mut and varying concentrations of *MEF2C* mRNA was put in and incubated at 37°C for 30min before adding 10% glycerol. For the specificity verification, 2 nM HEX labeled miR-638 and 1000 nM *MEF2C* mRNA and four competitor miRNAs (miR-150, miR-328-5p, miR-601, and miR-671-5p) were denatured at 75°C for 3min and renatured on ice for 10 min, respectively. Oligos were later mixed and incubated at 37°C for 30min before adding 10% glycerol. Samples were loaded on the 10% native polyacrylamide gel running at 100V for 40min before being scanned by the Amersham Typhoon Biomolecular Imager (Cytiva, USA). The curve fitting was processed by GraphPad Prism software in saturation binding, specific binding only.

NMM-enhanced fluorescence on miRNA with mRNA

0.25 μM miR-638/miR-638mut in 150 mM LiCl/KCl, 10 mM LiCac buffer (pH 7.0), and 0.1-0.5μM *MEF2C* mRNA was separately heated at 95 °C for 3 minutes and then renatured for 15 min at 37°C in

100 µl volume. 0.25 µM NMM was added before transferring to a 1 cm path-length quartz cuvette (Wuxi Jinghe Optical Instrument Co., China). The scanning process followed the method in Ligand-enhanced fluorescence mentioned above. Control experiments were performed with no NMM conditions, and data were normalized with controls and then smoothed over 5 data points in Microsoft Excel. [4]

Cell incubation, transfection, and dual reporter gene assay

The authenticated HEK293T cells were confirmed with no mycoplasma and incubated at 37 °C in the 5% CO_2 incubator in DMEM media (Gibco, USA). The 3' end of the Renilla luciferase gene psiCHECK-2 vector (Promega, USA) was modified by inserting the *MEF2C* native sequences between the XhoI and NotI restriction sites by Genewiz (China). 0.05 million HEK293T cells were seeded in 96-well plates (SPL, Korea) for each well. After 24 hours, each well was transfected with 10 ng plasmid and 10 pmol miRNA using Lipofectamine® 2000 and Lipofectamine® RNA iMAX, respectively (Thermo Fisher Scientific, USA). We lysed the cells in accordance with the Dual-Luciferase Reporter Assay System (Promega, USA) instructions 24 post-transfection and measured the plate using a SpectraMax iD5® (Molecular Devices, USA). The Renilla luciferase signal was normalized to the Firefly luciferase signal, and the data were processed using Microsoft Excel by averaging the results of three independent repeats.

RNA extraction and RT-qPCR

After 24 hours post-transfection, we lysed one million cells from the 6-well plate and subsequently purified the extracted RNA following the RNeasy Plus Mini Kit (Qiagen, Germany). We measured the concentration of total RNA by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). To generate cDNA, 100 ng total RNA from each transfection condition was reverse transcribed by the PrimeScript RT Master Mix (TAKARA, Japan). The concentration of the reverse transcribed cDNA was also measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Next, 40 ng cDNA from each sample was mixed with primers and Ssofast™ Evagreen (SYBR Green) qPCR master mix (Bio-Rad), and qPCR reactions were carried out under a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Finally, the Ct result was processed using Microsoft Excel, and the final data was achieved by the average results of three biological repeats.

Table S1. Predicted G4 scores among species**.**

Description	**sequence**	**cGcC**	**G4H**	**G4NN**
hsa-miR-638	AGGGAUCGCGGGCGGGUGGCGGCCU	4.875	1.12	0.9353
cja-mir-638	GGGACCGCGGGCGGGCGGCGGCCU	3.25	1	0.8563
mml-mir-638	AGGGAUCGCGGGCGGGCGGCGGCCU	4.3333	1.08	0.9164
ppy-mir-638	AGGGAUCGCGGGCGGUCGGCGGCCC	2.2	0.68	0.5921

Default threshold in corresponding G4 prediction programs (cGcC > 4.5, G4H > 0.9, G4NN >0.5). The green box indicated that it passes the threshold and has a high probability of forming rG4. For has-miR-638, the sequence passed the threshold for all 3 G4 prediction programs, suggesting it has a high chance of folding into the rG4 structure.

Table S2. Oligonucleotides used in this study.

Name	Sequence (5'-3')
hsa-miR-638	A**GGG**AUCGC**GGG**C**GGG**U**GG**C**GG**CCU
hsa-miR-638mut	AGGGAUCGCAAACAAAUAACAACCU
hsa-miR-Scramble	UUCUCCGAACGUGUCACGUTT
HEX-hsa-miR-638	HEX-A**GGG**AUCGC**GGG**C**GGG**U**GG**C**GG**CCU
HEX- hsa-miR-638mut	HEX-AGGGAUCGCAAACAAAUAACAACCU
hsa-miR-150-5p	UCUCCCAACCCUUGUACCAGUG
hsa-miR-328-5p	GGGGGGGCAGGAGGGGCUCAGGG
hsa-miR-601	UGGUCUAGGAUUGUUGGAGGAG
hsa-miR-671-5p	AGGAAGCCCUGGAGGGGCUGGAG
MEF2C mRNA	AAAUCAUUUUCCUCACCAUAGACUUGAUCCCAUCCUUACAACCCAUCCUUCUAA CUUGAUGUGUAUAAA
Short MEF2C plasmid	TTTCCTCACCATAGACTTGATCCCA
Full-length 3'UTR MEF2C plasmid	TCAGATTATTACTTACTAGTTTTTTTTTTTTTCTTGCAGTGTGTGTGTGTGCTA TACCTTAATGGGGAAGGGGGGTCGATATGCATTATATGTGCCGTGTGTGGAAAA AAAAAAAGTCAGGTACTCTGTTTTGTAAAAGTACTTTTAAATTGCCTCAGTGAT ACAGTATAAAGATAAACAGAAATGCTGAGATAAGCTTAGCACTTGAGTTGTACA ACAGAACACTTGTACAAAATAGATTTTAAGGCTAACTTCTTTTCACTGTTGTGC TCCTTTGCAAAATGTATGTTACAATAGATAGTGTCATGTTGCAGGTTCAACGTT ATTTACATGTAAATAGACAAAAGGAAACATTTGCCAAAAGCGGCAGATCTTTAC TGAAAGAGAGAGCAGCTGTTATGCAACATATAGAAAAATGTATAGATGCTTGGA CAGACCCGGTAATGGGTGGCCATTGGTAAATGTTAGGAACACACCAGGTCACCT GACATCCCAAGAATGCTCACAAACCTGCAGGCATATCATTGGCGTATGGCACTC ATTAAAAAGGATCAGAGACCATTAAAAGAGGACCATACCTATTAAAAAAAAATG TGGAGTTGGAGGGCTAACATATTTAATTAAATAAATAAATAAATCTGGGTCTGC ATCTCTTATTAAATAAAAATATAAAAATATGTACATTACATTTTGCTTATTTTC ATATAAAAGGTAAGACAGAGTTTGCAAAGCATTTGTGGCTTTTTGTAGTTTACT TAAGCCAAAATGTGTTTTTTTCCCCTTGATAGCTTCGCTAATATTTTAAACAGT CCTGTAAAAAACCAAAAAGGACTTTTTGTATAGAAAGCACTACCCTAAGCCATG AAGAACTCCATGCTTTGCTAACCAAGATAACTGTTTTCTCTTTGTAGAAGTTTT GTTTTTGAAATGTGTATTTCTAATTATATAAAATATTAAGAATCTTTTAAAAAA ATCTGTGAAATTAACATGCTTGTGTATAGCTTTCTAATATATATAATATTATGG TAATAGCAGAAGTTTTGTTATCTTAATAGCGGGAGGGGGGTATATTTGTGCAGT TGCACATTTGAGTAACTATTTTCTTTCTGTTTTCTTTTACTCTGCTTACATTTT ATAAGTTTAAGGTCAGCTGTCAAAAGGATAACCTGTGGGGTTAGAACATATCAC ATTGCAACACCCTAAATTGTTTTTAATACATTAGCAATCTATTGGGTCAACTGA CATCCATTGTATATACTAGTTTCTTTCATGCTATTTTTATTTTGTTTTTTGCAT TTTTATCAAATGCAGGGCCCCTTTCTGATCTCACCATTTCACCATGCATCTTGG AATTCAGTAAGTGCATATCCTAACTTGCCCATATTCTAAATCATCTGGTTGGTT TTCAGCCTAGAATTTGATACGCTTTTTAGAAATATGCCCAGAATAGAAAAGCTA TGTTGGGGCACATGTCCTGCAAATATGGCCCTAGAAACAAGTGATATGGAATTT ACTTGGTGAATAAGTTATAAATTCCCACAGAAGAAAAATGTGAAAGACTGGGTG CTAGACAAGAAGGAAGCAGGTAAAGGGATAGTTGCTTTGTCATCCGTTTTTAAT TATTTTAACTGACCCTTGACAATCTTGTCAGCAATATAGGACTGTTGAACAATC CCGGTGTGTCAGGACCCCCAAATGTCACTTCTGCATAAAGCATGTATGTCATCT ATTTTTTCTTCAATAAAGAGATTTAATAGCCATTTCAAGAAATCCCATAAAGAA CCTCTCTATGTCCCTTTTTTTAATTTAAAAAAAATGACTCTTGTCTAATATTCG TCTATAAGGGATTAATTTTCAGACCCTTTAATAAGTGAGTGCCATAAGAAAGTC AATATATATTGTTTAAAAGATATTTCAGTCTAGGAAAGATTTTCCTTCTCTTGG AATGTGAAGATCTGTCGATTCATCTCCAATCATATGCATTGACATACACAGCAA AGAAGATATAGGCAGTAATATCAACACTGCTATATCATGTGTAGGACATTTCTT

	ATCCATTTTTTCTCTTTTACTTGCATAGTTGCTATGTGTTTCTCATTGTAAAAG GCTGCCGCTGGGTGGCAGAAGCCAAGAGACCTTATTAACTAGGCTATATTTTTC TTAACTTGATCTGAAATCCACAATTAGACCACAATGCACCTTTGGTTGTATCCA TAAAGGATGCTAGCCTGCCTTGTACTAATGTTTTATATATTAAAAAAAAAAAT CTATCAACCATTTCATATATATCCCACTACTCAAGGTATCCATGGAACATGAAA GAATAACATTTATGCAGAGGAAAAACAAAAACATCCCTGAAAATATACACACTC ATACACACACACGCACAGGGGAATAAAATAAGAAAATCATTTTCCTCACCATAG ACTTGATCCCATCCTTACAACCCATCCTTCTAACTTGATGTGTATAAAATATGC AAACATTTCACAAATGTTCTTTGTCATTTCAAAATACTTTAGTATATCAATATC AGTAGATACCAGTGGGTGGGAAAGGGTCATTACATGAAAATATGAAGAAATAGC CATATTAGTTTTTTAACCTGCAATTTGCCTCAGCAACAAAGAAAAAGTGAATTT TTAATGCTGAAGATAAAGTAAGCTAAAGTACCAGCAGAAGCCTTGGCTATTTAT AGCAGTTCTGACAATAGTTTTATAAGAACATGAAGAGAACAGAATCACTTGAAA ATGGATGCCAGTCATCTCTTGTTCCCACTACTGAATTCTTATAAAGTGGTGGCA AGATAGGGAAGGGATAATCTGAGAATTTTTAAAAGATGATTTAATGAGAAGAAG CACAATTTTGATTTTGATGAGTCACTTTCTGTAAACAATCTTGGTCTATCTTTA CCCTTATACCTTATCTGTAATTTACCATTTATTGTATTTGCAAAGCTAGTATGG TTTTTAATCACAGTAAATCCTTTGTATTCCAGACTTTAGGGCAGAGCCCTGAGG GAGTATTATTTTACATAACCCGTCCTAGAGTAACATTTTAGGCAACATTCTTCA TTGCAAGTAAAAGATCCATAAGTGGCATTTTACACGGCTGCGAGTATTGTTATA TCTAATCCTATTTTAAAAGATTTTTGGTAATATGAAGCTTGAATACTGGTAACA GTGATGCAATATACGCAAGCTGCACAACCTGTATATTGTATGCATTGCTGCGTG GAGGCTGTTTATTTCAACCTTTTTAAAAATTGTGTTTTTTAGTAAAATGGCTTA TTTTTTCCCAAAGGTGGAATTTAGCATTTTGTAATGATGAATATAAAAATACCT GTCATCCCCAGATCATTTAAAAGTTAACTAAAGTGAGAATGAAAAAACAAAATT CCAAGACACTTTTTAAAAGAATGTCTGCCCTCACACACTTTTATGGATTTGTTT TTCTTACATACCCATCTTTTAACTTAGAGATAGCATTTTTTGCCCTCTTTATTT TGTTGTTTGTTTCTCCAGAGAGTAAACGCTTTGTAGTTCTTTCTTTAAAAAACA TTTTTTTTAAAGAAGAAGAAGCCACTTGAACCCTCAATAAAGGCTGTTGCCTAA GCATGGCATACTTCATCTGTTCTCATTTGTGCCATCTGCCGTGATGTCGTCACT TTTATGGCGTTAATTTCCTGCCACTACAGATCTTTTGAAGATTGCTGGAATACT GGTGTCTGTTAGAATGCTTCAGACTACAGATGTAATTAAAGGCTTTTCTTAATA TGTTTTAACCAAAGATGTGGAGCAATCCAAGCCACATATCTTCTACATCAAATT TTTCCATTTTGGTTATTTTCATAATCTGGTATTGCATTTTGCCTTCCCTGTTCA TACCTCAAATTGATTCATACCTCAGTTTAATTCAGAGAGGTCAGTTAAGTGACG GATTCTGTTGTGGTTTGAATGCAGTACCAGTGTTCTCTTCGAGCAAAGTAGACC TGGGTCACTGTAGGCATAGGACTTGGATTGCTTCAGATGGTTTGCTGTATCATT TTTCTTCTTTTTCTTTTCCTGGGGACTTGTTTCCATTAAATGAGAGTAATTAAA ATCGCTTGTAAATGAGGGCATACAAGCATTTGCAACAAATATTCAAATAGAGGC TCACAGCGGCATAAGCTGGACTTTGTCGCCACTAGATGACAAGATGTTATAACT AAGTTAAACCACATCTGTGTATCTCAAGGGACTTAATTCAGCTGTCTGTAGTGA ATAAAAGTGGGAAATTTTCAAAAGTTTCTCCTGCTGGAAATAAGGTATAATTTG TATTTTGCAGACAATTCAGTAAAGTTACTGGCTTTCTTAGTGAAAAAAAAAAA

Footnote: The Gs in miR-638 related to the G4 formation are in bold, while the mutated As in miR-638 are underlined. The seed region in miR-638 is in red, and the MRE in *MEF2C* is in pink.

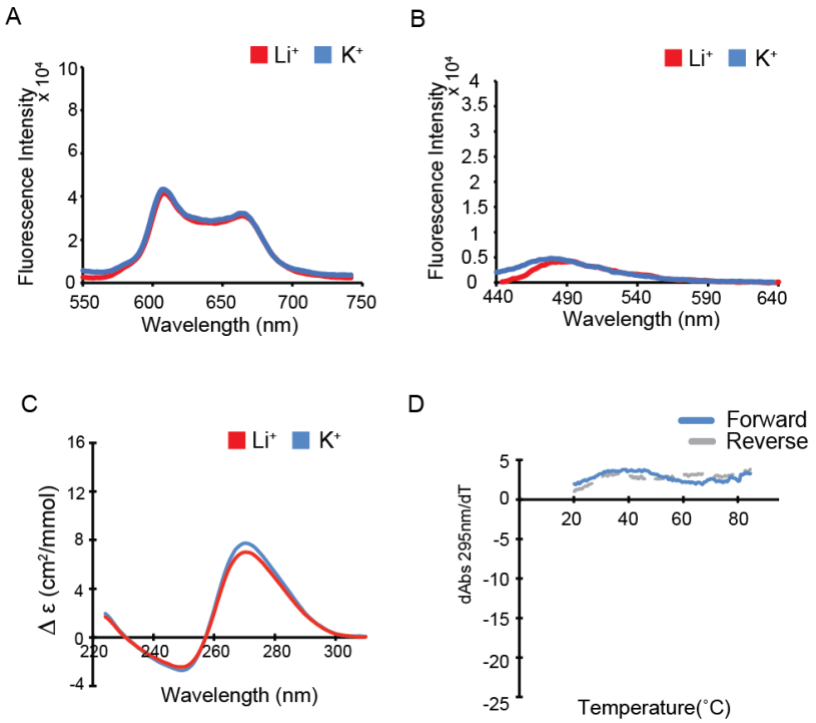

Figure S1. Biophysical assays reveal no G4 formation on miR-638mut. (A-B) NMM (A) and ThT (B) ligand-enhanced fluorescence on the miR-638mut. Spectra under 150 mM K^+ have no observable difference compared to the 150 mM Li^+ for both ligands. (C) CD-detected titration under K^+ has no detectable difference compared with Li^+ conditions, suggesting there is no rG4 formation in miR-638mut. (D) UV melting was monitored at 295 nm under 150 mM K^+ on miR-638mut. No G4-specific hypochromic shift was observed in the UV melting profile at 295nm.

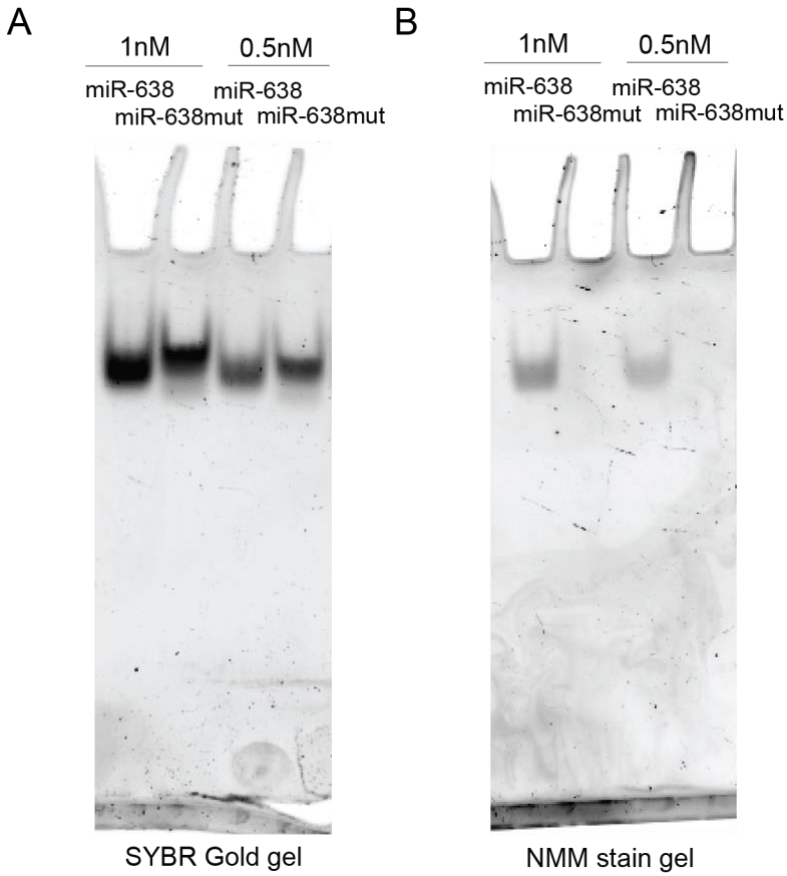

Figure S2. Full gel for native gel analysis on miR-638 and miR-638mut using SYBR gold (A) and NMM stain (B).

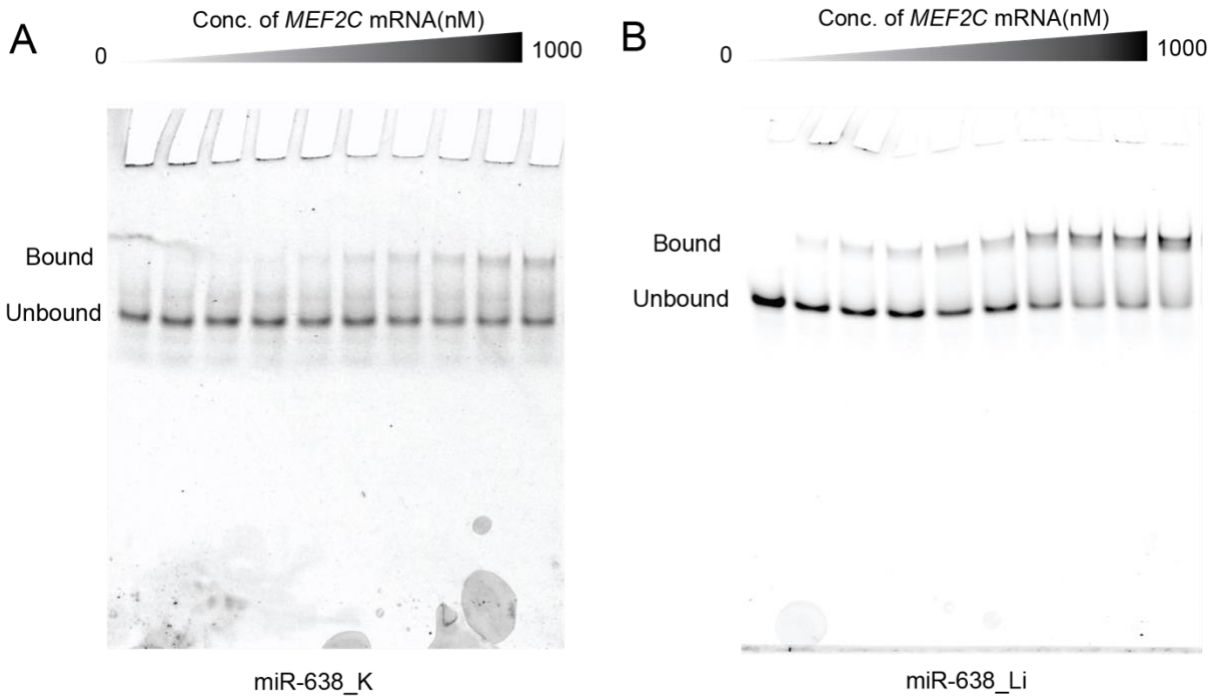

Figure S3. Full EMSA gel on miR-638 with MEF2C mRNA under 150 mM K^+ (A) and 150 mM Li^+ (B).

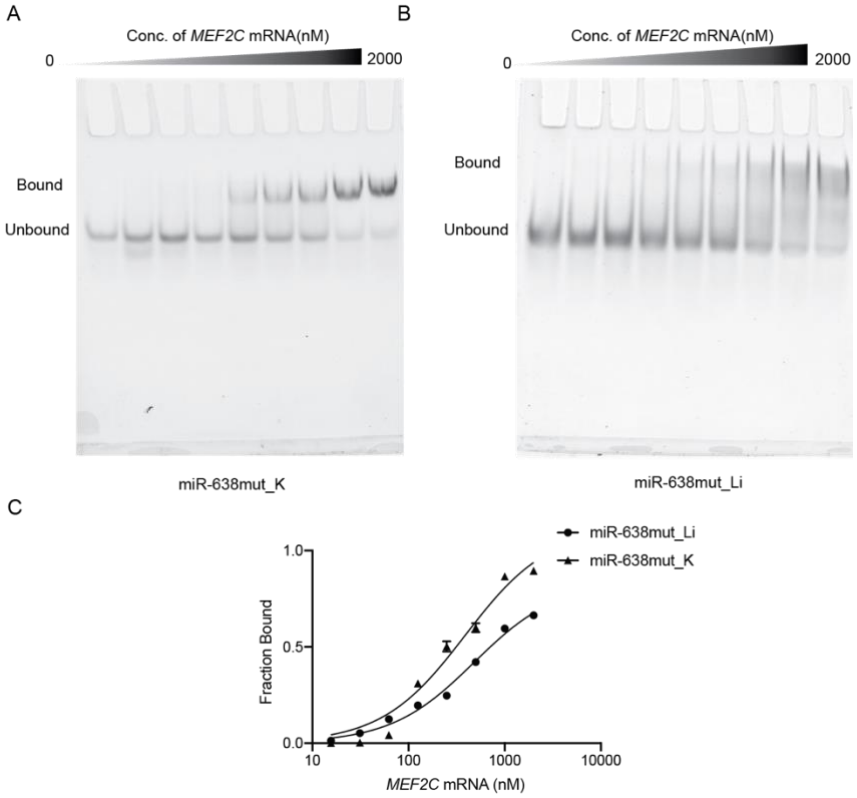

Figure S4. EMSA and binding curves on miR-638mut under K^+ and Li^+ conditions. (A-B) Full EMSA gel result under 150 mM K^+ (A, flipped for better demonstration) and Li^+ (B). (C) Binding curves generate K_d for miR-638mut to be 388.4 ± 90.8 nM under K^+, and 481.0 ± 70.3 nM under Li^+.

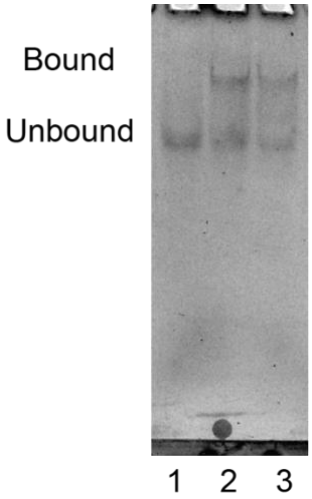

Figure S5. EMSA gel on miR-638 with *MEF2C* mRNA under 150 mM K^+, with four miRNA competitors added as controls. Lane 1 is with only miR-638. Lane 2 is with 2 nM miR-638 and 1000 nM *MEF2C.* Lane 3 is with 2 nM miR-638, 2 nM control miRNAs (0.5 nM for each control miRNA) and 1000 nM *MEF2C.*

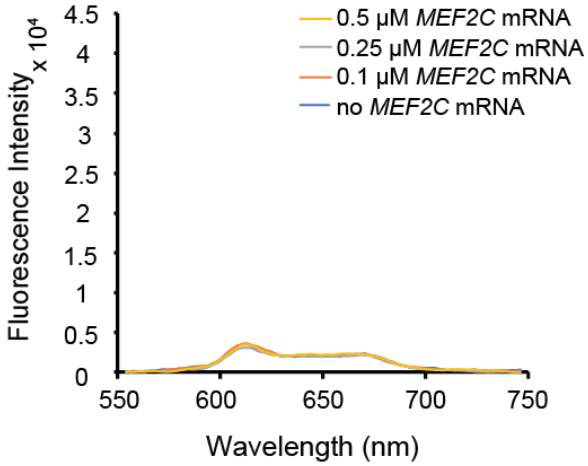

Figure S6. NMM-enhanced fluorescence on different concentrations of *MEF2C* mRNA. 0-0.5 μM *MEF2C* mRNA and 0.25 μM NMM was used to test the ligand-enhanced fluorescence. No fluorescence intensity change was detected, suggesting that there is no ligand-induced fluorescence under *MEF2C* mRNA.

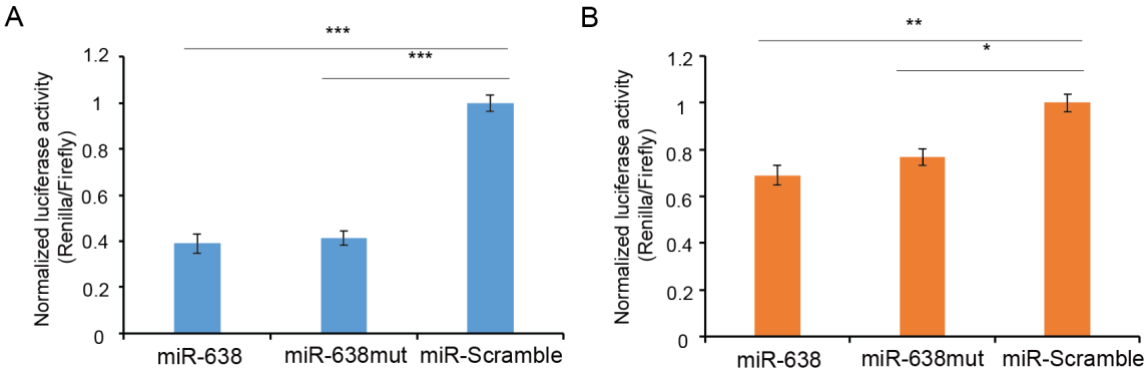

Figure S7. Reporter gene assay on short MEF2C plasmid and full-length 3'UTR MEF2C plasmid with miR-638, miR-638mut, and miR-Scramble transfection. (A) Normalised average luciferase signal from three biological repeats acquired 48 hours post-transfection with the miR-638/ miR-638mut/ miR-Scramble and the short MEF2C plasmid. ***p <0.001, signal under miR-638 and miR-638mut relative to miR-Scramble. (B) Normalised average luciferase signal from three biological repeats acquired 48 hours post-transfection with the miR-638/ miR-638mut/ miR-Scramble and full-length 3'UTR MEF2C plasmid. ** p <0.01, signal under miR-638 relative to miR-Scramble. * p <0.05, signal under miR-638mut relative to miR-Scramble.

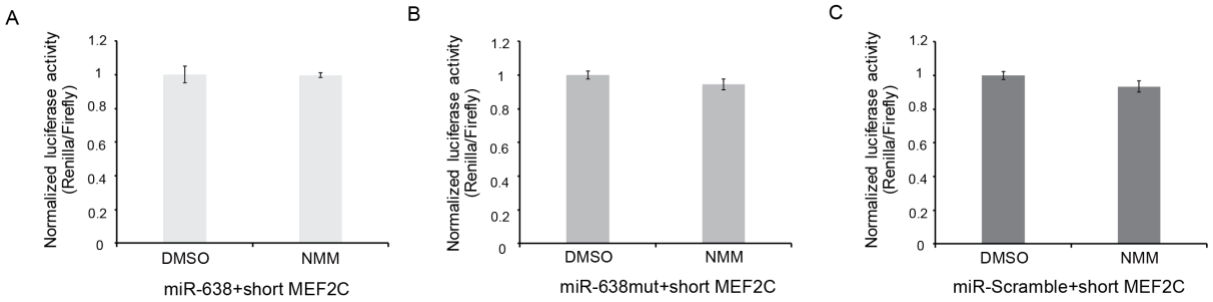

Figure S8. RT-qPCR result on short MEF2C plasmid with miR-638/miR-638mut/miR-Scramble transfection. (A-C) RT-qPCR data achieved by the average results of three biological repeats on miR-638/ miR-638mut/ miR-Scramble under DMSO and 40 μM NMM treatment, respectively. No significant difference was detected between DMSO and NMM for the three groups.

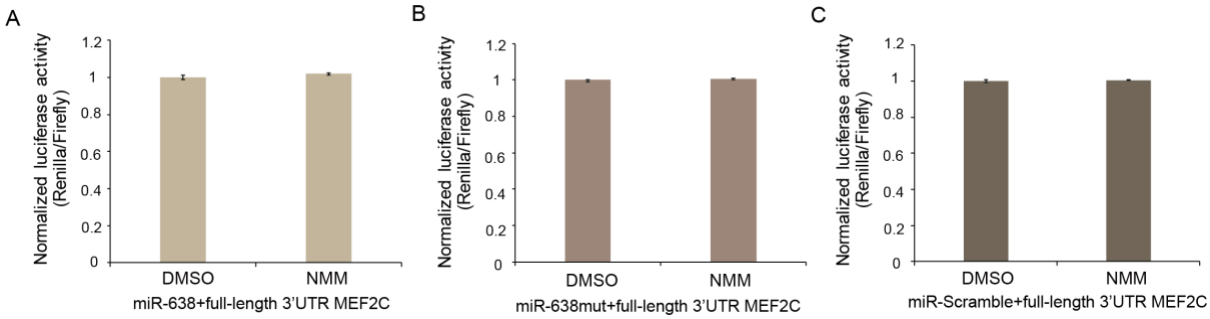

Figure S9. RT-qPCR result on full-length 3'UTR MEF2C plasmid with miR-638/miR-638mut/miR-Scramble transfection. (A-C) RT-qPCR data achieved by the average results of three biological repeats on miR-638/ miR-638mut/ miR-Scramble under DMSO and 40 μM NMM treatment, respectively. No significant difference was detected between DMSO and NMM for the three groups.

References

1. J. D. Beaudoin, R. Jodoin and J. P. Perreault, *Nucleic Acids Res*, 2014, **42**, 1209-1223.
2. A. Bedrat, L. Lacroix and J. L. Mergny, *Nucleic Acids Res*, 2016, **44**, 1746-1759.
3. J. M. Garant, J. P. Perreault and M. S. Scott, *Bioinformatics*, 2017, **33**, 3532-3537.
4. K. Lyu, S. B. Chen, C. Y. Chan, J. H. Tan and C. K. Kwok, *Chem Sci*, 2019, **10**, 11095-11102.