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# Supporting Information for

## Mismatch binding ligand upregulated back-splicing reaction producing circular RNA in a cellular model

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## Experimental Procedures

### General information:

Surface plasmon resonance (SPR) measurements were conducted using BIAcore T200 instrument (GE Healthcare). All oligonucleotides were purchased from Invitrogen. The original plasmid construct used in these experiments: pcDNA3.1(+) ZKSCAN1 nt 400-1782 delta440-500 delta1449-1735, was a gift from Jeremy Wilusz (Addgene plasmid # 60633 ; http://n2t.net/addgene:60633 ; RRID:Addgene_60633).[1] *Escherichia coli* (*E.* coli) strain DH5α was used to amplify plasmid DNA and the amplified plasmid DNA was extracted and purified using NucleoBond® Xtra Midi EF (Macherey-Nagel). HeLa cells (RIKEN BRC, RCB0007) were used for transfection and were maintained at 37 °C under 5% $CO_2$ in Dulbecco's modified eagle's medium (Sigma, D6429) supplemented with 10% (v/v) fetal bovine serum (MP Biomedicals) and penicillin-streptomycin (Gibco). The chemical compounds described in the paper, **NCD** and **QCD**, were synthesized as previously reported.[2] FuGENE HD™ transfection reagent (Promega) was used for plasmid transfection. For RT-qPCR experiments, cell lysis and reverse transcription was conducted using Superprep® II cell lysis & RT kit (TAKARA), and real-time PCR (qPCR) experiments were conducted using StepOne real-time PCR system (Applied Biosystem) using GoTaq® qPCR master mix (Promega). For Ribonuclease R (RNase R) treatment, cell lysis and total RNA extraction were conducted using ISOGEN (Nippon gene Co.), RNase R was purchased from Epicenter Technologies, and the reverse transcription was conducted using ReverTra Ace® qPCR RT Master Mix with gDNA remover (TOYOBO).

### SPR assay:

**1) Sensor preparation:** For the SPR assay, RNA containing the partial intronic sequence containing 5'-GGA-3'/5'-GGA-3' and 5'-UCC-3'/5'-GGA-3' was immobilized onto the sensor chip SA (Cytiva) where the surface was coated with streptavidin. The surface of the sensor chip was first washed three times with 50 mM NaOH and 1 M NaCl for 60 sec with a flow rate of 30 μL $min^{-1}$. Then the biotin-labeled RNAs, biotin-TEG-5'-GCUGAGAU GGA AGGCGUGAGCUUUUGCUCACACCU GGA AUCCCAGC-3' and biotin-TEG-5'- GCUGAGAU UCC AGGCGUGAGCUUUUGCUCACACCU GGA AUCCCAGC-3' (The 5'-GGA-3'/5'-GGA-3' and 5'-UCC-3'/5'-GGA-3' regions are underlined), were immobilized onto the surface under the following conditions: 1 μM RNA in 10 mM HEPES, 500 mM NaCl, pH 7.4, the flow rate of 5 μL $min^{-1}$ for the flow of 60 sec. The amount of RNA immobilized on the sensor chip SA was 1217 RU and 1075 RU respectively.

**2) SPR analysis protocol:** SPR analysis for the binding of **NCD** and **QCD** to the RNA-immobilized surface was conducted on BIAcore T-200 and carried out by subsequently flowing 0.0625, 0.125, 0.25, 0.5, and 1 μM of each compound for 60 sec of contacting time in HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05 % v/v Surfactant P20, pH 7.4) (Cytiva) with the flow rate of 30 μL $min^{-1}$ at 25 °C followed by dissociation of bound compounds by flowing running buffer for 120 sec. Surface regeneration was carried out after the assay of each compound using 1.2 mM NaOH, 0.2 M NaCl, and 0.1 mM EDTA solution, with a contact time of 180 sec and a flow rate of 30 μL $min^{-1}$. Kinetic parameters of the binding of **NCD** and **QCD** to the RNA-immobilized surface were obtained by using the single-cycle kinetics method.

### Plasmid construct preparation:

The desired mutation was introduced via PCR mutagenesis into the region coding for the circZKSCAN1 expressing pre-mRNA of the original construct (pcDNA3.1(+) ZKSCAN1 nt 400-1782 delta440-500 delta1449-1735, **p-UAC**). The specific location of the introduced mutation is shown in Fig. S1A. First, a guanine point mutation was introduced into one of the complementary intronic sequences (Fig. S1A, yellow arrow 1000-1035 nt region), using the primers (5'-GTA GCT CAC ACC TGG AAT CCC AGC AGC GG-3') and (5'- CCG CTG CTG GGA TTC CAG GTG TGA GCT AC-3') and *PfuUltra* High-fidelity DNA polymerase (Agilent) to produce the intermediate construct. The PCR product was then treated with DpnI before it was transformed into *E.* coli DH5α cells for amplification and isolated, as described above in **General information**. Additional nucleotide mutation was then further introduced into the intermediate construct in the other complementary intronic sequence (Fig. S1A, yellow arrow 1-40 nt region). For **p-GGA** plasmid, the primer pair (5'-GAA TTC AAA GTG CTG AGA TGG AAG GCG TGA GCC ACC ACC-3') and (5'- GGT GGT GGC TCA CGC CTT CCA TCT CAG CAC TTT GAA TTC-3') was used, while for **p-UCC** plasmid, the primer pair (5'- GAA TTC AAA GTG CTG AGA TTC CAG GCG TGA GCC ACC ACC-3') and (5'- GGT GGT GGC TCA CGC CTG GAA TCT CAG CAC TTT GAA TTC-3') was used. The respective constructs were then amplified via *E.* coli DH5α cells and collected as described previously, and the final sequence of the region coding for **pre-mGGA** and **pre-mUCC** are shown in Fig. S1C and D.

### Reverse-transcription quantitative PCR (RT-qPCR experiments):

The HeLa cells were plated in a 96-well plate at a density of 1 $x10^4$ cells/well and were incubated for 4 hrs to allow the cells to adhere to the well bottom. It was then transfected with **p-GGA** and **p-UCC** expressing plasmid obtained previously using Fugene HD™ with the ratio (FuGENE : plasmid = 5:2, v/w) according to the manufacturer's instructions. After 24 hrs incubation under 37 °C with 5% $CO_2$, the medium was exchanged with fresh medium containing **NCD** or **QCD** (**NCD**: 0, 0.5, 1, 3, and 5 μM; **QCD**: 5 μM), and the cells were further incubated for 24 hrs. The transfected cells were lysed and the total RNA was reverse transcribed into cDNA using the Superprep® II cell lysis & rt kit according to the manufacturer's instructions. The resulting cDNA was diluted 5-fold before performing qPCR analysis.

qPCR experiments were conducted with StepOne real-time PCR system (Applied Biosystem) and GoTaq® qPCR master mix (Promega) using the cDNA as the template. Each 10 μl reaction contained 1 μl of the cDNA solution, 0.2 μl each of forward- and reverse- primers (10 μM), 0.1 μl of 100x CXR dye, and 5 μl of GoTaq® qPCR Master Mix. A negative control without cDNA template or samples without reverse transcriptase was included in each assay. The primers used for the detection of beta-actin and the various target circRNAs are listed below in **Table S1**.

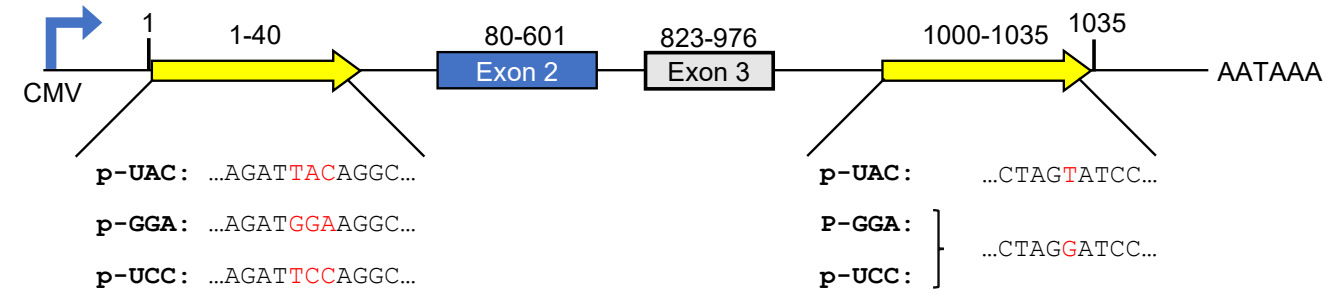
Relative fold change of circRNA was calculated using the comparative $\Delta\Delta C_t$ method[3] and beta-actin was used as endogenous control. Of the 9 data points obtained for each sample; the outliers at the minimum and maximum values were omitted. The qPCR products were analyzed by 8% (29:1, acylamide/bis-acrylamide) polyacrylamide gel electrophoresis to check the amplicon size. The specificity of primer pairs was also verified with the presence of a single peak in the melting curve after PCR amplification and sequencing of the amplicon.

### Ribonuclease R (RNase R) treatment:

HeLa cells plated in a 24-well plate (5 x$10^4$ cells/well) were first transfected with the **p-GGA** expression plasmid and treated in the same manner as described above. Total RNA was extracted using the ISOGEN RNA extraction kit, according to manufacturer's instructions. The obtained total RNA was then treated with DNase I, followed by ethanol precipitation. The RNA pellet was dissolved in water, and 360 ng of the total RNA was then incubated for 15 min at 37 °C with or without 1U RNase R. The resulting solution was directly subjected to reverse transcription using ReverTra Ace® qPCR RT Master Mix with gDNA remover, according to manufacturer's instructions. The expression was then analyzed by qPCR as mentioned above, the experiment was conducted 3 independent times.

## Supplementary Figures

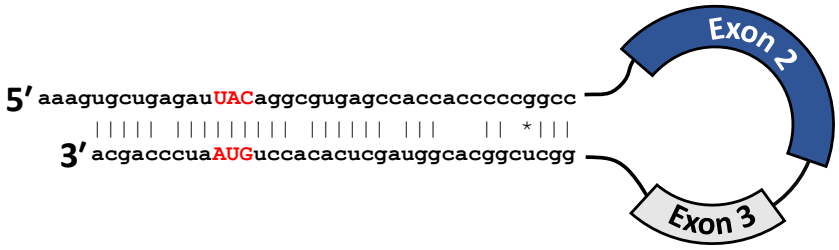
**A**




| Plasmid | Resulting RNA transcript | Resulting circRNA |
|---|---|---|
| p-UAC | pre-mUAC | circZKSCAN1 containing a single nucleotide difference at 901 |
| p-GGA | pre-mGGA | |
| p-UCC | pre-mUCC | |

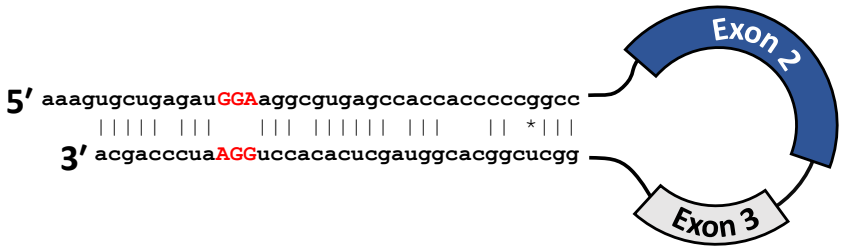
**B**

aaagtgctgagatTACaggcgtgagccaccacccccggccCACTTTTTGTAAAGGTACGTACTAATGACTTTTTTTTTATACTTCAGGAATA
GTAAAGAAACACATCATAAAACCTCCCAGGACATAAAGGTGAGCACAGACCCTGTTTGGATCAAGTCAGTTCCTGGAGCCTGAATGATGACT
<-3′-TGGAGGGTCCTGTATTTCCACTC-5′: circZKSCAN1_Rev
GCTGAATCACGGGAAGCCACGGGTCTGTCCCCACAGGCTGCACAGGAGAAGGATGGTATCGTAATAGTGAAGGTGGAAGAGGAAGATGAGGA
AGACCACATGTGGGGGCAGGATTCCACCCTACAGGACACGCCTCCTCCAGACCCAGAGATATTCCGCCAACGCTTCAGGCGCTTCTGTTACC
AGAACACTTTTGGGCCCCGAGAGGCTCTCAGTCGGCTGAAGGAACTTTGTCATCAGTGGCTGCGGCCAGAAATAAACACCAAGGAACAGATC
CTGGAGCTTCTGGTGCTAGAGCAGTTTCTTTCCATCCTGCCCAAGGAGCTCCAGGTCTGGCTGCAGGAATACCGCCCCGATAGTGGAGAGGA
GGCCGTGACCCTTCTAGAAGACTTGGAGCTTGATTTATCAGGACAACAGGTAAAAAGAGGTGAAACCTATTATGTGTGAGCAGGGCACAGAC
GTTGAAACTGGAGCCAGGAGAAGTATTGGCAGGCTTTAGGTTATTAGGTGGTTACTCTGTCTTAAAAATGTTCTGGCTTTCTTCCTGCATCC
ACTGGCATACTCATGGTCTGTTTTTAAATATTTTAATTCCCATTTACAAAGTGATTTACCCACAAGCCCAACCTGTCTGTCTTCAGGTCCCA
circZKSCAN1_Fw: 5′-CAGTTCAGGAGTCCTCGAGCC-3′->
GGTCAAGTTCATGGACCTGAGATGCTCGCAAGGGGGATGGTGCCTCTGGATCCAGTTCAGGAGTCCTCGAGCCTTGACCTTCATCACGAGGC
CACCCAGTCCCACTTCAAACATTCGTCTCGGAAACCCCGCCTCTTACAGTCACGAGGTAAGAAGCAAGGAAAAGAATTAggctcggcacggt
agctcacacctGTAatccca



**C**

**aaagtgctgagatGGAaggcgtgagccaccacccccggcc**CACTTTTTGTAAAGGTACGTACTAATGACTTTTTTTTTTATACTTCAG**GAATA**
**GTAAAGAAACACATCATAAAACCTCCCAGGACATAAAGGTGAGCACAGACCCTGTTTGGATCAAGTCAGTTCCTGGAGCCTGAATGATGACT**
**GCTGAATCACGGGAAGCCACGGGTCTGTCCCCACAGGCTGCACAGGAGAAGGATGGTATCGTAATAGTGAAGGTGGAAGAGGAAGATGAGGA**
**AGACCACATGTGGGGGCAGGATTCCACCCTACAGGACACGCCTCCTCCAGACCCAGAGATATTCCGCCAACGCTTCAGGCGCTTCTGTTACC**
**AGAACACTTTTGGGCCCCGAGAGGCTCTCAGTCGGCTGAAGGAACTTTGTCATCAGTGGCTGCGGCCAGAAATAAACACCAAGGAACAGATC**
**CTGGAGCTTCTGGTGCTAGAGCAGTTTCTTTCCATCCTGCCCAAGGAGCTCCAGGTCTGGCTGCAGGAATACCGCCCCGATAGTGGAGAGGA**
**GGCCGTGACCCTTCTAGAAGACTTGGAGCTTGATTTATCAGGACAACAG**GTAAAAAGAGGTGAAACCTATTATGTGTGAGCAGGGCACAGAC
GTTGAAACTGGAGCCAGGAGAAGTATTGGCAGGCTTTAGGTTATTAGGTGGTTACTCTGTCTTAAAAATGTTCTGGCTTTCTTCCTGCATCC
ACTGGCATACTCATGGTCTGTTTTTAAATATTTTAATTCCCATTTACAAAGTGATTTACCCACAAGCCCAACCTGTCTGTCTTCAG**GTCCCA**
**GGTCAAGTTCATGGACCTGAGATGCTCGCAAGGGGGATGGTGCCTCTGGATCCAGTTCAGGAGTCCTCGAGCCTTGACCTTCATCACGAGGC**
**CACCCAGTCCCACTTCAAACATTCGTCTCGGAAACCCCGCCTCTTACAGTCACGAG**GTAAGAAGCAAGGAAAAGAATTA**ggctcggcacggt**
**agctcacacctGGAatcccagca**




**D**

**aaagtgctgagatTCCaggcgtgagccaccacccccggcc**CACTTTTTGTAAAGGTACGTACTAATGACTTTTTTTTTTATACTTCAG**GAATA**
**GTAAAGAAACACATCATAAAACCTCCCAGGACATAAAGGTGAGCACAGACCCTGTTTGGATCAAGTCAGTTCCTGGAGCCTGAATGATGACT**
**GCTGAATCACGGGAAGCCACGGGTCTGTCCCCACAGGCTGCACAGGAGAAGGATGGTATCGTAATAGTGAAGGTGGAAGAGGAAGATGAGGA**
**AGACCACATGTGGGGGCAGGATTCCACCCTACAGGACACGCCTCCTCCAGACCCAGAGATATTCCGCCAACGCTTCAGGCGCTTCTGTTACC**
**AGAACACTTTTGGGCCCCGAGAGGCTCTCAGTCGGCTGAAGGAACTTTGTCATCAGTGGCTGCGGCCAGAAATAAACACCAAGGAACAGATC**
**CTGGAGCTTCTGGTGCTAGAGCAGTTTCTTTCCATCCTGCCCAAGGAGCTCCAGGTCTGGCTGCAGGAATACCGCCCCGATAGTGGAGAGGA**
**GGCCGTGACCCTTCTAGAAGACTTGGAGCTTGATTTATCAGGACAACAG**GTAAAAAGAGGTGAAACCTATTATGTGTGAGCAGGGCACAGAC
GTTGAAACTGGAGCCAGGAGAAGTATTGGCAGGCTTTAGGTTATTAGGTGGTTACTCTGTCTTAAAAATGTTCTGGCTTTCTTCCTGCATCC
ACTGGCATACTCATGGTCTGTTTTTAAATATTTTAATTCCCATTTACAAAGTGATTTACCCACAAGCCCAACCTGTCTGTCTTCAG**GTCCCA**
**GGTCAAGTTCATGGACCTGAGATGCTCGCAAGGGGGATGGTGCCTCTGGATCCAGTTCAGGAGTCCTCGAGCCTTGACCTTCATCACGAGGC**
**CACCCAGTCCCACTTCAAACATTCGTCTCGGAAACCCCGCCTCTTACAGTCACGAG**GTAAGAAGCAAGGAAAAGAATTA**ggctcggcacggt**
**agctcacacctGGAatcccagca**

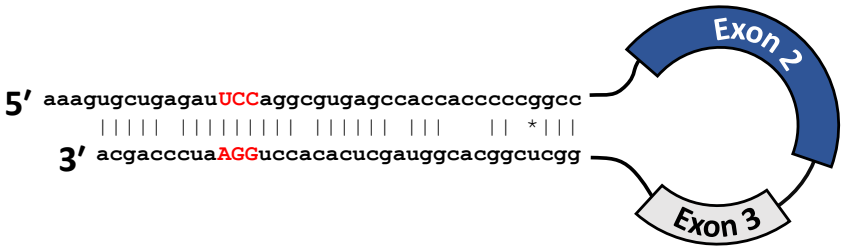



**Figure S1. A)** Specific mutation introduced to generate the **pre-mGGA** and **pre-mUCC** expressing construct **p-GGA** and **p-UCC** respectively, from the original design, **p-UAC**, previously reported by Wilusz et al.,[1] the yellow arrows denote the RCMs in intronic sequences, the mutated nucleotides are highlighted in red, and the length of the sequences is denoted underneath individual elements. **B)** Whole sequence of the region coding for the original circZKSCAN1 in **p-UAC** (top), and the predicted resulting **pre-mUAC** hairpin structure (bottom). The divergent primer alignment is also shown, the arrow indicate the direction of PCR amplification. **C)** The whole sequence of the region coding for the **p-GGA** (top) and the predicted resulting **pre-mGGA** hairpin structure (bottom). **D)** The whole sequence of the region coding for the **p-UCC** (top) and the predicted resulting **pre-mUCC** hairpin structure (bottom). Characters in blue denote the exon 2 region, and the ones highlighted in gray denote the exon 3 region. The yellow highlighted lower-case letters in bold denote the RCMs which is predicted to form the stem of the hairpin. The single nucleotide difference in exon 3 is highlighted in red, in reference genome, it is expected to be T instead of C. The introduced mutations are highlighted in bold uppercase red characters.

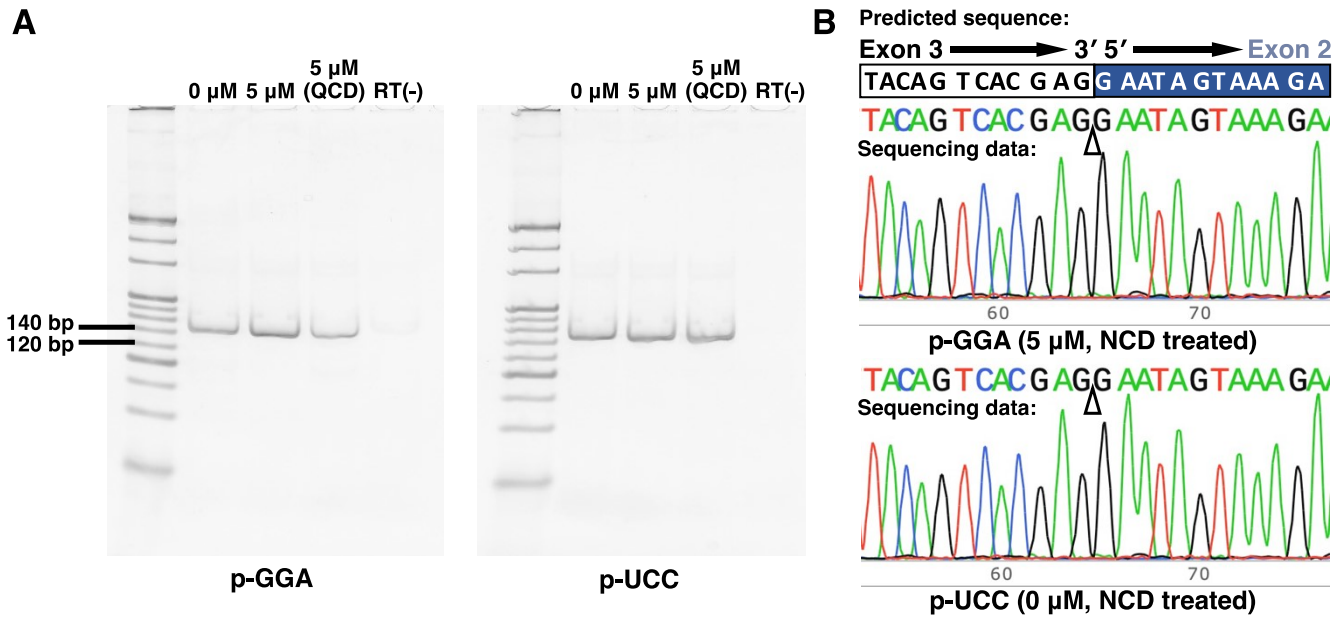



**Figure S2. (A)** PAGE of qPCR product obtained from cells transfected with **p-GGA** and **p-UCC**(left and right, 0 and 5 μM **NCD** sample, and 5 μM **QCD** sample and No-RT sample), and **(B)** the representative sequencing result of qPCR product obtained from cells transfected with **p-GGA,** (5 μM **NCD** sample, top), and **p-UCC** (0 μM **NCD** sample, bottom) the black triangle denotes the position of the junction site in circZKSCAN1.

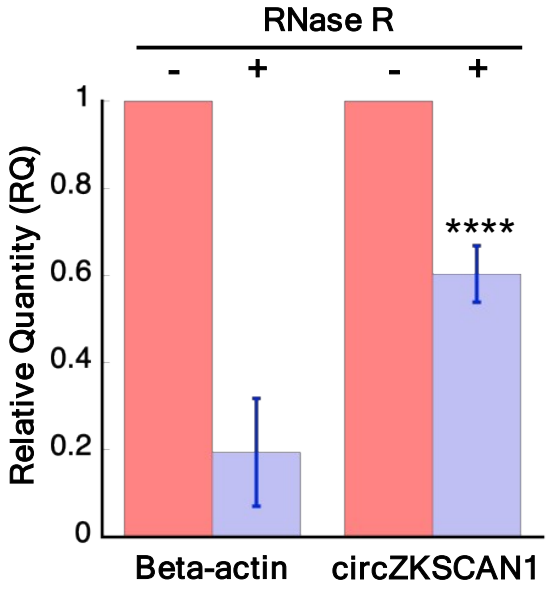



**Figure S3.** RNase R treatment of total RNA obtained from cells transfected with **p-GGA** (5 μM **NCD**) followed by RT-qPCR detection of beta-actin and circZKSCAN1**.** (****: $p < 0.02$, the significance of circZKSCAN1 RNase R (+) sample was obtained against the beta-actin RNase R (+) sample, using a two-tailed t-test, n=3)

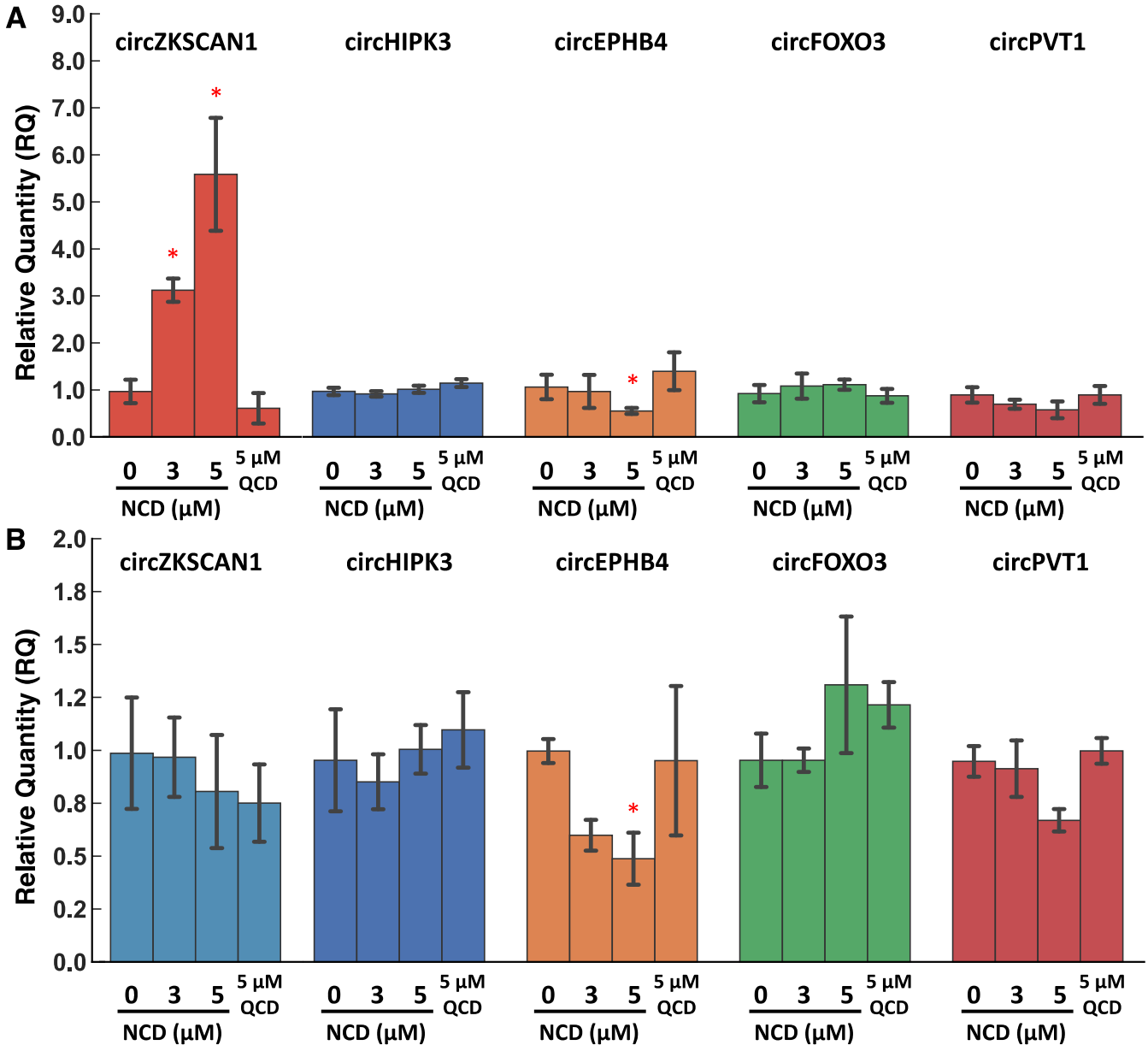



**Figure S4.** The relative change in expression of other commonly studied endogenously expressed circRNAs under the two highest treated **NCD** concentration (3 and 5 μM) and **QCD** (5 μM) in **A) p-GGA** transfected cells and **B) p-UCC** transfected cells. The relative expression of circZKSCAN1 as discussed in the main text, was included for comparison. (*: $p < 0.05$, fold change ≥ 2, the significance was obtained against their respective **NCD** 0 μM treated sample, using a two-tailed t-test, n=4)

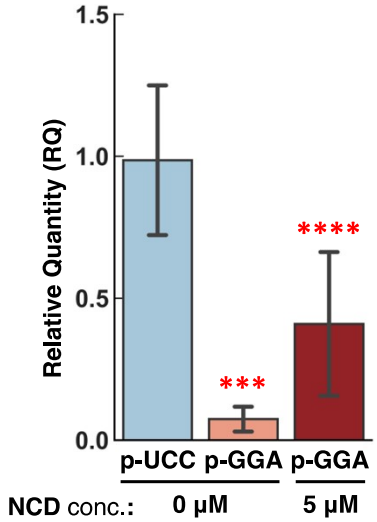



**Figure S5.** Relative expression of circZKSCAN1 in cells transfected with **p-GGA** when compared to **p-UCC**. RQ value was obtained by normalizing the $\Delta C_t$ value against the **p-UCC** transfected cell (**NCD** 0 mM) sample, obtained from RT-qPCR. (**: $p < 3x10^{-6}$, ***: $p < 0.005$, the significance was obtained against the **NCD** 0 µM treated **p-UCC** transfected cell sample, using two-tailed t-test, n=7).

**Table S1.** Primers used for RT-qPCR

| Probe name | Sequence |
|---|---|
| Beta_actin_mRNA_Fw | 5'-CTCTTCCAGCCTTCCTTCCT-3' |
| Beta_actin_mRNA_Rev | 5'-AGCACTGTGTTGGCGTACAG-3' |
| circZKSCAN1_Fw | 5'-CAGTTCAGGAGTCCTCGAGCC-3' |
| circZKSCAN1_Rev | 5'-CTCACCTTTATGTCCTGGGAGGT-3' |
| circHIPK3_Fw | 5'-TATGTTGGTGGATCCTGTTCGGCA-3' |
| circHIPK3_Rev | 5'-TGGTGGGTAGACCAAGACTTGTGA-3' |
| circEPHB4_Fw | 5'-ATTCCCTCACAGCCTCATTAGGGT-3' |
| circEPHB4_Rev | 5'-TGCCCGTCATGATTCTCACAGAGT-3' |
| circFOXO3_Fw | 5'-GTGGGGAACTTCACTGGTGCTAAG-3' |
| circFOXO3_Rev | 5'-GGGTTGATGATCCACCAAGAGCTCTT-3' |
| circPVT1_Fw | 5'-GGTTCCACCAGCGTTATTC-3' |
| circPVT1_Rev | 5'-CAACTTCCTTTGGGTCTCC-3' |

**p-GGA**

| NCD concentration | 0 μM | 0.5 μM | 1 μM | 3 μM | 5 μM | 5 μM QCD |
|---|---|---|---|---|---|---|
| Relative Quantity (RQ values) | 0.764 | 0.643 | 1.140 | 4.331 | 7.051 | 1.052 |
| | 1.052 | 0.648 | 1.226 | 3.091 | 4.843 | 0.348 |
| | 1.239 | 1.012 | 1.241 | 3.319 | 4.312 | 1.063 |
| | 0.878 | 0.838 | 0.932 | 3.322 | 5.188 | 0.427 |
| | 1.918 | 0.657 | 1.182 | 2.586 | 7.687 | 0.297 |
| | 0.483 | 0.525 | 1.273 | 3.423 | 4.482 | 0.287 |
| | 0.497 | 0.485 | 2.466 | 2.663 | 5.529 | 6.931 |
| | 1.210 | 1.156 | 1.654 | 2.915 | 8.312 | 0.225 |
| | 1.129 | 1.713 | 2.652 | 3.112 | 3.092 | 0.795 |
| Average (after removing outliers) | 0.967 | 0.783 | 1.455 | 3.121 | 5.585 | 0.610 |
| Standard Deviation | 0.250 | 0.212 | 0.442 | 0.246 | 1.202 | 0.325 |
| p-value | | 0.193 | 0.037 | 3.74E-09 | 8.61E-07 | 0.054 |

**Table S2.** The Relative Quantity values used for Fig. 4 A and B

**Table S3.** The formula and raw Ct values used to generate RQ values in Table S2.

[3]Formula for:

$$\Delta Ct = C_{t(circZKSCAN1)} - C_{t(Beta\text{-}actin)}; \quad \Delta\Delta C_t = \Delta C_t - (C_{t(Average\ 0\ mM\ \mathbf{NCD}\ circZKSCAN1)} - C_{t(Average\ Ct\ Beta\text{-}actin)}); \quad RQ = 2^{-\Delta\Delta Ct}$$

| **p-GGA** | Batch 1 | | | | | Batch 2 | | | | | Batch 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NCD** conc. | Beta-actin Ct | circZKSCAN1 Ct | ΔCt | ΔΔCt | RQ | Beta-actin Ct | circZKSCAN1 Ct | ΔCt | ΔΔCt | RQ | Beta-actin Ct | circZKSCAN1 Ct | ΔCt | ΔΔCt | RQ |
| 0 ∝M | 19.548 | 27.523 | 7.975 | 0.388 | 0.764 | 18.922 | 27.762 | 8.840 | 0.187 | 0.878 | 18.805 | 28.980 | 10.175 | 1.007 | 0.497 |
| | 19.648 | 27.162 | 7.514 | -0.073 | 1.052 | 19.051 | 26.765 | 7.714 | -0.939 | 1.918 | 18.906 | 27.799 | 8.892 | -0.276 | 1.210 |
| | 19.508 | 26.787 | 7.279 | -0.309 | 1.239 | 19.047 | 28.751 | 9.704 | 1.051 | 0.483 | 18.903 | 27.896 | 8.993 | -0.174 | 1.129 |
| 0.5 ∝M | 19.510 | 27.735 | 8.225 | 0.637 | 0.643 | 18.962 | 27.870 | 8.909 | 0.255 | 0.838 | 18.691 | 28.901 | 10.210 | 1.042 | 0.485 |
| | 19.362 | 27.575 | 8.213 | 0.626 | 0.648 | 19.018 | 28.276 | 9.258 | 0.605 | 0.657 | 19.011 | 27.970 | 8.958 | -0.209 | 1.156 |
| | 19.482 | 27.052 | 7.570 | -0.017 | 1.012 | 19.062 | 28.644 | 9.582 | 0.929 | 0.525 | 18.940 | 27.331 | 8.391 | -0.777 | 1.713 |
| 1 ∝M | 19.510 | 26.908 | 7.398 | -0.189 | 1.140 | 18.970 | 27.725 | 8.755 | 0.102 | 0.932 | 18.822 | 26.687 | 7.866 | -1.302 | 2.466 |
| | 19.437 | 26.731 | 7.293 | -0.294 | 1.226 | 19.021 | 27.433 | 8.412 | -0.241 | 1.182 | 18.904 | 27.346 | 8.442 | -0.726 | 1.654 |
| | 19.473 | 26.748 | 7.275 | -0.312 | 1.241 | 18.962 | 27.268 | 8.305 | -0.348 | 1.273 | 18.884 | 26.644 | 7.760 | -1.407 | 2.652 |
| 3 ∝M | 19.749 | 25.221 | 5.473 | -2.115 | 4.331 | 18.950 | 25.871 | 6.921 | -1.732 | 3.322 | 18.983 | 26.738 | 7.755 | -1.413 | 2.663 |
| | 19.430 | 25.389 | 5.959 | -1.628 | 3.091 | 19.098 | 26.380 | 7.282 | -1.371 | 2.586 | 19.002 | 26.626 | 7.624 | -1.543 | 2.915 |
| | 19.551 | 25.407 | 5.856 | -1.731 | 3.319 | 19.029 | 25.907 | 6.878 | -1.775 | 3.423 | 19.029 | 26.559 | 7.530 | -1.638 | 3.112 |
| 5 ∝M | 19.458 | 24.225 | 4.767 | -2.818 | 7.051 | 18.976 | 25.213 | 6.278 | -2.375 | 5.188 | 19.344 | 26.044 | 6.701 | -2.467 | 5.529 |
| | 19.190 | 24.501 | 5.311 | -2.276 | 4.843 | 19.013 | 24.733 | 5.711 | -2.942 | 7.687 | 19.381 | 25.494 | 6.113 | -3.055 | 8.312 |
| | 19.663 | 25.142 | 5.479 | -2.108 | 4.312 | 19.070 | 25.559 | 6.489 | -2.164 | 4.482 | 19.327 | 26.867 | 7.539 | -1.628 | 3.092 |
| 5 ∝M QCD | 19.908 | 27.422 | 7.514 | -0.074 | 1.052 | 19.626 | 29.588 | 9.882 | 1.229 | 4.427 | 19.433 | 25.888 | 6.375 | -2.793 | 6.931 |
| | 19.855 | 28.965 | 9.110 | 1.523 | 0.348 | 19.509 | 29.911 | 10.402 | 1.749 | 0.297 | 19.191 | 30.549 | 11.319 | 2.151 | 0.225 |
| | 19.981 | 27.480 | 7.500 | -0.088 | 1.063 | 19.629 | 30.086 | 10.456 | 1.803 | 0.287 | 19.472 | 28.971 | 9.499 | 0.331 | 0.795 |
| Average Ct Beta-actin | 19.570 | | 0.731 | | 0.551 | 19.106 | | 0.606 | | 0.991 | 19.057 | | 0.582 | | 0.470 |
| | | | 0.914 | | 0.867 | | | 0.797 | | 1.013 | | | 1.091 | | 0.725 |
| Average Ct **NCD** 0 ∝M | 27.157 | | 1.130 | | 1.119 | 27.759 | | 0.972 | | 0.758 | 28.225 | | 1.120 | | 0.516 |
| | | | 1.226 | | 1.486 | | | 1.781 | | 1.121 | | | 0.214 | | 0.768 |
| | | | 0.366 | | 0.741 | | | 1.220 | | 1.667 | | | 0.507 | | 1.151 |
| | | | 1.441 | | 1.956 | | | 1.703 | | 0.991 | | | 1.116 | | 1.405 |
| Average (after removing outliers) | | | 0.986 | | 1.281 | | | 1.152 | | 0.979 | | | 0.805 | | 0.751 |
| Standard Deviation | | | 0.263 | | 0.381 | | | 0.327 | | 0.170 | | | 0.267 | | 0.182 |
| p-value | | | | | 0.145 | | | 0.352 | | 0.955 | | | 0.260 | | 0.097 |

**p-UCC**

| NCD concentration | 0 μM | 0.5 μM | 1 μM | 3 μM | 5 μM | 5 μM QCD |
|---|---|---|---|---|---|---|
| Relative Quantity (RQ values) | 1.132 | 1.433 | 0.889 | 0.734 | 1.118 | 0.706 |
| | 0.501 | 1.365 | 0.930 | 1.246 | 0.681 | 0.629 |
| | 1.270 | 2.493 | 1.556 | 0.653 | 0.541 | 0.760 |

| p-UCC | Batch 1 | | | | | Batch 2 | | | | | Batch 3 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **NCD** conc. | Beta-actin Ct | circZKSCAN1 Ct | ΔCt | ΔΔCt | RQ | Beta-actin Ct | circZKSCAN1 Ct | ΔCt | ΔΔCt | RQ | Beta-actin Ct | circZKSCAN1 Ct | ΔCt | ΔΔCt | RQ |
| 0 ∝M | 18.908 | 23.243 | 4.336 | -0.179 | 1.132 | 18.821 | 21.448 | 2.627 | 0.452 | 0.731 | 18.839 | 24.340 | 5.501 | -0.294 | 1.226 |
| | 19.233 | 24.746 | 5.512 | 0.998 | 0.501 | 18.898 | 21.203 | 2.305 | 0.129 | 0.914 | 18.650 | 25.897 | 7.247 | 1.451 | 0.366 |
| | 19.266 | 23.435 | 4.169 | -0.345 | 1.270 | 18.909 | 20.909 | 2.000 | -0.176 | 1.130 | 18.795 | 24.064 | 5.269 | -0.527 | 1.441 |
| 0.5 ∝M | 19.103 | 23.099 | 3.996 | -0.519 | 1.433 | 18.916 | 21.951 | 3.035 | 0.860 | 0.551 | 18.803 | 24.028 | 5.225 | -0.571 | 1.486 |
| | 19.407 | 23.472 | 4.065 | -0.449 | 1.365 | 18.925 | 21.306 | 2.382 | 0.206 | 0.867 | 18.715 | 24.942 | 6.227 | 0.432 | 0.741 |
| | 19.398 | 22.594 | 3.196 | -1.318 | 2.493 | 18.885 | 20.898 | 2.013 | -0.163 | 1.119 | 18.807 | 23.634 | 4.827 | -0.968 | 1.956 |
| 1 ∝M | 19.222 | 23.906 | 4.685 | 0.171 | 0.889 | 18.962 | 21.859 | 2.897 | 0.722 | 0.606 | 18.783 | 23.746 | 4.963 | -0.833 | 1.781 |
| | 19.398 | 24.016 | 4.619 | 0.104 | 0.930 | 18.935 | 21.439 | 2.504 | 0.328 | 0.797 | 18.826 | 24.335 | 5.509 | -0.287 | 1.220 |
| | 19.396 | 23.272 | 3.876 | -0.638 | 1.556 | 18.933 | 21.150 | 2.217 | 0.041 | 0.972 | 18.967 | 23.995 | 5.028 | -0.768 | 1.703 |
| 3 ∝M | 19.042 | 24.003 | 4.961 | 0.447 | 0.734 | 18.890 | 21.078 | 2.189 | 0.013 | 0.991 | 18.946 | 24.577 | 5.631 | -0.165 | 1.121 |
| | 19.310 | 23.507 | 4.197 | -0.317 | 1.246 | 18.969 | 21.126 | 2.158 | -0.018 | 1.013 | 18.929 | 23.988 | 5.059 | -0.737 | 1.667 |
| | 19.416 | 24.545 | 5.129 | 0.615 | 0.653 | 18.967 | 21.543 | 2.576 | 0.400 | 0.758 | 18.902 | 24.711 | 5.809 | 0.013 | 0.991 |
| 5 ∝M | 19.138 | 23.490 | 4.353 | -0.161 | 1.118 | 18.950 | 21.907 | 2.956 | 0.781 | 0.582 | 18.996 | 27.017 | 8.021 | 2.225 | 0.214 |
| | 18.628 | 23.696 | 5.068 | 0.554 | 0.681 | 18.920 | 20.970 | 2.050 | -0.126 | 1.091 | 19.219 | 25.995 | 6.776 | 0.980 | 0.507 |
| | 19.322 | 24.721 | 5.399 | 0.885 | 0.541 | 19.309 | 21.322 | 2.013 | -0.163 | 1.120 | 19.422 | 25.060 | 5.638 | -0.158 | 1.116 |
| 5 ∝M QCD | 19.634 | 24.650 | 5.016 | 0.502 | 0.706 | 19.486 | 22.751 | 3.265 | 1.089 | 0.470 | 19.220 | 25.397 | 6.177 | 0.381 | 0.768 |
| | 19.672 | 24.856 | 5.184 | 0.670 | 0.629 | 19.466 | 22.105 | 2.639 | 0.464 | 0.725 | 19.208 | 24.801 | 5.593 | -0.203 | 1.151 |
| | 19.797 | 24.706 | 4.910 | 0.396 | 0.760 | 19.057 | 22.187 | 3.130 | 0.954 | 0.516 | 19.459 | 24.764 | 5.305 | -0.491 | 1.405 |
| Average Ct Beta actin | 19.294 | | | | | 19.011 | | | | | 18.972 | | | | |
| Average Ct **NCD** 0 ∝M | 23.808 | | | | | 21.187 | | | | | 24.767 | | | | |

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