Supplementary Information

8-Oxo-7,8-dihydroguanine in the context of a gene promoter G-quadruplex is an on-off switch for transcription

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Materials and Methods

Plasmid Construction

The plasmids were constructed from the psiCHECK2 plasmid (Promega) that has coding sequences for the Renilla luciferase (Rluc) and firefly luciferase (luc) genes. The luc gene is regulated by the HSV-TK promoter that was not modified and used as the internal standard to conduct the Dual-Glo luciferase assay (Promega). The Rluc gene was originally regulated by the SV40 early enhancer/promoter, which we modified by replacing the TATA-box with the potential G-quadruplex sequences (PQS) of interest. Additionally, the PQS was flanked by recognition sequences for the Nt.BspQ1 nicking endonuclease. Insertion of the PQS and nicking endonuclease recognition sequences was achieved using restriction free cloning. The cloning experiment was conducted in a 25-µL reaction with 1x Phusion reaction buffer (NEB), 200 µM dNTPs, 1 µM each of the forward and reverse primers, 1,000 ng of psiCHECK2 plasmid, 3% DMSO, and 0.5 units of Phusion DNA polymerase. The PCR was initiated with a 98 °C denaturation step for 30 sec followed by 30 cycles of PCR consisting of a 98 °C denaturation step for 20 sec, a 55 °C annealing step for 30 sec, a 72 °C extension step for 5 min, and a post step consisting of 98 °C for 8 sec followed by 72 °C for 5 min. After the 30 cycles, a final extension step of 72 °C for 5 min was conducted. Following PCR, the samples were digested with Dpn1 by adding 2.3 µL of Dpn1 reaction buffer and 5 units of Dpn1 to the PCR reaction vessel. The Dpn1 reaction was conducted for 2 hr at 37 °C followed by heat quenching at 80 °C for 20 min.

The PCR and Dpn1 digested reaction was then transformed using NEB 5-alpha competent *E. coli* following the manufacturer's protocol. After transformation, 50-100 µL from the transformation was spread on a pre-warmed agar plate containing ampicillin (100 µg/mL). The plates were incubated overnight at 37 °C. Next, individual colonies were picked and each grown overnight at 37 °C in 5 mL of lysogeny broth media containing ampicillin (100 µg/mL). The plasmid DNA from each grown sample was then purified using a miniprep kit (Qiagen) following the manufacturer's protocols. Last, ~200 ng of plasmid and 1 µM sequencing forward primer in 15 µL of 10 mM Tris (pH 8.0) was submitted for Sanger sequencing at the DNA sequencing core at the University of Utah.

Insertion of site-specific modifications into the plasmids was achieved following literature protocols.[1,2] Specifically, 5 µg of plasmid with Nt.BspQ1 recognition sequences flanking the site in which the DNA base modification will be inserted was placed in 50 µL of 1x Nt.BspQ1 reaction buffer with 5 units of Nt.BspQ1. The reaction was placed at 50 °C for 60 min followed by heat quenching at 80 °C for 20 min. Next, 1 nmole (~1000x) of 5`-phosphorylated oligomer with the site-specific modification was added to the quenched reaction. The modified oligomers were made by solid-phase synthesis in the DNA/Peptide core facility at the University of Utah. They were cleaved, deprotected, and HPLC purified following protocols previously outlined.[3] The plasmid oligomer mixture was thermal cycled by heating at 80 °C for 2 min followed by cooling on ice for 2 min, which was repeated four times. The nicks were then sealed using T4 DNA ligase, by adding 6 µL of 10x ligase buffer and 800 units of ligase. The ligation reaction was left at 20 °C for 4 h. To induce supercoiling into the plasmids, they were treated with 5 units of gyrase for 1 h (see Supplementary Fig. 1 for a schematic of this method). The modified, supercoiled plasmids were purified from the protein and excess insert using an Ultra Clean PCR cleanup kit (Mo Bio) following the manufacturer's protocol. Plasmid concentrations were determined by nanodrop UV-vis measurements.

To confirm the DNA modifications were introduced into the plasmid, we applied a protocol established in our laboratory, in which the modification was removed by a DNA glycosylase to yield a ligatable gap.[4] By ligating the gap, Sanger sequencing provided a characteristic nucleotide loss at the modification site to confirm the presence of the modification. Complete details of the method can be found in our recent publication.[4]

Primer Sequences

Insertion of 5 track *VEGF* PQS in the coding strand
5`- CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACG CTC TTC CGG GGC GGG CCG GGG GCG GGG TCC CGG CGG GGC GCT CTT CTG CAG AGG CCG AGG CCG CCT CGG CCT CTG AGC T

5`- AGC TCA GAG GCC GAG GCG GCC TCG GCC TCT GCA GAA GAG CGC CCC GCC GGG ACC CCG CCC CCG GCC CGC CCC GGA AGA GCG TCA GCC ATG GGG CGG AGA ATG GGC GGA ACT G

Insertion of 5 track *VEGF* PQS in the template strand
5`-CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACA GAA GAG CGC CCC GCC GGG ACC CCG CCC CCG GCC CGC CCC GGA AGA GCG CAG AGG CCG AGG CCG CCT CGG CCT CTG AGC T

5`- AGC TCA GAG GCC GAG GCG GCC TCG GCC TCT GCG CTC TTC CGG GGC GGG CCG GGG GCG GGG TCC CGG CGG GGC GCT CTT CTG TCA GCC ATG GGG CGG AGA ATG GGC GGA ACT G

Insertion of modified PQS negative *VEGF* in the coding strand
5`- CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACG CTC TTC CTG GGC GGA CCT TGG GCG GAA TCC CGG CTG GGC GCT CTT CTG CAG AGG CCG AGG CCG CCT CGG CCT CTG AGC T

5`- AGC TCA GAG GCC GAG GCG GCC TCG GCC TCT GCA GAA GAG CGC CCA GCC GGG ATT CCG CCC AAG GTC CGC CCA GGA AGA GCG TCA GCC ATG GGG CGG AGA ATG GGC GGA ACT G

Insertion of modified PQS negative *VEGF* in the template strand
5`- AGC TCA GAG GCC GAG GCG GCC TCG GCC TCT GCG CTC TTC CTG GGC GGA CCT TGG GCG GAA TCC CGG CTG GGC GCT CTT CTG TCA GCC ATG GGG CGG AGA ATG GGC GGA ACT G

5`- CAG TTC CGC CCA TTC TCC GCC CCA TGG CTG ACA GAA GAG CGC CCA GCC GGG ATT CCG CCC AAG GTC CGC CCA GGA AGA GCG CAG AGG CCG AGG CCG CCT CGG CCT CTG AGC T

Sequencing Primer
5`- TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTC ATG CAT CTC AAT TAG TCA GCA ACC ATA GT

Cell Culture Studies

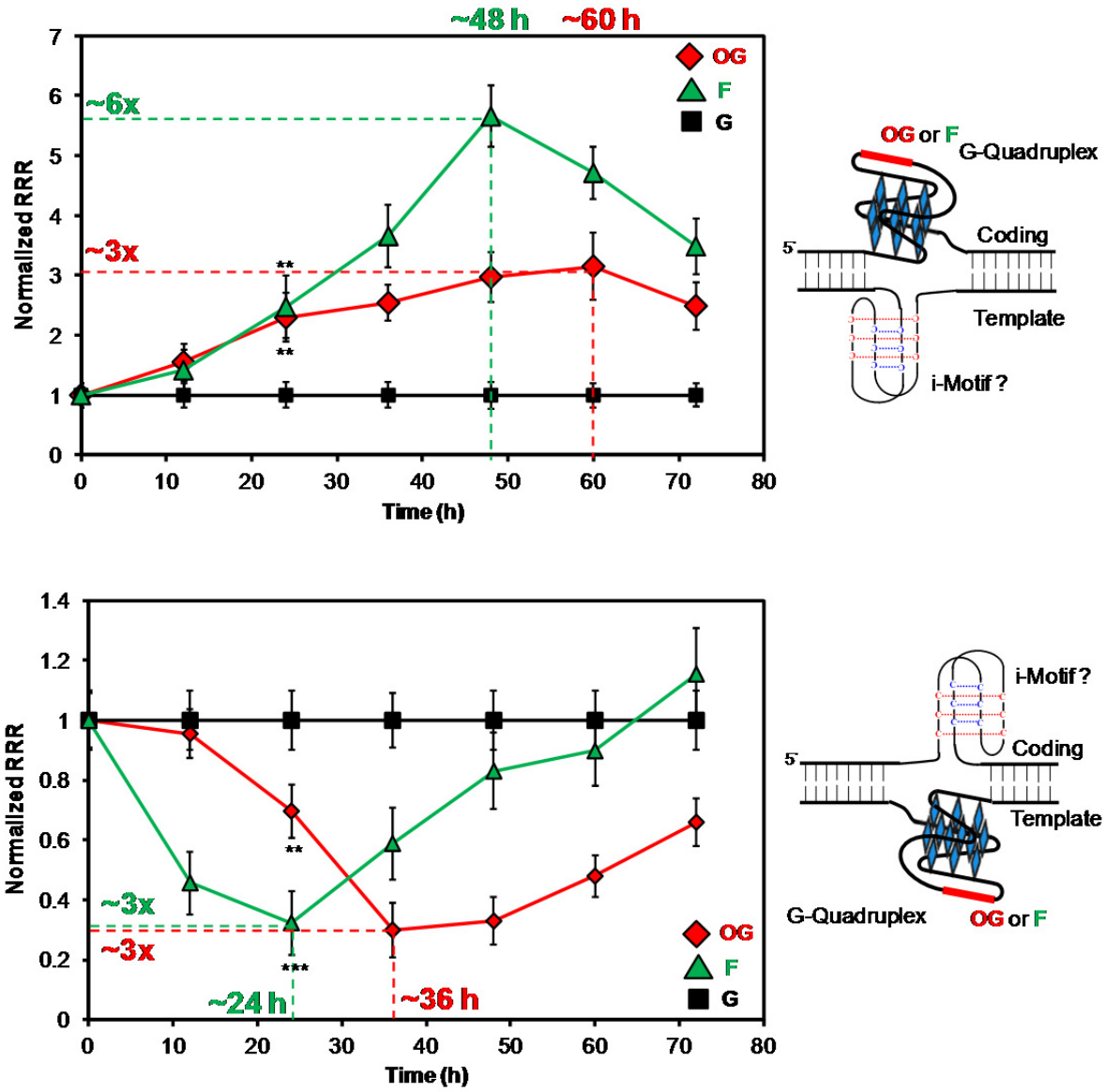
All cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 μg/mL gentamicin, 1x glutamax, and 1x non-essential amino acids. The cells were grown at 37 °C with 5% CO_2 at ~80% relative humidity and were split when they reached 70-80% confluence. The $Ogg1^{-/-}$ MEFs were previously developed,[5] and deposited in the Ximbio sharing program. The wild-type glioblastoma cells (U87 MG) were purchased from ATCC.

The transfection experiments were conducted in white, 96-well plates by seeding 2 x 10^4 cells per well and then allowing them to grow for 24 h. After 24 h, the cells were transfected with 200-400 ng of plasmid using X-tremeGene HP DNA transfection agent (Roch) following the manufacturer's protocol in Opti-MEM media. All transfection experiments were conducted at least 4 times. Next, 48 h post transfection, the Dual-Glo luciferase (Promega) assay was conducted following the manufacturer's protocol.

To conduct the siRNA knock down of APE1 or CSB (ERCC6), FlexiTube siRNAs (Qiagen) were transfected into the glioblastoma cells with x-tremeGene HP DNA transfection agent (Roche) at 1 nM, 5 nM and 50 nM concentration 24 h prior to the plasmid of interest.

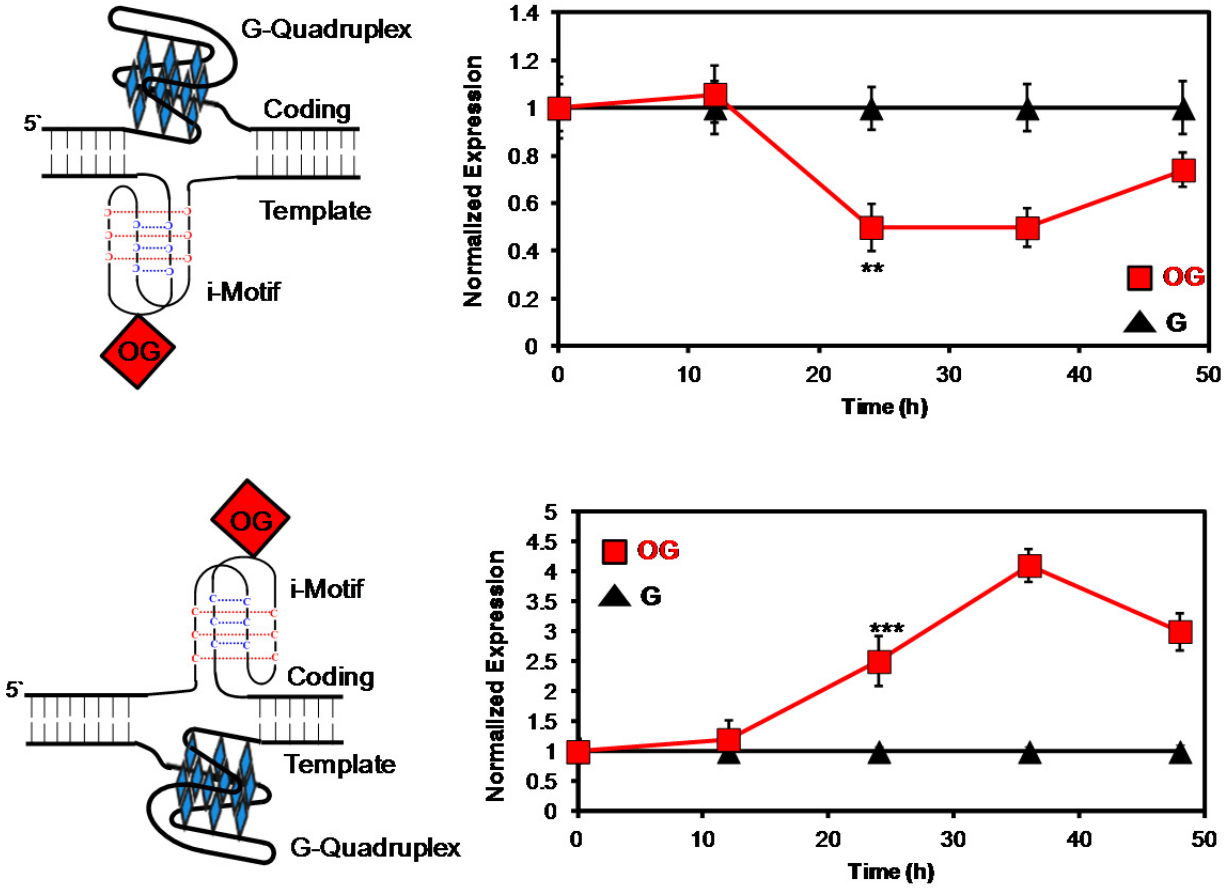
The data were analyzed by converting the luminescence measured into normalized relative response ratios (RRR), which is the luminescence of Rluc divided by the luminescence of luc (i.e., RRR = Rluc/luc). To obtain the normalized expression values reported, each RRR was divided by the RRR for the wild type sequence in that data set, for example, normalized expression = RRR_{OG12}/RRR_{WT}. The error bars represent 95% confidence intervals obtained from the data.

Figure S1. Time-course analyses for luciferase expression in glioblastoma cells.



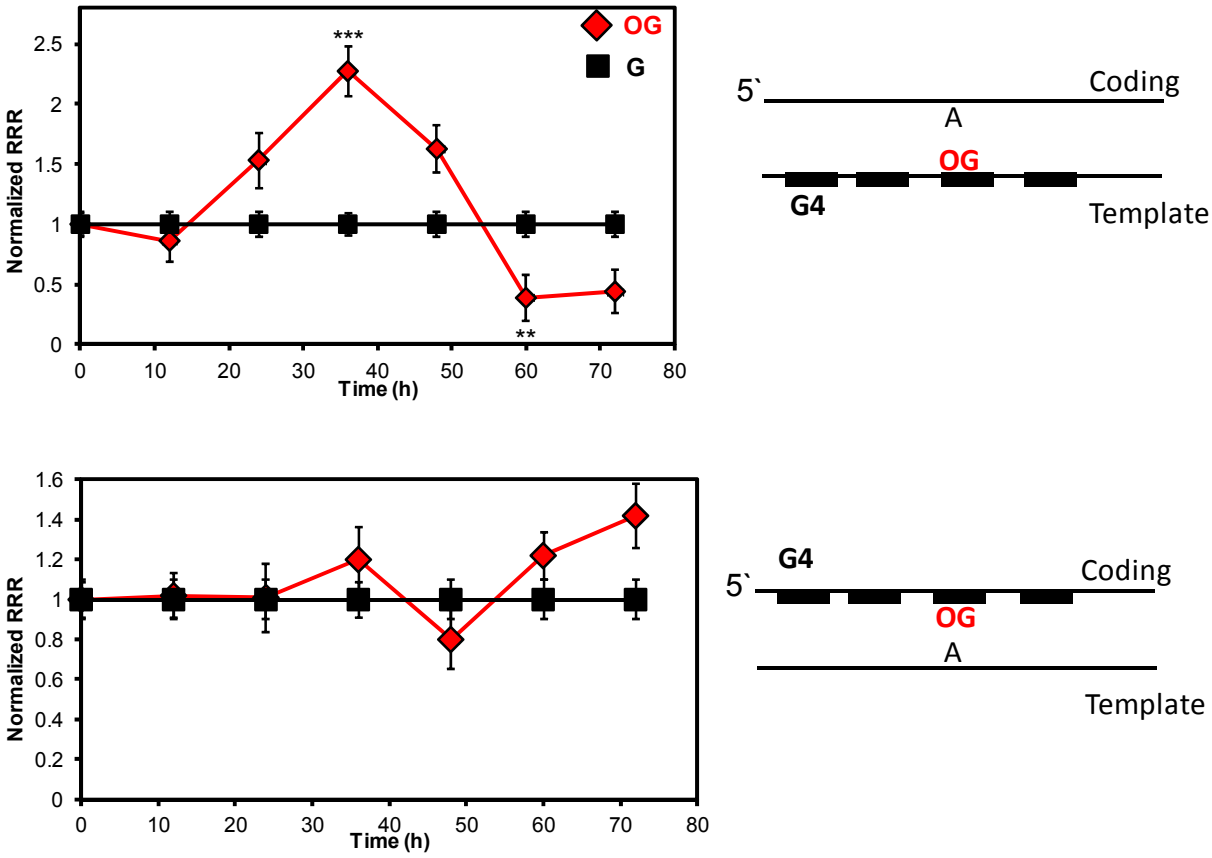
On the basis of a Student's t test, the time in which significance at $^{**}P<0.01$ or $^{***}P<0.001$ was first observed are marked on each plot.

Figure S2. Time-course analyses for luciferase expression with OG in an i-motif context.



On the basis of a Student's t test, the time in which significance at $^{**}P<0.01$ or $^{***}P<0.001$ was first observed are marked on each plot.

Figure S3. Time-course analyses for luciferase expression with an OG:A base pair in glioblastoma cells.



On the basis of a Student's t test, the time in which significance at $^{**}P<0.01$ was first observed are marked on each plot.

References

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