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Supplemental information

ciRS-7 and miR-7 regulate ischemia-induced neuronal death via glutamatergic signaling

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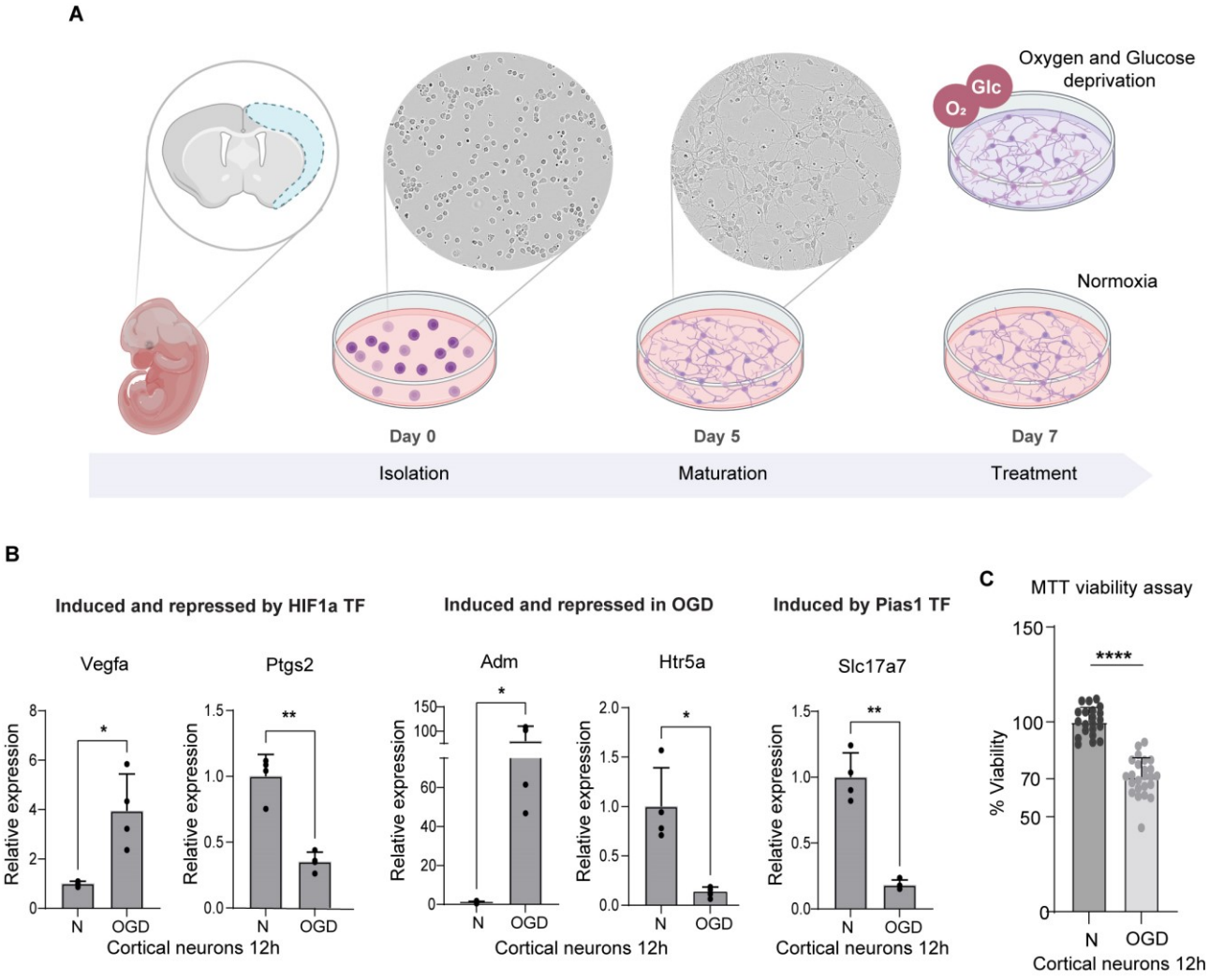
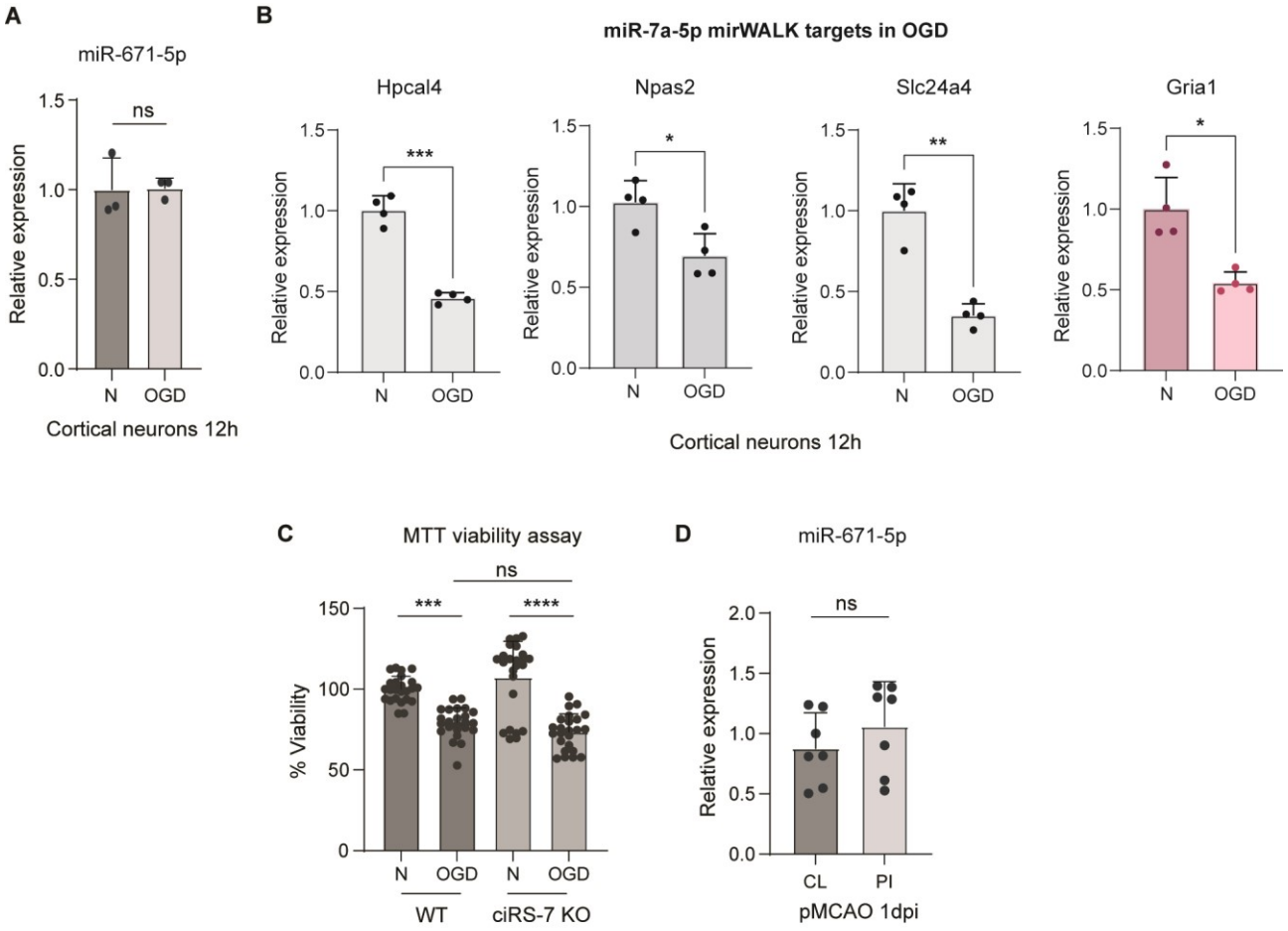
SUPPLEMENTARY FIGURES



Supplementary figure S1. OGD experimental setup, Related to Figure 1.

(A) Schematic illustration of the cortical neuron culture experimental design. After E15 embryonic cortices dissection (Day 0), isolated neurons were plated and matured (Day 5) and were subsequently treated with oxygen and glucose deprivation for 12 hours (Day 7). **(B)** Bar plot illustrating the expression levels detected by quantitative PCR of genes that showed differential expression in the RNA-seq dataset of cortical neurons treated with OGD for 12 hours compared to their normoxic counterparts. In alignment with Figure 1C IPA analysis, the result validate downstream effect of HIF1a induction by upregulation and downregulation of its targets Vegfa and Ptgs2 in response to

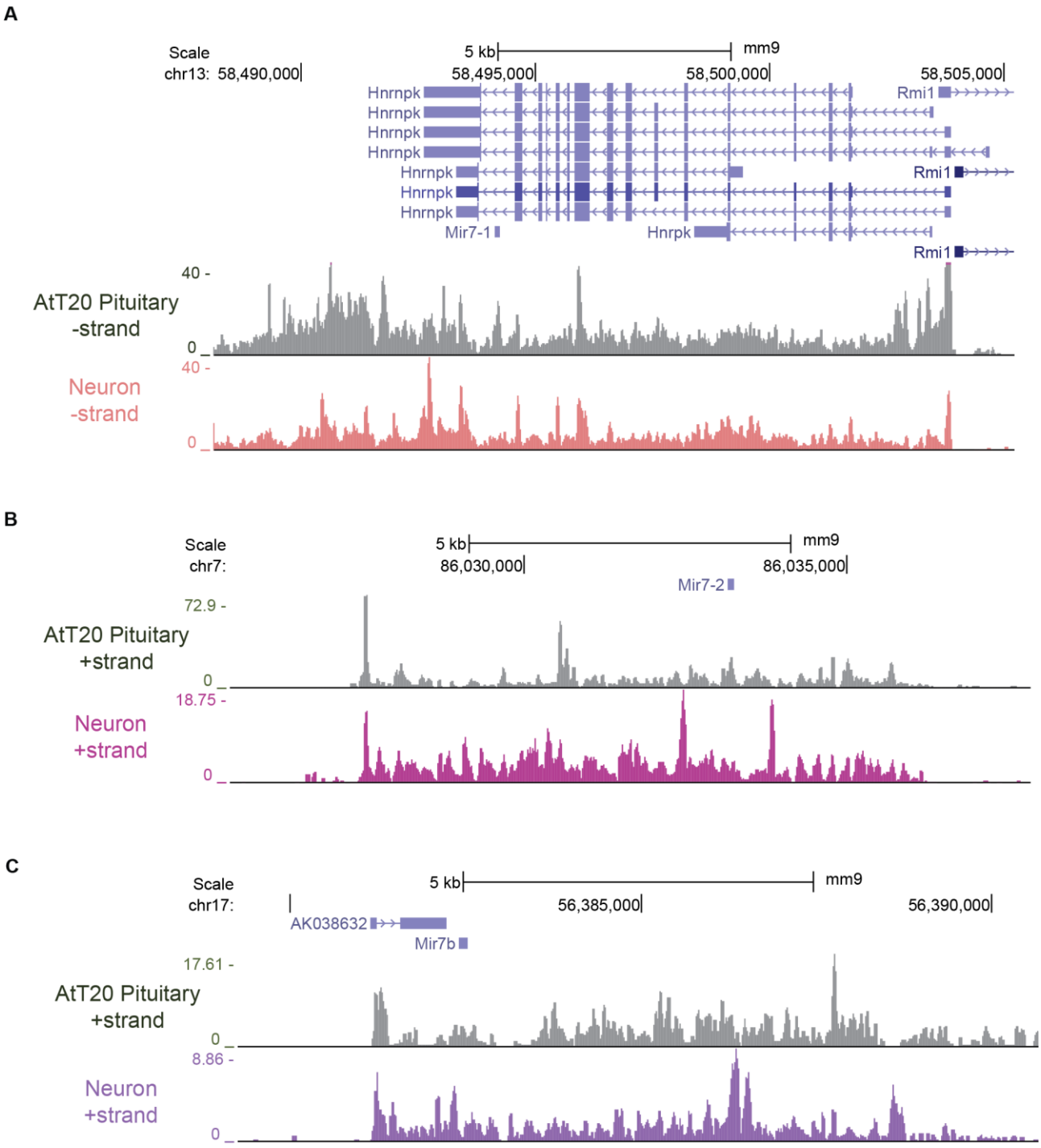
OGD. Additionally, Adm and Htr5a were found to be upregulated and downregulated, respectively, in the RNA-seq dataset. The gene Slc17a7 is anticipated to be activated by the Pias1 transcription factor in the Figure 1C analysis. However, due to Pias1 downregulation in OGD, there is a corresponding decrease in the expression level of its target, Slc17a7 (n = 4 biological replicates, * = p-value < 0.05, ** = p-value < 0.01, calculated with paired t-test; data as mean ± SD). **(C)** Bar plot of relative absorbance in MTT viability assay of wild-type cortical neurons treated with normoxic (complete medium and normal humidified cell culture incubator) or OGD conditions (no glucose, no pyruvate, 1%O2) for 12 hours. Data expressed as survival percentage normalized on wild-type normoxic (n = 4 biological replicates; **** = p-value < 0.0001 calculated with calculated with unpaired t-test; data as mean ± SD).



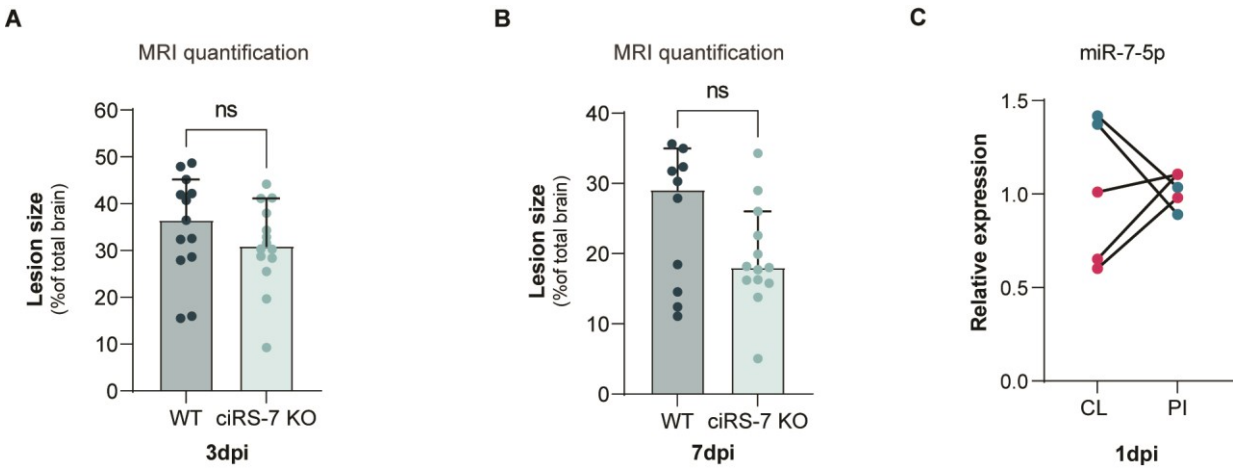
Supplementary figure S2. ciRS-7 expression level is not anticorrelated to miR-671-5p and does not affect OGD induced cell death, Related to Figure 2,3 and 4.

(A) Bar plot of the quantification by quantitative PCR of miR-671-5p in wild-type cortical neurons treated with normoxic (complete medium and normal humidified cell culture incubator) or OGD conditions (no glucose, no pyruvate, 1%O2) for 12 hours. Relative expression normalized on the average expression of normoxic conditions (n = 3; p-value calculated with paired t-test; data as mean ± SD). **(B)** Bar plot illustrating the expression levels detected by quantitative PCR of genes that showed differential expression in the RNA-seq dataset of cortical neurons treated with OGD for 12 hours compared to their normoxic counterparts. In alignment with the RNA-seq data the predicted miR-7 targets are downregulated in OGD conditions (n = 4 biological replicates, * = p-value < 0.05, ** = p-value < 0.01, ** = p-value < 0.001 calculated with paired t-test; data as mean ± SD).

(C) Bar plot of relative absorbance in MTT viability assay of wild-type and ciRS-7 KO cortical neurons treated with normoxic (complete medium and normal humidified cell culture incubator) or OGD conditions (no glucose, no pyruvate, 1%O2) for 12 hours. Data expressed as survival % normalized on wild-type normoxic (n = 4 biological replicates; *** = p-value < 0001 **** = p-value < 0 calculated with one-way ANOVA test corrected with Tukey's post-hoc test; data as mean ± SD). **(D)** Bar plot of the quantification by quantitative PCR of miR-671-5p in 3-4 months old BALB/c mice subjected to pMCAO surgery. Cortical peri-ischemic (PI) and contralateral (CL) samples were collected at 1 day post-surgery, timepoint in which ciRS-7 is significantly downregulated. Relative expression normalized on the average expression of contralateral healthy hemisphere (n = 6-7; p-value calculated with paired t-test; data as mean ± SD).

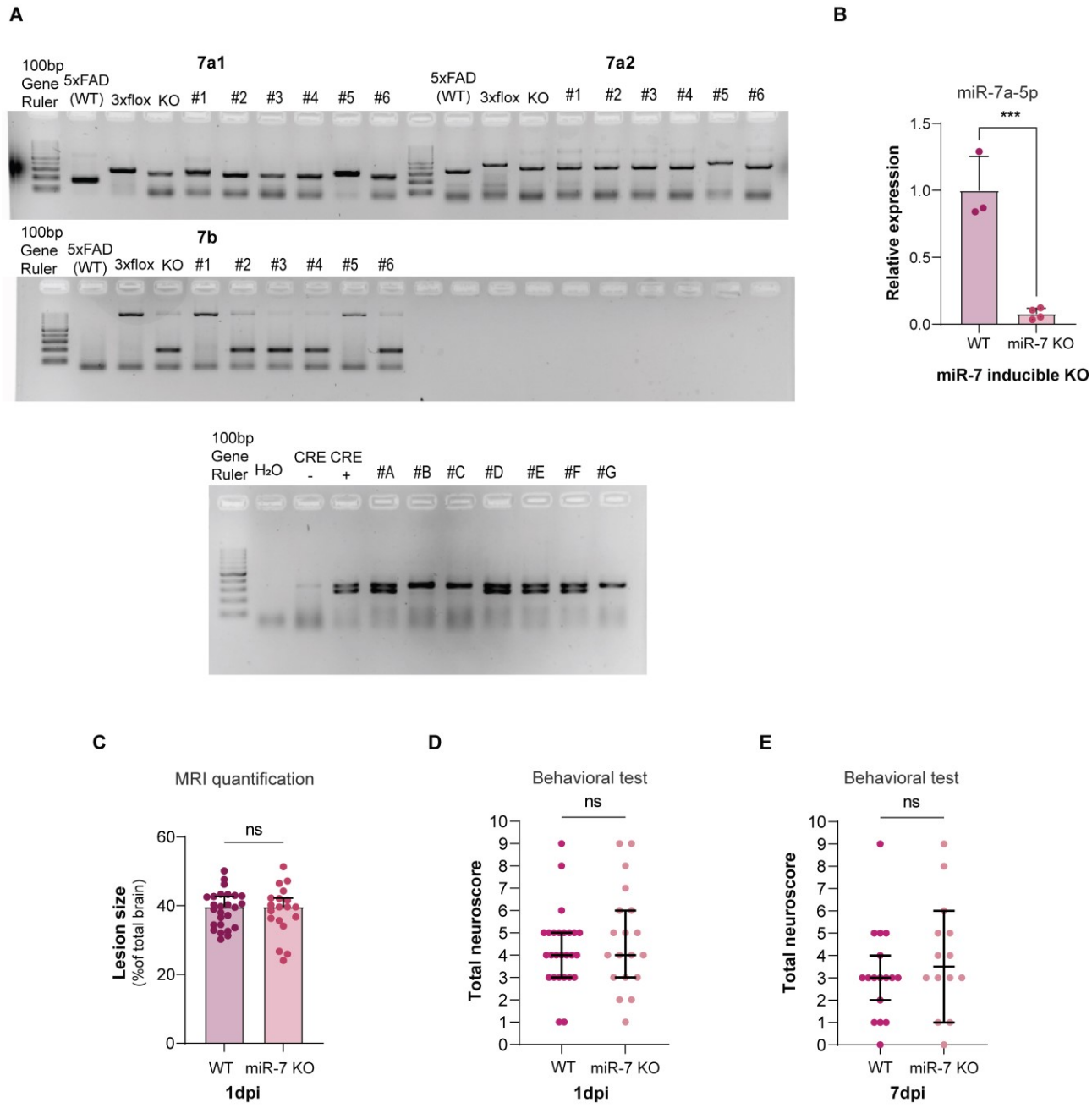


Supplementary figure S3. miR-7 loci expression in Neurons and Pituitary cells, related to Figure 2. The GRO-seq coverage tracks for the three miR-7 loci (miR-7a-1, miR-7a-2, and miR-7-b) in the UCSC genome browser are shown in panels A, B, and C, respectively. These tracks were sourced from GSE64515 (Pituitary) and GSE215210 (Neurons) as presented in Supplementary Table S3.



Supplementary figure S4. tMCAO at later timepoint is not influenced by ciRS-7 and does not induce miR-7 in wild-type mice, Related to Figure 4.

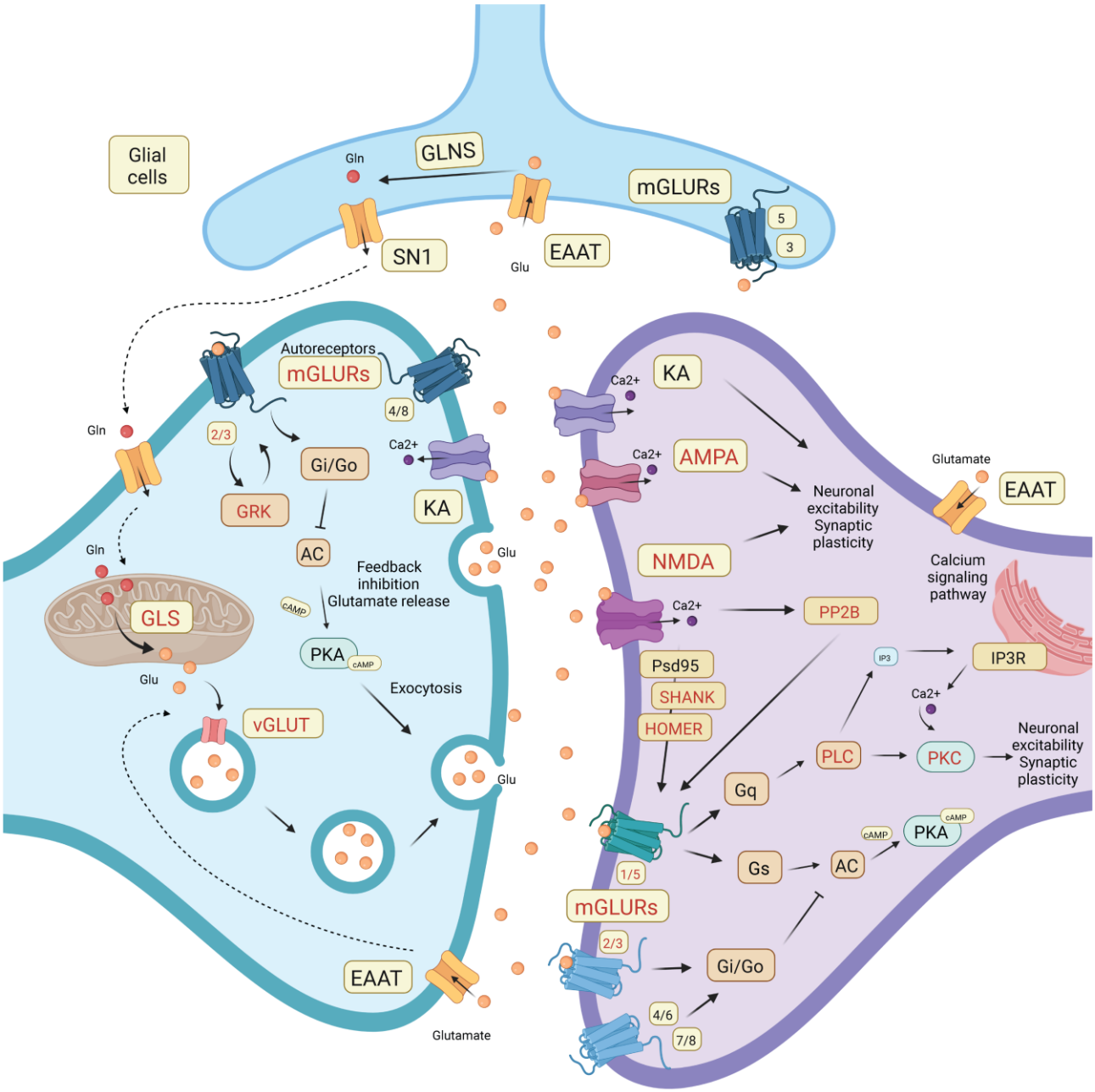
(A) Bar plot of MRI quantification of the lesion size of wild-type and ciRS-7 KO mice subjected of tMCAO at 3 day post-surgery. Data expressed as lesion percentage on total brain size adjusted for oedema (n = 12-14 per group; p-value calculated with Mann–Whitney test; data as median with 95% confidence interval). **(B)** Bar plot of MRI quantification of the lesion size of wild-type and ciRS-7 KO mice subjected of tMCAO at 7 day post-surgery. Data expressed as lesion percentage on total brain size adjusted for oedema (n = 10-13 per group; p-value calculated with Mann–Whitney test; data as median with 95% confidence interval). **(C)** Before-after plot of the quantification by quantitative PCR of miR-7 expression in tMCAO wild-type ischemic mice at one day post ischemia (1dpi). The plot highlights high variability of miR-7 levels in the peri-ischemic (PI) cortex based on the basal expression in the contralateral (CL) cortex. Mice with increase of miR-7 expression in peri-ischemic cortex are highlighted in magenta, mice with decrease of miR-7 expression in the peri-ischemic cortex are highlighted in blue (n = 5).



Supplementary figure S5. miR-7 KO animals do not show acute effects on ischemic stroke outcome, Related to Figure 5.

(A) (*top*) Nucleic acid (DNA) agarose gel of genotyping from ear samples of miR-7 KO animals in which all miR-7 loci (7a1, 7a2, 7b) are embedded in between loxP sequences (3xflox) regions and can be recombined through activation of Cre-recombinase upon tamoxifen administration. Both Cre positive and Cre negative mice were administered tamoxifen and checked for induced recombination and spontaneous recombination. 5XFAD represent another mouse strain lacking loxP sequences and

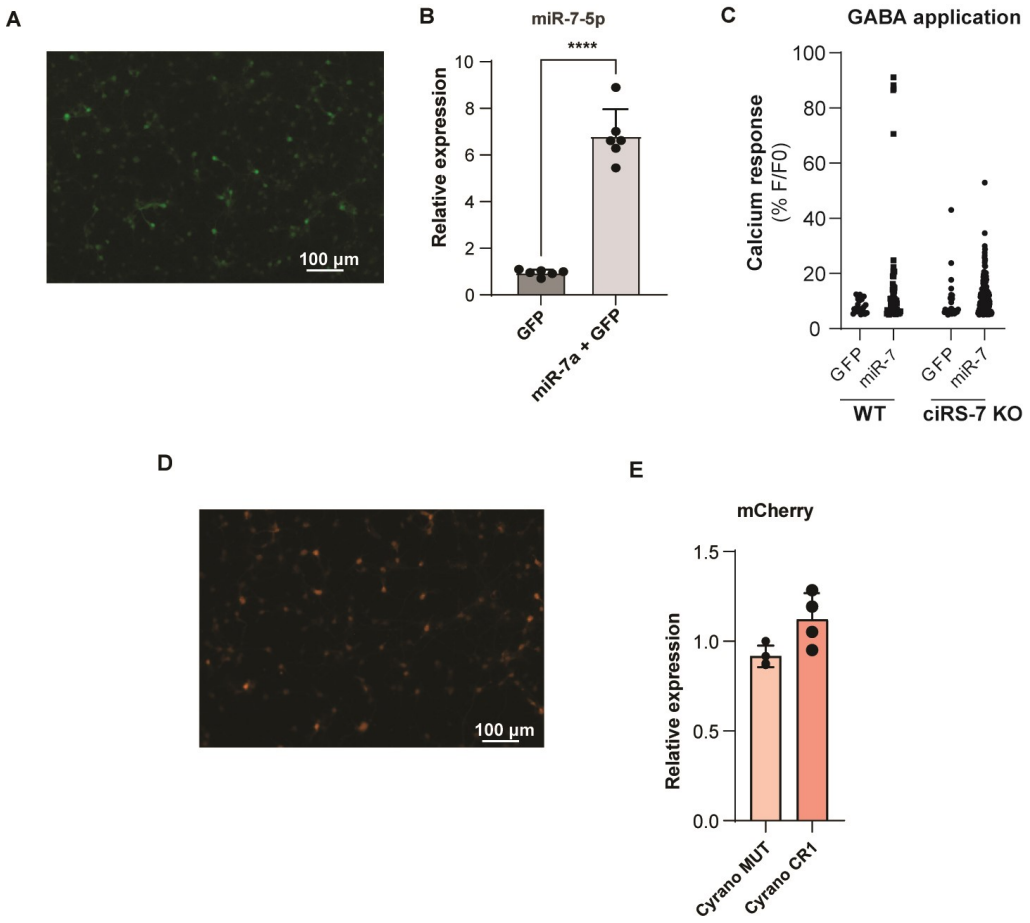
3xflox is a miR-7 KO mouse strain that has unrecombined loxP regions in the miR-7 loci. Mice were screened for recombination 2 weeks upon tamoxifen injections (#1-#7) in the three loci. Number 4 (#4) represent a Cre positive mouse which has fully recombined all the three loci, thus it was included in the animal study as KO. Number #6 is an animal lacking Cre expression but that received treatment with tamoxifen, none of the loci spontaneously recombined, thus it was included in the animal study as WT. (*bottom*) Nucleic acid (DNA) agarose gel of genotyping to determine the presence of Cre recombinase. Cre- present a band of IL-10 which was used as positive control of the PCR reaction. Only CRE+ mice should be able to recombine, if spontaneous recombination is detected in Cre-, the mice are excluded from the study. **(B)** Bar plot of the quantification by quantitative PCR of miR-7-5p in 3-4 months old miR-7 KO mice subjected to tMCAO surgery. Cerebellar miR-7 expression analysis validates the abolishment of miR-7 in CRE-positive mice injected with tamoxifen in respect of Cre-negative injected with the same drug. miR-7 KO mice expresses around 90% less miR-7 than wild-type. (n = 3-4 per group; *** = p-value < 0001 calculated with unpaired t-test; data as mean ± SD). **(C)** Bar plot of MRI quantification of the lesion size of wild-type and miR-7 KO mice subjected of tMCAO at 1 day post-surgery. Data expressed as lesion percentage on total brain size adjusted for oedema (n = 19-26 per group; p-value calculated with Mann–Whitney test; data as median with 95% confidence interval). **(D)** Scatter dot plot of cognitive deficit and motor impairment verified by neuroscore behavioral test in wild-type and miR-7 KO mice subjected of tMCAO at 1 day post-surgery. Data expressed as total neuroscore values (n = 19-26 per group; data as median with 95% confidence interval). **(E)** Scatter dot plot of cognitive deficit and motor impairment verified by neuroscore behavioral test in wild-type and miR-7 KO mice subjected of tMCAO at 7 day post-surgery. Data expressed as total neuroscore values (n = 8-11 per group; data as median with 95% confidence interval).



Supplementary figure S6. miR-7 targets extensively regulate glutamatergic signaling, Related to Figure 5.

Schematic illustration of miR-7 glutamatergic neuronal targets in ischemic stroke obtained by the overlap of miR-7 validated targets in pyramidal neurons obtained by HITS-CLIP-seq analysis and downregulated genes in ciRS-7 KO tMCAO ischemic animals, conditions in which miR-7 is upregulated. The figure is adapted from KEGG pathway glutamatergic signaling in which miR-7 targets are highlighted in red. In black are represented the genes involved in the pathway but

not targeted by miR-7.

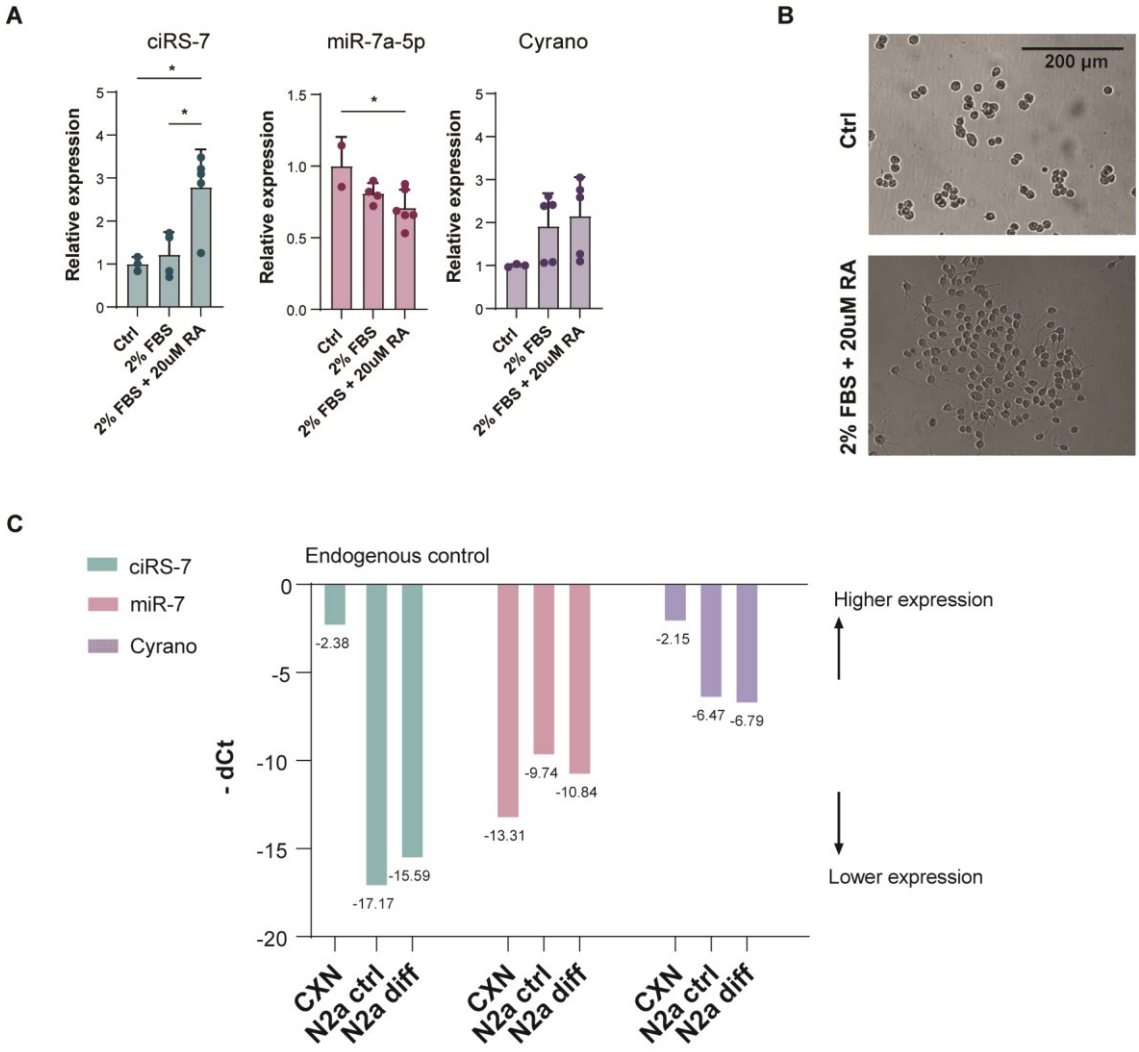


Supplementary figure S7. miR-7 overexpression resembling physiological levels in stroke does not alter GABA response, Related to Figure 5 and 6.

(A) Representative fluorescence image of the efficiency of infection and expression of cortical neurons with GFP or miR-7a-GFP lentivirus. **(B)** Bar plot of the quantification by quantitative PCR of miR-7a-5p expression in wild-type cortical neurons at 7 days post-infection with GFP-miR-7a (miR-7a) or GFP only (GFP) lentivirus. Relative expression normalized on the average expression of GFP infected neurons conditions (n = 6; **** = p-value < 0.0001 calculated with paired t-test; data as mean ± SD). **(C)** Calcium imaging analysis of the unaffected cellular response to GABA in wild-type and ciRS-7 KO cortical neurons infected with GFP or miR-7a-GFP lentivirus

(n = 5 biological replicates; signal included > 5% of baseline). (**D**) Representative fluorescence image of the efficiency of infection and expression of cortical neurons with MUT-mCherry or CR1-mCherry

lentivirus. (**E**) Bar plot of the quantification by quantitative PCR of mCherry expression in wild-type cortical neurons at 7 days post-infection with MUT-mCherry (Cyrano MUT) or CR1-mCherry (Cyrano CR1) lentivirus. Relative expression normalized on the average expression of MUT infected neurons conditions.



Supplementary figure S8. The buffer effect on miR-7 depends on the expression of ciRS-7, Related to Figure 5.

(A) Bar plot of the quantification by quantitative PCR of ciRS-7 (blue), miR-7 (magenta), Cyrano (purple) in Neuro-2a neuroblastoma murine cell line (N2a) in control growth conditions (Ctrl), starvation (2% FBS) and differentiation media with retinoic acid (2% FBS and 20uM RA) at 5 days time-point (n = 3; * = p-value < 0.05 calculated with unpaired t-test; data as mean ± SD). **(B)** Representative bright field microscopy image of the phenotype Neuro-2a cells in control media (Ctrl) and differentiated with 2% FBS and 20uM retinoic acid (2% FBS and 20uM RA) at 5 days time-point. **(C)** Bar plot of the quantification by quantitative PCR of ciRS-7 (blue), miR-7 (magenta), Cyrano (purple) in wild-type primary cortical neurons (CXN) and Neuro-2a cells undifferentiated (N2a ctrl) and differentiated with 2% FBS and 20uM retinoic acid (N2a diff). The data is presented

as the negative difference in Ct values of the gene of interest relative to the same endogenous control gene (-dCt). A higher negative difference indicates lower expression of the gene of interest compared to the control highly expressed gene, while a lower negative difference suggests higher expression of the gene of interest. The arrows schematically represent these concepts, and each unit of difference represents a power of two changes in expression (n = 4-5 replicate per group; data as mean of the dCt of each sample type).