# Supplemental Materials

***Molecular Biology of the Cell***

Selvi et al.

**SUPPORTING INFORMATION**

# CARM1 regulates astroglial lineage through transcriptional regulation of Nanog and post-transcriptional regulation by miRNAs.

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**Methods:**

**Human Embryonic stem cell culture maintenance:**

a) Matrigel coating: Matrigel (BD Biosciences 354277) solution was prepared in DMEM media (Invitrogen) in the ratio of 0.1ml matrigel per 10 ml of media. Tissue culture flasks and culture dishes were coated with matrigel and left for a minimum of 45 minutes at room temperature. Prior to use, the matrigel solution was discarded and the tissue cultureplate/dish/flask was rinsed with 1X PBS and maintained without drying at all times.

b) Feeder-free culturing of ES cells: BG01V cells were purchased from Global stem (GSC-1103). The frozen cells were thawed at 37ºC, and transferred to DMEM media containing knockout serum (KSR, Invitrogen) followed by centrifugation at 700 rpm at room temperature for 5 minutes. The cells were then revived in MEF (mouse embryonic fibroblast) conditioned medium (Globalstem), containing 4 ng/ml bFGF (basic fibroblast growth factor, Invitrogen). After three days, a media change using MEF conditioned medium supplemented with bFGF was given. Colonies were observed after five days of culturing. Confluent cultures were trypsinized using 0.05% trypsin EDTA (Invitrogen) for 2 minutes at 37ºC, followed byneutralization using DMEM with KSR and centrifuged at 700 rpm for 5 minutes at roomtemperature. Cell count was routinely performed during each trypsinization and seeded as perexperimental requirements.

**Embryoid body formation:** The embryoid body formation was initiated with an ES cell count of 1 X $10^6$ cells/ml. The ES cells were resuspended in conditioned medium (Globalstem) containing 1% PVA (Sigma).This suspension was added to low-attachment petri plates (Tarsons) and incubated at 37°C for 48 hours without disturbing the plates and differentiation was carried out as described below.

**Differentiation:** The EBs were allowed to grow in size and were visualized at regular intervals under the light microscope. Once the clumping of cells was clearly evident, the EBs were transferred to DMEM medium (Invitrogen) supplemented with 20% KSR (Invitrogen) and 10% FBS (Biological Industries) onto 0.1% gelatin pre-coated dishes. EBs could also be set up with the differentiation medium in which case the formation of the EB was slower.

Media changes were given every 5-7 days with the differentiation media. Compound treatment was generally given between the day 2 and day 4 of EB formation for 48 hours time point.

**Alkaline phosphatase staining:** Alkaline phosphatase staining was performed to determine the pluripotency status. Cells that were in culture for a minimum of 5 days were used for the staining purpose.The staining was performed using an alkaline phosphatase staining kit (MilliporeSCR004).

**Immunofluorescence Analysis:** Cells were grown on cover slips coated with poly-lysine at 37°C in a 5% CO2 incubator. After the indicated treatment, the cells were washed with 1X PBS and fixed with 4% paraformaldehyde (in PBS) for 20 minutes at room temperature. The cells were then permeabilized using 0.5% Triton X-100 (in PBS) for 10 minutes and subsequently washed thrice with 1X PBS for 10 minutes. The non-specific sites were blocked using 5% FBS (in PBS) for 45 minutes at 37°C. The immunostaining was performed with primary antibody atappropriate dilutions for 1 hour at room temperature. The cells were washed with wash buffer (1% FBS in 1X PBS) 4 times, 3 minutes each, and incubated with a secondary antibody tagged with a fluorescent dye at appropriate dilutions for 1 hour at room temperature. After being washed with wash buffer, the nuclei were stained with Hoechst (1 µg/ml) for 20 minutes. The cover slips were washed twice with 1XPBS and were inverted onto a microscopic slide over 2 µl

of 70% glycerol (in PBS) and visualized using confocal microscopy. The antibodies used were Mouse monoclonal anti-GFAP [2A5] Abcam-ab4648, Rabbit polyclonal anti-Histone H3 (asymmetric di methyl R17) - ChIP Grade-Abcam-ab8284, Mouse Monoclonal anti-Sox1-R&D Systems-MAB3369, Mouse monoclonal anti-Human/Mouse Brachyury--Millipore-04-135.

**Whole transcriptome and miRNA profiling:** High quality total RNA from TBBD treated and DMSO treated cells/ EBs was profiled for whole genome gene expression using Affymetrix Human Exon 1.0 ST GeneChip as per manufacturer recommended protocols. Same total RNA was profiled for whole genome miRNA using Agilent Human miRNA profiling microarray as per manufacturer recommended protocols. Raw data was obtained in .cel format for Affymetrix GeneChip and .txt format for Agilent miRNA microarray. Microarray data was submitted in NCBI GEO with the accession ID XXXXX

**Microarray Data Analysis:** Gene expression microarray data was normalized using RMA method followed by median to all the samples. miRNA expression microarray data was normalized to 90th Percentile followed by median to all the samples using GeneSpring GX v 12.0 from Agilent Technologies INC, Santa Clara. Identification of statistically significant differentially expressed genes and miRNA was done using Volcano plot method by applying Student T-test with Benjamini Hochberg FDR correction. Genes and miRNA that were 2 fold and above up or down regulated with a p-value of less than or equal to 0.05 was considered as significant for downstream biological analysis.

**Biological analysis of differentially expressed genes and miRNA:** GOElite (www.genmapp.org/go_elite) tool was used to identify statistically significant dysregulated pathways and Gene Ontologies encompassing the differentially expressed genes. miRWalk

database (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html) was used to identify the validated targets of differentially expressed miRNAs. Further, GOElite analysis was done for validated targets of differentially expressed miRNA to identify dysregulated pathways and gene ontologies.

**Transcriptional and Post-transcriptional regulatory network modeling:** Feed forward and Feed Back loop analysis of differentially expressed transcription factor: gene: miRNA was done using data obtained from Circuits DB (http://biocluster.di.unito.it/circuits/) and validated miRNA targets. Cytoscape V 8.2 was used to model the transcriptional and post transcriptional regulatory networks.

**miRNA cloning and expression**: Pre-miR-92a and the scrambled construct was cloned into pSuper-puro vector as per manufacturer's instructions. The construct was transfected into SH-SY5Y cells, and RNA extraction was done after 36 hours of transfection, for Northern blot analysis and qRT-PCR analysis.

**Zebrafish maintenance and breeding**: The embryos were grown until the tail bud stage and dechorionated using pronase (2mg/ml final concentration). The dechorionated embryos were treated with various concentrations of TBBD (50 μM, 100 μM, 150 μM, 200 μM) until 24 hpf and 48 hpf and were then used for imaging, immunostaining and cryosectioning. Following cryosectioning, mouse monoclonal anti-GFAP antibody-Abcam- ab4648 and rabbit polyclonal anti-TUBB3 antibody-Abcam- ab18207 were used to stain the cryosections.

**Morpholino experiments**: Custom made lissamine tagged splice-blocking, morpholino antisense oligonucleotides targeting zebrafish CARM1 were obtained from Gene-tools Inc, USA.

Working dilutions of morpholino was prepared in Danieau solution with 0.1% phenol red and was injected into one-cell stage zebrafish embryos using Femtojet system from Eppendorf.

For the rescue of sensory response, capped mouse CARM1 mRNA was synthesized *in vitro* using T7 mMessage mMachine kit (Ambion). Capped CARM1 mRNA (150pg) was injected either alone or with the morpholino against CARM1 into one-cell stage zebrafish embryos to analyze the rescue phenotype.

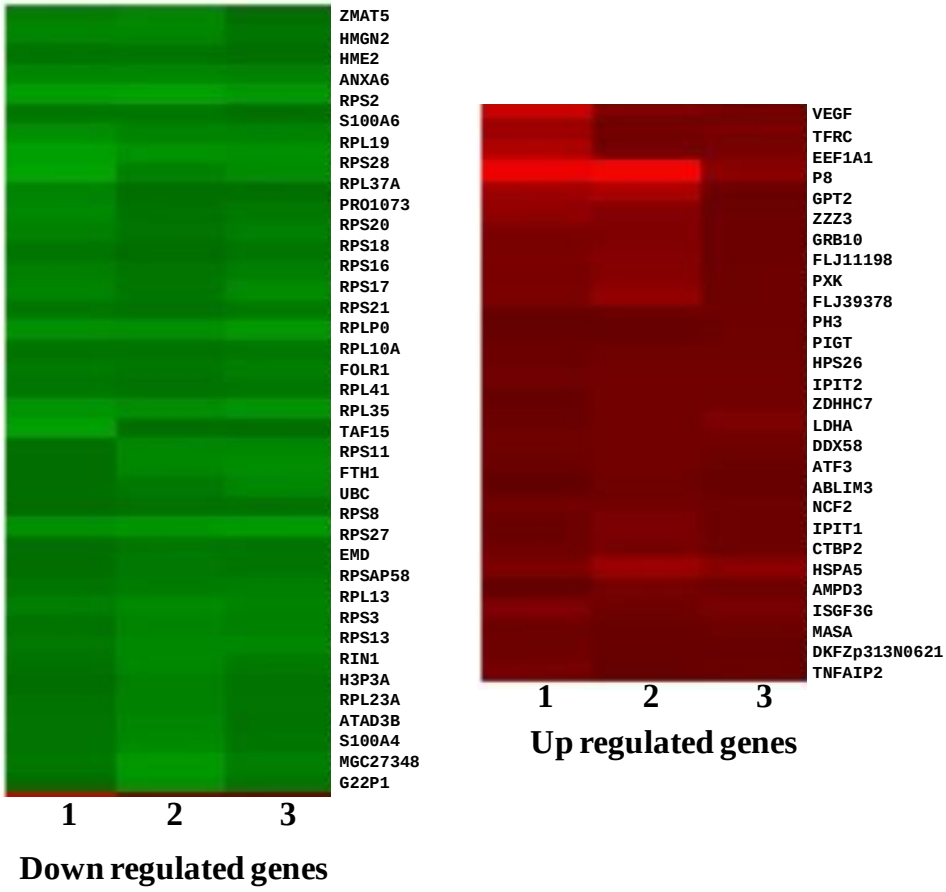
**Whole mount immunostaining of zebrafish embryos:** The embryos were fixed in 4% paraformaldehyde overnight at 4°C and then permeabilized using 1% Tween20 in 1X PBS. Following blocking with 10% foetal bovine serum in PBS containing 1% Tween20, the embryos were incubated with the primary antibodies in the same buffer. The antibodies used were rabbit polyclonal anti-Histone H3 (asymmetric di methyl R17) - ChIP Grade-Abcam-ab8284, rabbit polyclonal anti-dimethyl-Histone H3 (Arg26) Antibody-Millipore-07-215, rabbit polyclonal anti-PRMT4 antibody-Abcam-ab84370. After washes and incubation with the fluorescent dye tagged secondary antibody, the immunostained embryos were imaged using confocal microscopy.

**Response to stimulus:** 200 10 hpf embryos in replicates were treated with DMSO or with TBBD (50µM, 100µM, 150µM and 200µM) or were left untreated. After 48, 72 and 96 hours of treatments, the larvae (n=100) were touched with a needle under the microscope and their response to touch was assayed. The larvae that do not respond to touch i.e., those that do not dart away were scored and tabulated. Similar measurement was done with control, CARM1 morphants, and with CARM1 mRNA injected embryos.

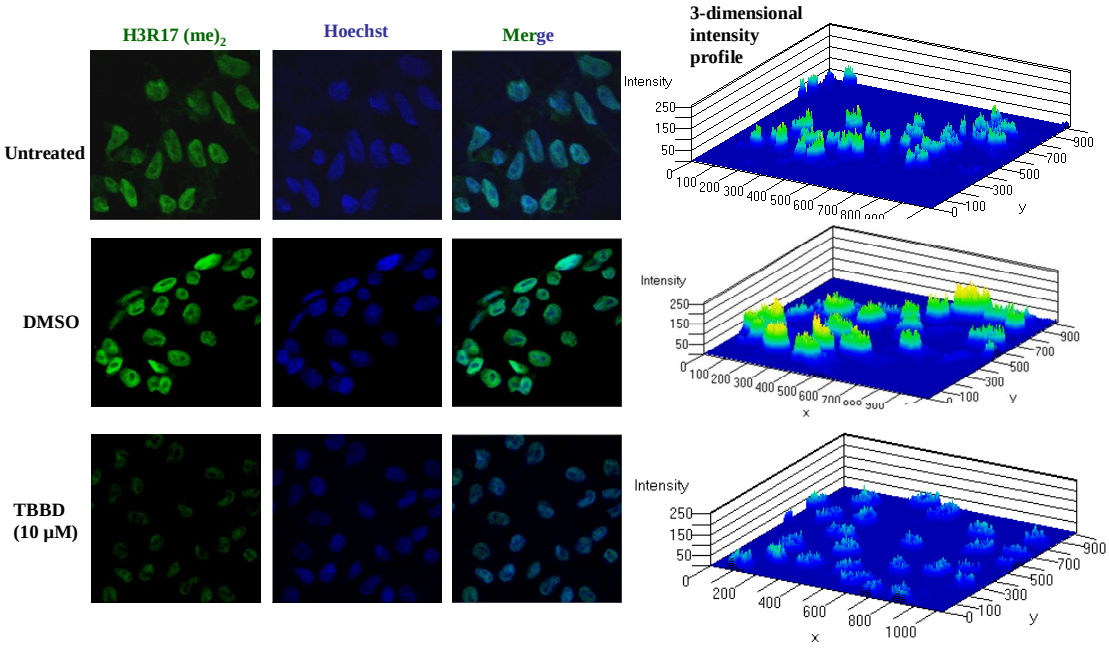
**pERK levels analysis:** U373 cells were treated with 50 µM TBBD for 10 minutes, 45 minutes or 24 hours; cells treated with DMSO for the same periods of time were used as control. Cell

lysates were resolved on a 10% SDS-PAGE gel and subjected to immunoblotting analysis as described in Selvi *et al.*, 2010. ERK and pERK antibodies from Cell Signalling Technologies was used for the analysis.

**Supporting figures:**



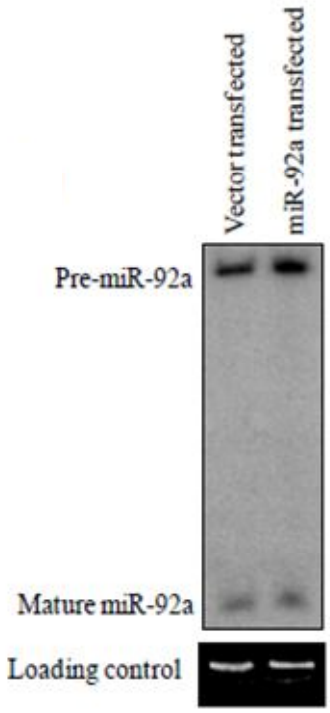

**Supporting Figure 1: Histone H3R17 methylation modulates global gene expression.** A combined heatmap representing the upregulated and downregulated genes upon TBBD treatment in HeLa cells. The three samples are biological replicates of treated samples compared to DMSO control.



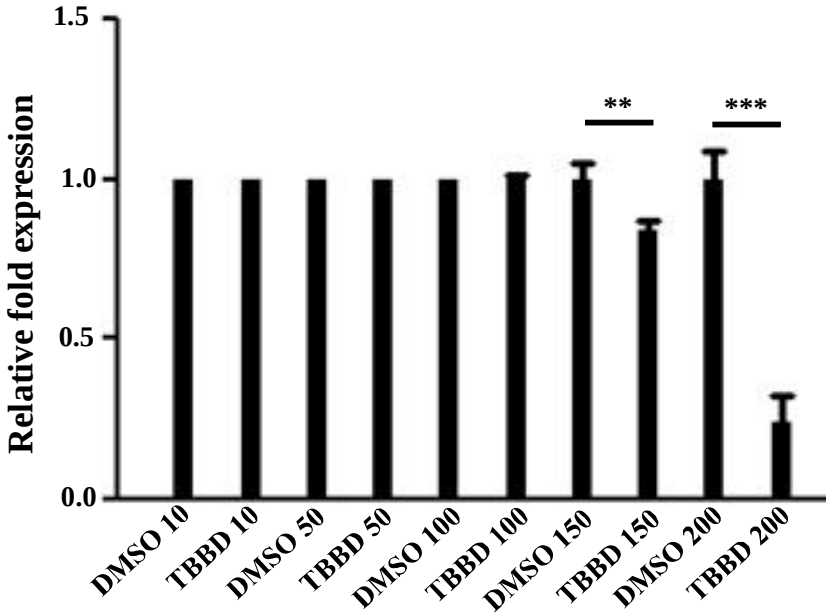

**Supporting Figure 2: Inhibition of histone H3R17 methylation in BGO1V cells.** Immunofluorescence analysis of human embryonic stem cell line, BGO1V, untreated, treated with DMSO or 10 µM TBBD as indicated with H3R17 methylation antibody, nuclear staining with Hoechst and the merged image of the two, depicts the decrease in H3R17 methylation upon TBBD treatment. The intensity is plotted in the graphs on right.

| | | | |
|---|---|---|---|
| hsa-miR-367 | **0.964** | hsa-miR-1202 | **-1.459** |
| hsa-miR-1246 | **-2.219** | hsa-miR-302a* | **-1.447** |
| hsa-miR-33b* | **-1.929** | hsa-miR-1260 | **-1.399** |
| hsa-miR-365 | **-1.904** | hsa-miR-720 | **-1.344** |
| hsa-miR-1234 | **-1.763** | hsa-miR-302d | **-1.341** |
| hsa-miR-363 | **-1.731** | hsa-miR-1274b | **-1.326** |
| hsa-miR-1274a | **-1.689** | hsa-miR-1238 | **-1.304** |
| hsa-miR-1308_v15.0 | **-1.637** | hsa-miR-302b | **-1.195** |
| hsa-miR-424 | **-1.628** | hsa-miR-92a | **-1.169** |
| hsa-miR-10a | **-1.621** | hsa-miR-130a | **-1.16** |
| hsa-miR-575 | **-1.592** | hsa-miR-1207-5p | **-1.164** |
| hsa-miR-16 | **-1.584** | hsa-miR-302a* | **-1.151** |
| hsa-miR-15b | **-1.577** | hsa-miR-1238 | **-1.123** |
| hsa-miR-575 | **-1.555** | hsa-miR-302c | **-1.122** |
| hsa-miR-20b | **-1.512** | hsa-miR-19a | **-1.02** |
| hsa-miR-188-5p | **-1.494** | hsa-miR-17 | **-1.069** |
| hsa-miR-1290 | **-1.482** | hsa-miR-33b* | **-1.065** |

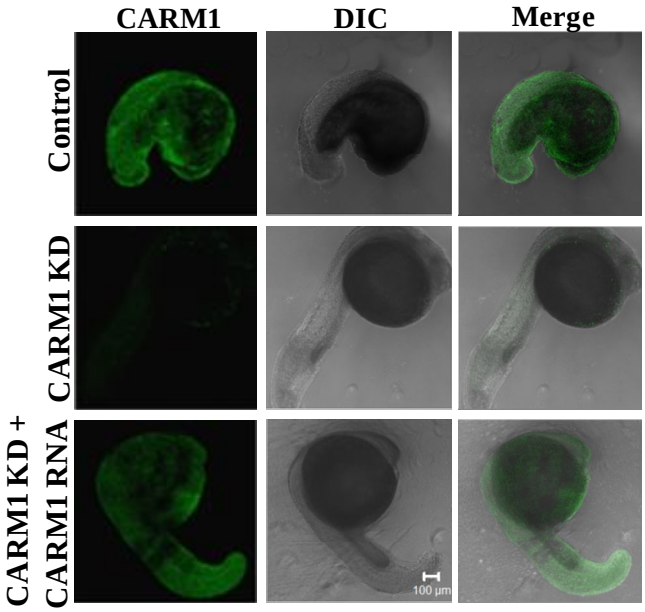
**Supporting Figure 3: miRNA changes in TBBD treated hESC derived EBs.** Tabular representation of the miRNA profile that was altered upon TBBD treatment of the BG01V derived EBs. Except for a single miRNA that showed upregulation (shown in red), majority of the miRNAs showed a downregulation in expression.



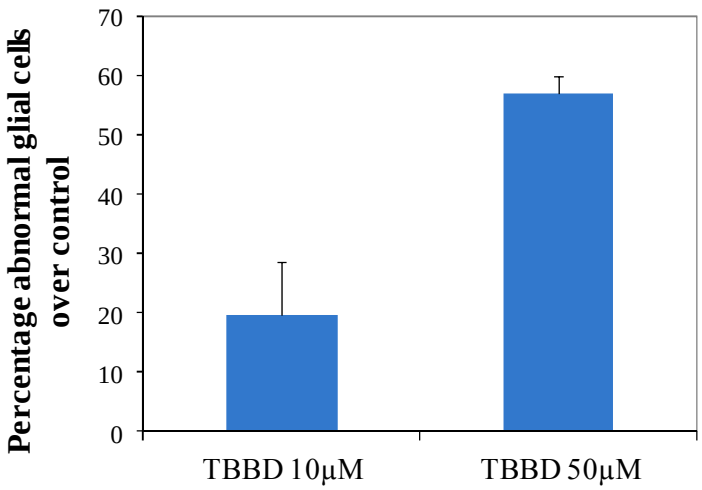

**Supporting Figure 4: miR92a overexpression in SH SY5Y cells.** Northern blot analysis showing the overexpression of premature and mature miR92a following transfection of pSUPER-pre-miR92a in SH-SY5Y cells, compared to vector transfected control cells.



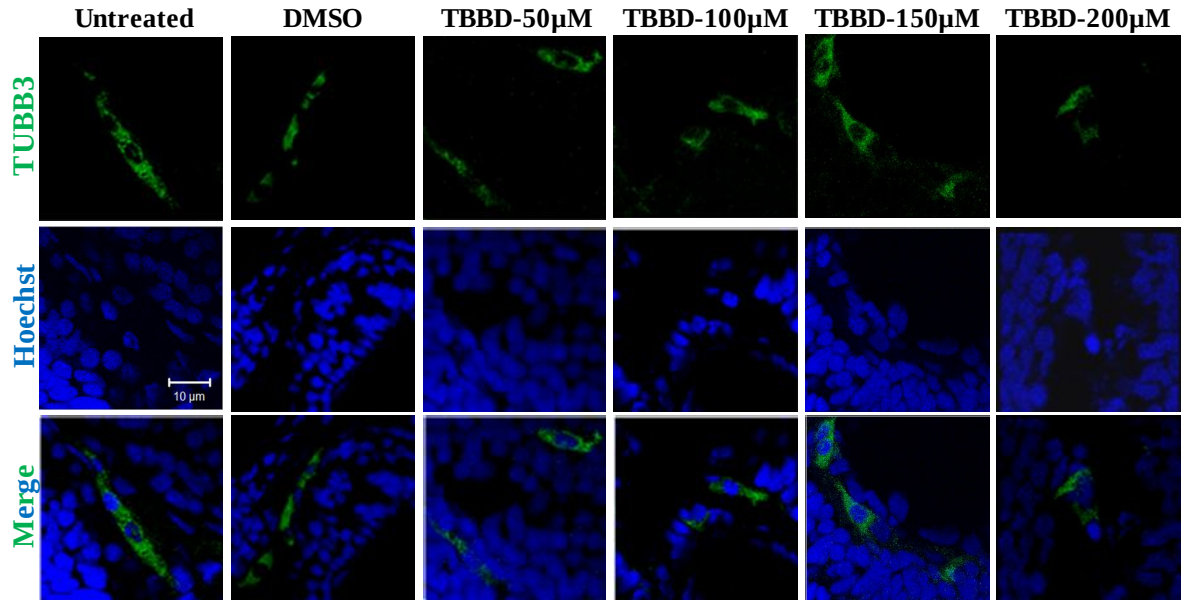

**Supporting Figure 5: Histone H3R17 methylation is essential for the early neuronal progenitor *Sox1* expression in zebrafish embryos**. Realtime PCR quantification of *Sox1* expression in zebrafish embryos treated with TBBD at different concentrations from 10 hpf for 24 hours. DMSO treated Zf embryos (lanes 1,3,5,7,9) or TBBD treated (lane 2-10 µM, lane 4- 50 µM, lane 6-100 µM, lane 8-150 µM, lane 10- 200 µM). At doses beyond 150 µM concentration of TBBD, a dose dependent decrease of *Sox1* expression is observed. Two-tailed p value: D150 vs T150: 0.009; D200 vs T200: 0.0004 (other values not significant).



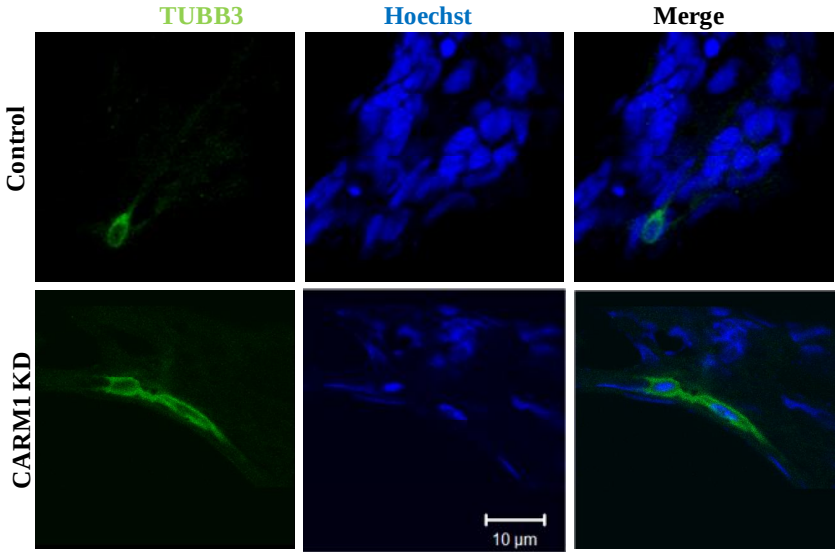

**Supporting Figure 6: Expression of CARM1 in 24 hpf embryos**; CARM1 morphants showed much lesser expression of CARM1 protein compared to control embryos, this decrease in expression was restored upon injection of *in vitro* transcribed CARM1 RNA.



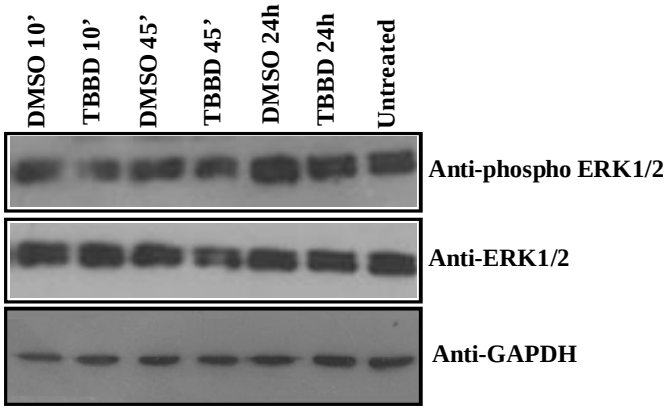

**Supporting Figure 7: Quantification of abnormal glial cells upon TBBD treatment.**Analysis of GFAP expression in U373 MG cells treated with TBBD for 24 hours, compared to DMSO treated cells.The quantification of the abnormal GFAP localization in the U373 MG cells is shown in the graph.The abnormally stained cells were divided by the number of the total cells and plotted. A paired t-test revealed $p < 0.018$.The error bar represents the standard deviation of replicates.



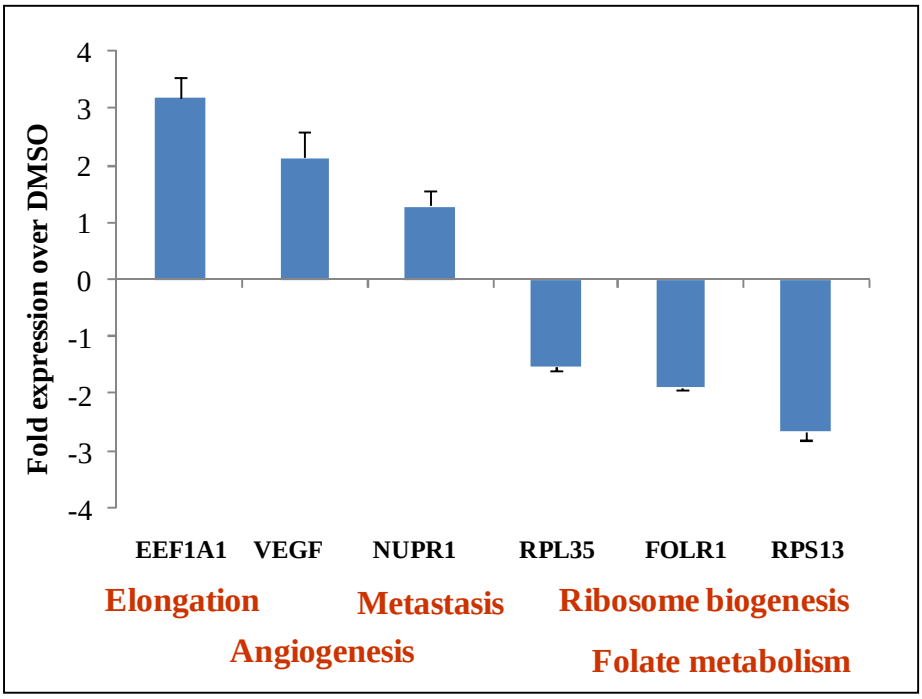

**Supporting Figure 8: TBBD treatment does not affect TUBB3 localisation.** Immunofluorescence analysis of TUBB3 expression and localisation in zebrafish embryos treated with TBBD from 10 hpf for 48 hours. The embryos were fixed following treatment with different concentrations of TBBD as indicated in the panels, subjected to cryosectioning and analyzed after staining.




**Supporting Figure 9: TUBB3 localisation is not affected in CARM1 morphants.** Immunofluorescence analysis of TUBB3 expression and localisation in 48 hpf CARM1 morphants. The embryos were fixed, subjected to cryosectioning and analyzed after staining.




**Supporting Figure 10: Phospho ERK levels in TBBD treated glial cells.** Immunoblotting of U373-MG cell lysates obtained from control, DMSO or TBBD treatment for the indicated time points with antibodies against phospho-ERK1/2. ERK1/2, and GAPDH were used as loading controls.




**Supporting Figure 11: Validation of differentially altered genes by using real-time PCR.** *EEF1A1*, *VEGF* and *NUPR1*/P8 represent upregulated genes. *RPL35*, *FolR1* and *RPS13* represent downregulated genes. The expression of these genes was compared to control DMSO treated HeLa cells. The qRT PCR analysis revealed a similar fold change as observed in the microarray analysis.

**Supporting sequence information:**

Sequence containing Nanog binding site on miR17-92 promoter:

>hg19_wgEncodeRegTfbsClusteredV2_NANOG_(SC-33759)range=chr13:92001244-92001453

5'CCGCCGGTCGCCGCGCGGCTGCCGCCGGGAAACGGGTTGGGGGGGTTGCCGCGTC

CGGCGGGGCCTGACTCTGACCCGCCGCCCCCTGGCGGCTACGCGGAGAATCGCAGG

GCCGCGCTCCCCCTTGTGCGACATGTGCTGCCGGCCCGGGCTCCATGAGCGTGGCGG

GCACTTTGCAGTCTCGGGTGTTCCTGCCCGGTCTTCTGTTCC 3'

**Primer information: Primer sequences are written in the 5'-3' direction.**

Table 1: qRT-PCR primers:

| Gene ID | Forward primer (5'-3') | Reverse primer (5'-3') |
|---|---|---|
| NUPR1 | CTCATCATGCCTATGCCCACT | GCAGCAGCTTCTCTCTTGGTG |
| FOLR1 | ACTGAGCTTCTCAATGTCTGC | GTAGGAAACATCCTTATGGG |
| VEGF | CAAATCACTGTGGATTTTGGAAAC | GGTCACTCACTTTGCCCCTGTCGC |
| EEF1A1 | ATCACCATTGATATCTCCTT | CACCAACACCAGCAGCAAC |
| Oct4 | TCCTGGAGGGCCAGGAATCGG | CATCGGAGTTGCTCTCCAC |
| NANOG | CACGGAGACTGTCTCTCCTC | GAGAGTTCTTGCATCTGCTG |
| Sox1 | TCAAACGGCCCATGAACGCCTTC | TCCGGGTGCTCCTTCATGTGC |
| CDK17 | GGAAGTCAGACTATTGATGAATCATTG | CATTGCCATGAAGGAGCCAAGGCTC |
| MAT2B | AGCTGTCGGCTGGTGGAGGAGGAAG | AACATGGGGCTGAAAATCATGAATG |
| NF1 | GTGGTCAGCCGCTTCGACGAGCAGC | TTCCAGTGTATCCAATATAATCAACTG |
| HAND2 | ACATCGCCTACCTCATGGAC | TGGTTTTCTTGTCGTTGCTG |
| CIC | GACGGCGGAGAAGTAGACAG | ACGCTGAGAACGAGAAGCTC |
| BMP4 | AAAGTCGCCGAGATTCAGGG | GACGGCACTCTTGCTAGGC |
| Otx2 | CATGCAGAGGTCCTATCCCAT | AAGCTGGGGACTGATTGAGAT |
| Pax6 | AACGATAACATACCAAGCGTGT | GGTCTGCCCGTTCAACATC |

Table 2: ChIP primers:

| Gene ID | Forward primer (5’-3’) | Reverse primer (5’-3’) |
|---|---|---|
| Nanog promoter | ACGAGACATAGACTATCTGCCTGAA | GCACTCTGCTCCTGGGTCTG |
| Nanog EJC | AGATGCCTCACACGGAGACT | GAAAAAGGGGTTTCCAGAGG |
| miR17-92 | GTGCTCCTGATTGGGCTTCTTTTCTCAG | TAAACCAAAACGCATGCCCGAGC |