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Supplementary Materials for

The nuclear DICER–circular RNA complex drives the deregulation of the glioblastoma cell microRNAome

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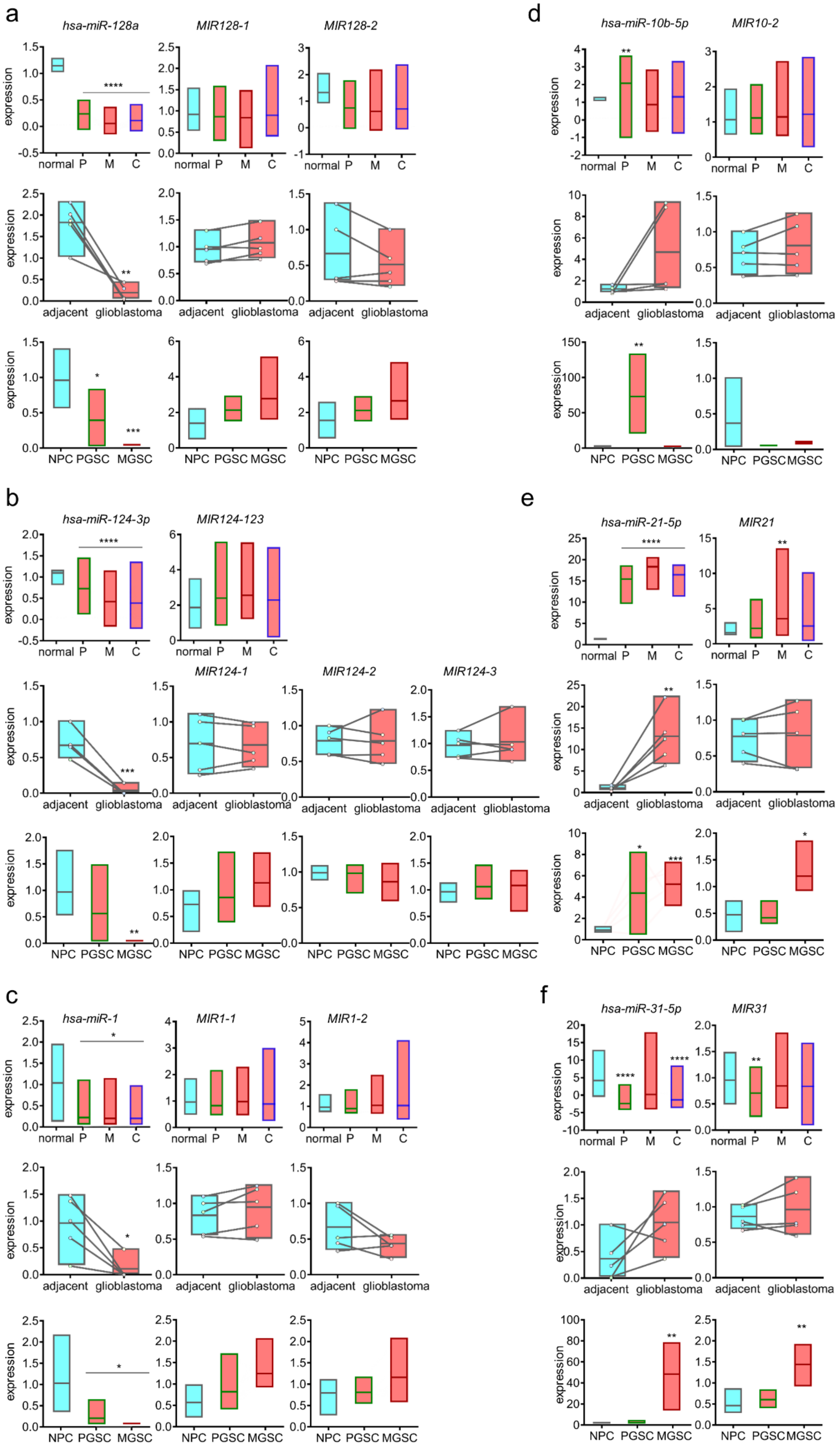
Table S4

Other Supplementary Material for this manuscript includes the following:

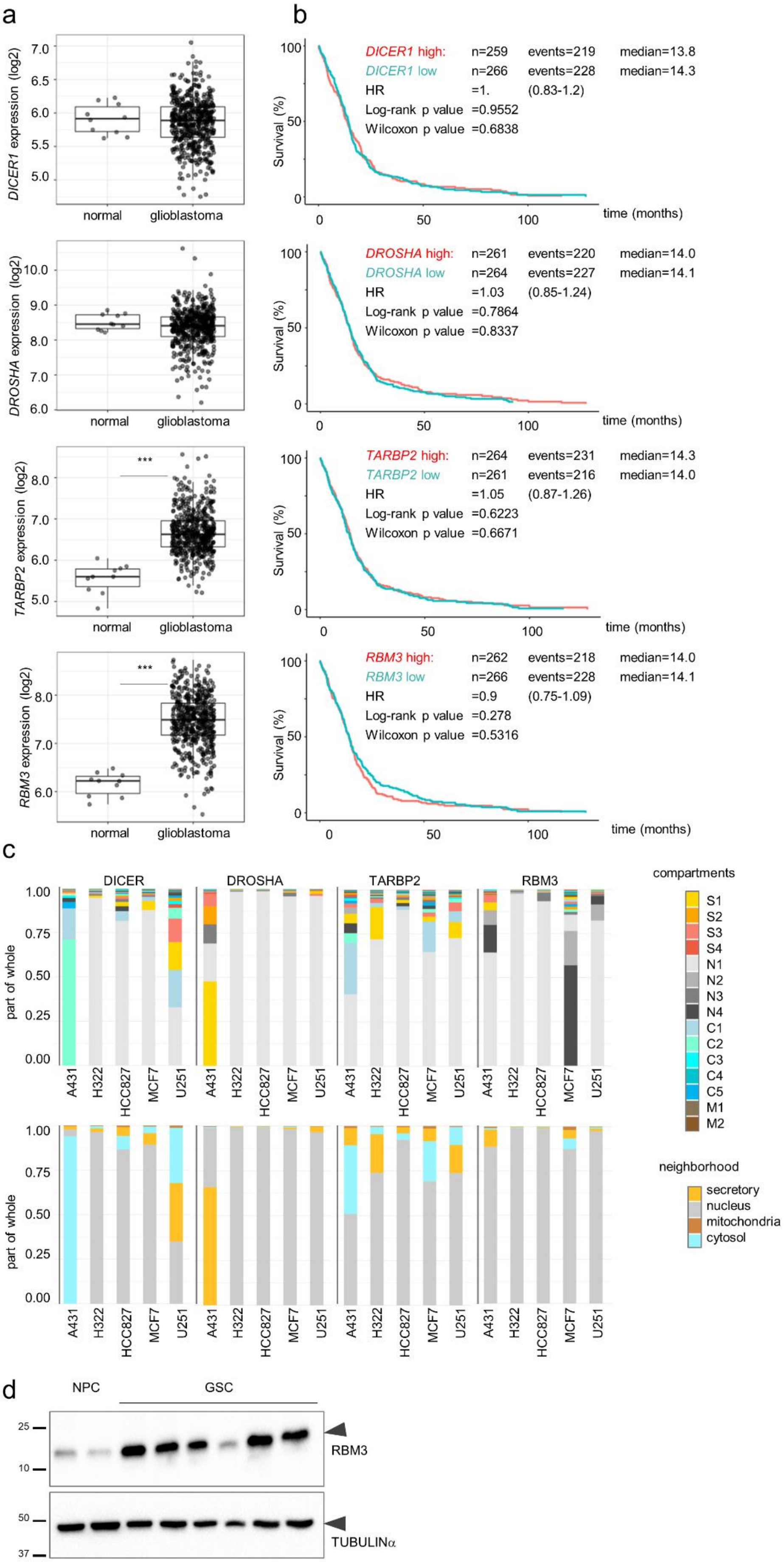
(available at advances.sciencemag.org/cgi/content/full/6/51/eabc0221/DC1)

Tables S1 to S3


a
hsa-miR-128a
MIR128-1
MIR128-2
expression
normal P M C
****
adjacent glioblastoma
**
NPC PGSC MGSC
*
***
b
hsa-miR-124-3p
MIR124-123
****
MIR124-1
MIR124-2
MIR124-3
***
**
c
hsa-miR-1
MIR1-1
MIR1-2
*
d
hsa-miR-10b-5p
MIR10-2
**
e
hsa-miR-21-5p
MIR21
f
hsa-miR-31-5p
MIR31
****

Supplementary Figure S1 related to Figure 1.
Mature, but not precursor microRNAs are suppressed in glioblastomas and GSCs.

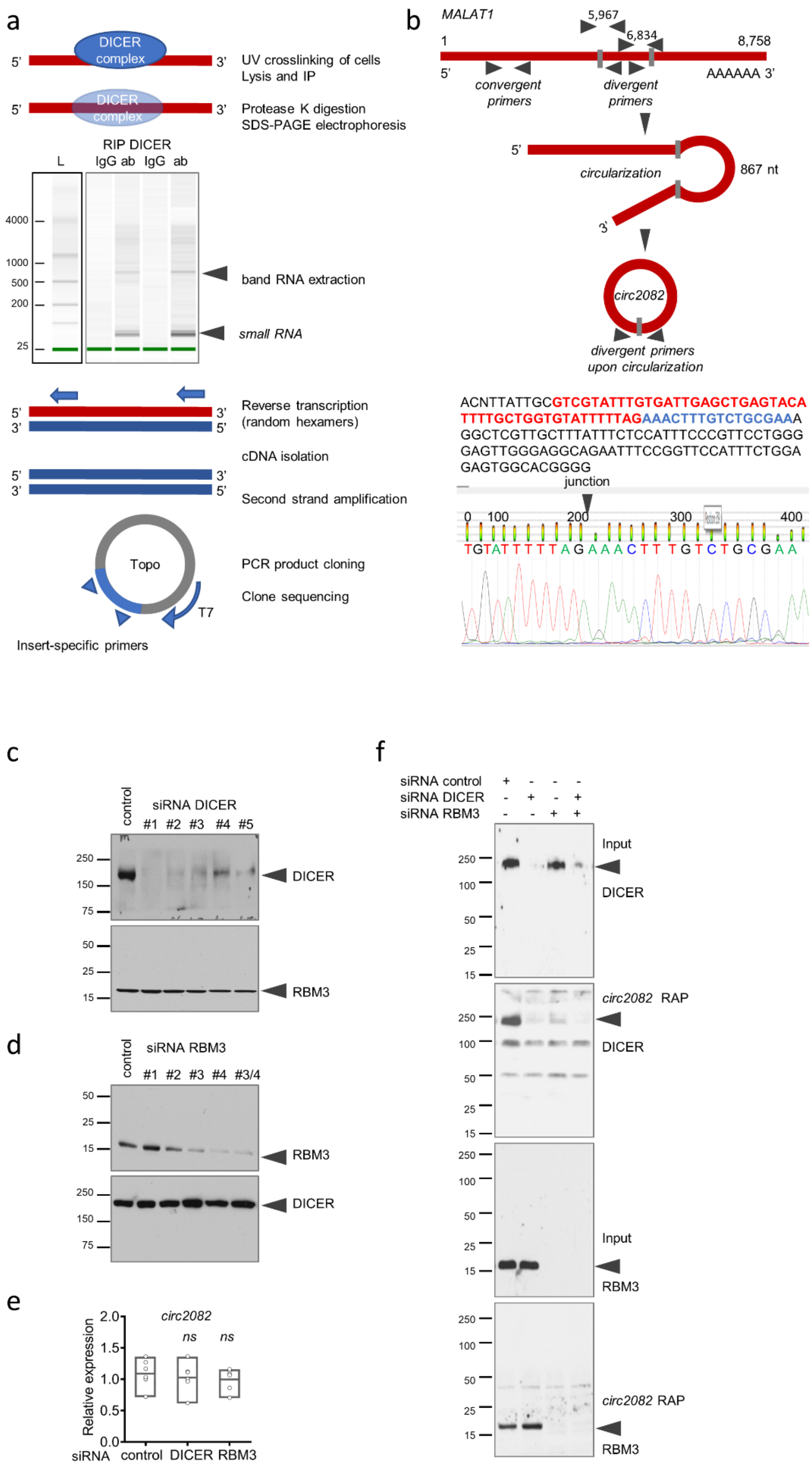
a-f: Validation of data shown on the main **Fig. 1a-i** by averaged expression of selected mature microRNAs and microRNA precursors by TCGA data analysis (**a-f:** upper panels, blue bars – healthy individuals, red bars – glioblastoma patients from three dominant subtypes: P – proneural, M - mesenchymal, and C – classical subtypes, n=82, 145, and 134, respectively) and qPCR analysis (**a-f:** middle panels, blue bars - adjacent, non-pathological brain, red bars - glioblastoma tissue; matching lines identify pairs, n=5 each; **a-f:** lower panels, blue bars – NPCs, green bars – P GSCs (P), and red bars – M GSCs (M) subtypes, n=5 per group). Mature and precursor microRNAs: *hsa-miR-128a*, *MIR128-1*, *MIR-128-2* (**a**), *hsa-miR-124-3p*, *MIR-124-1*, *MIR-124-2*, *MIR-124-3* (**b**), *hsa-miR-1*, *MIR1-1*, *MIR-1-2* (**c**), *hsa-miR-10b-5p*, *MIR10-2* (**d**), *hsa-miR-21-5p*, *MIR21* (**e**), *hsa-miR-31-5p*, *MIR31* (**f**). Data are shown as mean ± SD (matching pairs identified by lines; p-value: * <0.05, ** <0.01, *** <0.001, **** <0.0001).


a
DICER1 expression (log2)
7.0
6.5
6.0
5.5
5.0
normal
glioblastoma
DROSHA expression (log2)
10.0
9.0
8.0
7.0
6.0
normal
glioblastoma
TARBP2 expression (log2)
***
8.0
7.0
6.0
5.0
normal
glioblastoma
RBM3 expression (log2)
***
8.0
7.0
6.0
normal
glioblastoma
b
DICER1 high: n=259 events=219 median=13.8
DICER1 low n=266 events=228 median=14.3
HR =1. (0.83-1.2)
Log-rank p value =0.9552
Wilcoxon p value =0.6838
Survival (%)
100
50
0
0
50
100
time (months)
DROSHA high: n=261 events=220 median=14.0
DROSHA low n=264 events=227 median=14.1
HR =1.03 (0.85-1.24)
Log-rank p value =0.7864
Wilcoxon p value =0.8337
TARBP2 high: n=264 events=231 median=14.3
TARBP2 low n=261 events=216 median=14.0
HR =1.05 (0.87-1.26)
Log-rank p value =0.6223
Wilcoxon p value =0.6671
RBM3 high: n=262 events=218 median=14.0
RBM3 low n=266 events=228 median=14.1
HR =0.9 (0.75-1.09)
Log-rank p value =0.278
Wilcoxon p value =0.5316
c
DICER
DROSHA
TARBP2
RBM3
part of whole
1.00
0.75
0.50
0.25
0.00
A431
H322
HCC827
MCF7
U251
compartments
S1
S2
S3
S4
N1
N2
N3
N4
C1
C2
C3
C4
C5
M1
M2
neighborhood
secretory
nucleus
mitochondria
cytosol
d
NPC
GSC
25
10
RBM3
50
37
TUBULINα

Supplementary Figure S2 related to Figure 2.

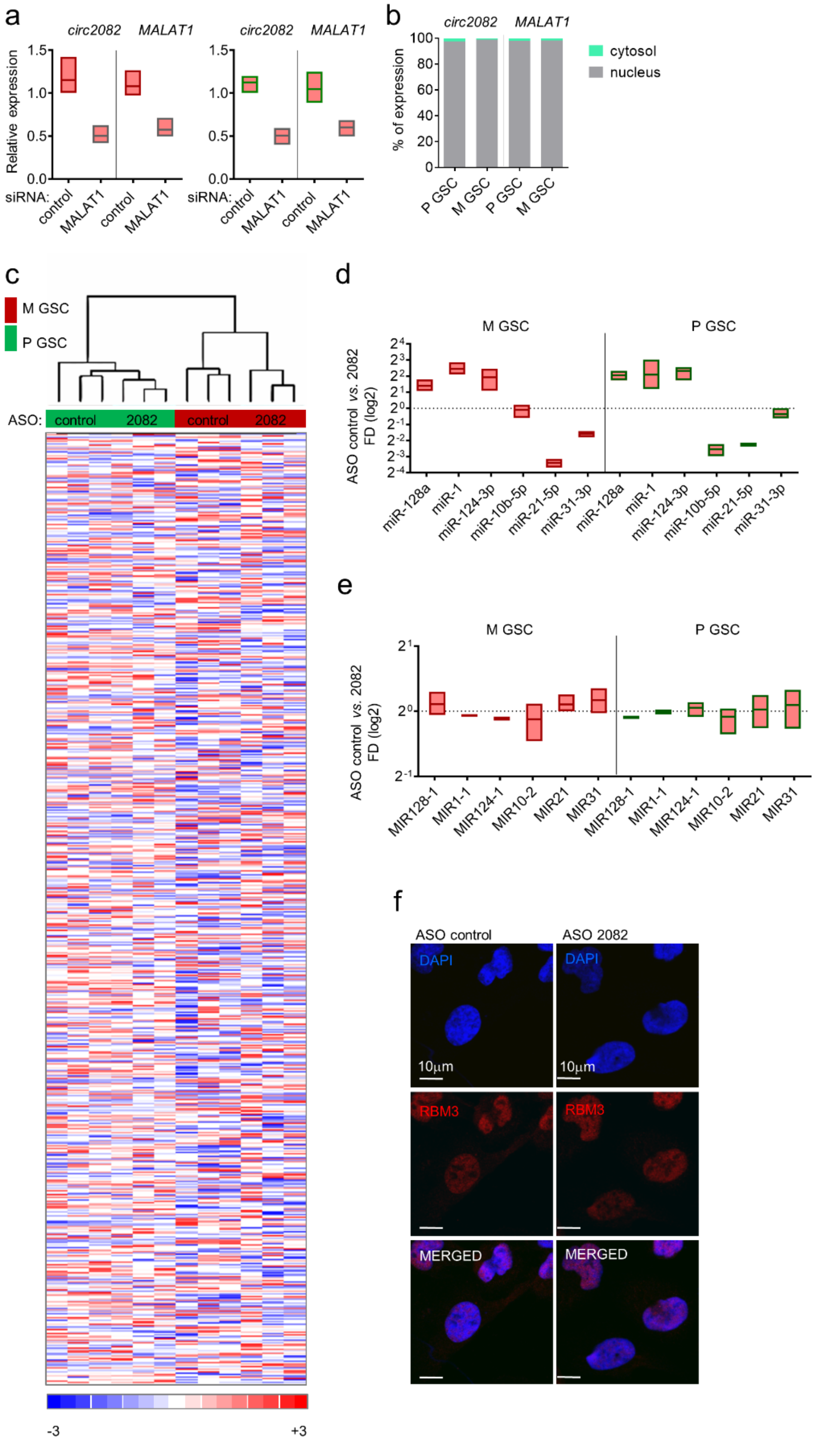
Genes encoding for proteins vital for microRNA processing are not linked to glioblastoma patients' survival.

a: The scatter plot analysis of DICER, DROSHA, TARBP2, and RBM3 expression in the TCGA dataset of glioblastoma patients (n=528) vs. healthy individuals (n=10), p-value: *** <0.001.
b: Kaplan-Meier survival analysis of DICER, DROSHA, TARBP2 and RBM3 expression association with patient survival in TCGA dataset of glioblastoma patients.
c: Bar analysis of cell-line-specific localization of DICER, DROSHA, TARBP2, and RBM3 in Subcell Barcode dataset based on the mass-spectroscopy analysis; S1 - Golgi, endo/lysosome, S2 - ER, peroxisome, S3 - ER, mito.ribosome, S4 - plasma membrane, N1 - nucleosol high density, ribosome, N2 - nucleus, speckles, N3 - nucleus, nucleolus, N4 – nucleosol, C1, C2, C3, C4 - cytosol, cytoskeleton, M1 - mitochondria matrix, M2 - mitochondria membrane.
d: Western blot analysis of RBM3 in the whole-cell lysate of NPCs (n=2) and GSCs (n=6). TUBULINα was used as a loading control.

a
DICER complex
5'
3'
UV crosslinking of cells
Lysis and IP
Protease K digestion
SDS-PAGE electrophoresis
RIP DICER
L
IgG ab IgG ab
4000
1000
500
200
25
band RNA extraction
small RNA
Reverse transcription
(random hexamers)
cDNA isolation
Second strand amplification
Topo
PCR product cloning
Clone sequencing
T7
Insert-specific primers
b
MALAT1
1
5,967
6,834
8,758
AAAAAA 3'
convergent primers
divergent primers
circularization
867 nt
circ2082
divergent primers upon circularization
ACNTTATTGCGTCGTATTTGTGATTGAGCTGAGTACA
TTTTGCTGGTGTATTTTTAGAAACTTTGTCTGCGAAA
GGCTCGTTGCTTTATTTCTCCATTTCCCGTTCCTGGG
GAGTTGGGAGGCAGAATTTCCGGTTCCATTTCTGGA
GAGTGGCACGGGG
junction
0 100 200 300 400
TGTATTTTTAGAAACTTTGTCTGCGAA
c
control
siRNA DICER
#1 #2 #3 #4 #5
250
150
75
DICER
50
25
15
RBM3
d
control
siRNA RBM3
#1 #2 #3 #4 #3/4
50
25
15
RBM3
250
150
75
DICER
e
circ2082
ns
ns
Relative expression
2.0
1.5
1.0
0.5
0.0
siRNA control DICER RBM3
f
siRNA control + - - -
siRNA DICER - + - +
siRNA RBM3 - - + +
Input
DICER
circ2082 RAP
DICER
Input
RBM3
circ2082 RAP
RBM3
250
100
50
25
15

Supplementary Figure S3 related to Figure 3.
Circ2082 that originates from *MALAT1* binds directly to RBM3 and forms complex with DICER.

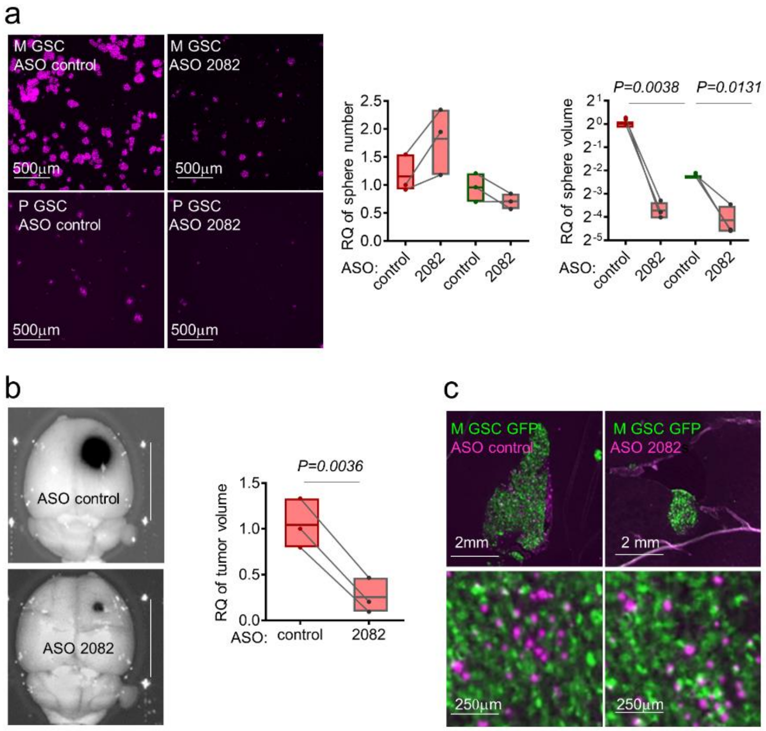
a: The schematic representation of the experimental flowchart. Protein-RNA immunoprecipitation (RIP) of UV-crosslinked GSC nuclear lysates with DICER antibodies was followed by RNA isolation and analysis by Total RNA Platform Agilent Bioanalyzer. The representative RNA profile is shown (n=2). Arrows indicate *circ2082* and small RNA peaks. The ladder indicates RNA size (nt) on the left.
b: The schematic representation of linear *MALAT1* and circularization event leading to the emergence of *circ2082* (top). Primers used throughout the study to distinguish between linear *MALAT1* and *circ2082* are marked by arrows. The junction site sequence and sequencing result are shown (bottom).
c-d: Western blot analysis of DICER (**c**) and RBM3 (**d**) in the whole-cell lysate of GSC (n=5) upon their respective siRNA-mediated knockdown using indicated antibodies is shown. Both antibodies were used as a loading control upon the knockdown of their respective protein partners; arrows indicate DICER and RBM3 bands. Corresponding molecular weight is shown on the left.
e: Box scatter plot of qPCR analysis of *circ2082* expression with mean ±SD, and relative quantification scale is shown (n=5). GSCs were transfected with control, DICER, or RBM3 siRNA.
f: Western blot analysis of DICER and RBM3 in GSCs transfected with control, DICER, and/or RBM3 siRNA. UV-crosslinked GSC nuclear lysate inputs and RNA Antisense Purification (RAP) with the anti-*circ2082* probe are shown; arrows indicate DICER and RBM3 bands. Corresponding molecular weight is shown on the left.


a
circ2082
MALAT1
circ2082
MALAT1
Relative expression
1.5
1.0
0.5
0.0
siRNA:
control
MALAT1
control
MALAT1
siRNA:
control
MALAT1
control
MALAT1
b
circ2082
MALAT1
% of expression
100
80
60
40
20
0
P GSC
M GSC
P GSC
M GSC
cytosol
nucleus
c
M GSC
P GSC
ASO:
control
2082
control
2082
-3
+3
d
M GSC
P GSC
ASO control vs. 2082 FD (log2)
miR-128a
miR-1
miR-124-3p
miR-10b-5p
miR-21-5p
miR-31-3p
miR-128a
miR-1
miR-124-3p
miR-10b-5p
miR-21-5p
miR-31-3p
e
M GSC
P GSC
ASO control vs. 2082 FD (log2)
MIR128-1
MIR1-1
MIR124-1
MIR10-2
MIR21
MIR31
MIR128-1
MIR1-1
MIR124-1
MIR10-2
MIR21
MIR31
f
ASO control
ASO 2082
DAPI
DAPI
10μm
10μm
RBM3
RBM3
MERGED
MERGED

Supplementary Figure 4 related to Figure 4.

ASO treatment that silences *circ2082* but not *MALAT1* affects mature but not precursor microRNAs.

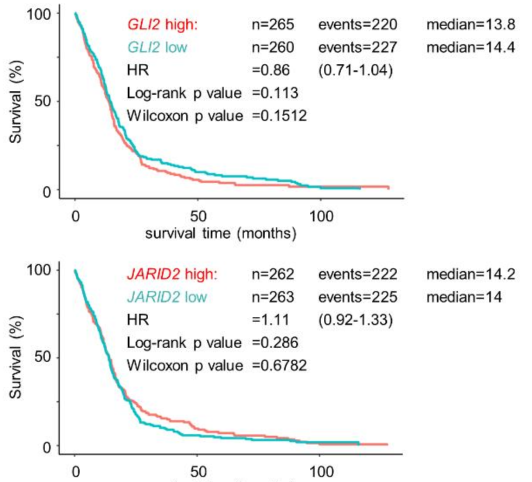
a: Box plot of qPCR analysis for *circ2082* or linear *MALAT1* with mean ±SD and relative quantification scale is shown. GSC (of indicated subtypes: red – M GSCs, green – P GSCs, n=3 per subtype) were transfected with control siRNA or *MALAT1* siRNA.
b: QPCR analysis of *circ2082* and linear *MALAT1* relative percentage of expression in the nuclear and cytoplasmic fraction of GSCs.
c: Heatmap with an unsupervised hierarchical clustering for GSCs transfected with ASO control or *circ2082* (n=3 per subtype) based on mRNA transcripts (p-value < 0.05) is shown. The color bars indicate corresponding clusters.
d, e: Box plot of qPCR analysis for indicated mature microRNAs (**d**) and microRNA precursors (**e**) with mean ±SD and relative quantification scale is shown. GSC (of indicated subtypes, n=3 per subtype) were transfected with ASO control or *circ2082*.
f: Representative images of cultured GSC (n=3 per subtype) transfected with ASO control or *circ2082* are shown. DAPI staining (blue) and RBM3 immunohistochemistry staining (red) analysis was performed. Scale bars: 10µm.

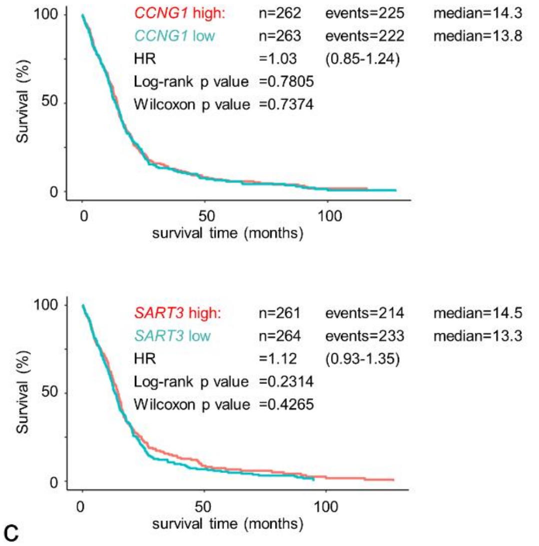

a
M GSC
ASO control
M GSC
ASO 2082
P GSC
ASO control
P GSC
ASO 2082
500μm
500μm
500μm
500μm
RQ of sphere number
2.5
2.0
1.5
1.0
0.5
0.0
ASO:
control
2082
control
2082
P=0.0038
P=0.0131
RQ of sphere volume
2^1
2^0
2^{-1}
2^{-2}
2^{-3}
2^{-4}
2^{-5}
ASO:
control
2082
control
2082
b
ASO control
ASO 2082
P=0.0036
RQ of tumor volume
1.5
1.0
0.5
0.0
ASO:
control
2082
c
M GSC GFP
ASO control
M GSC GFP
ASO 2082
2mm
2 mm
250μm
250μm

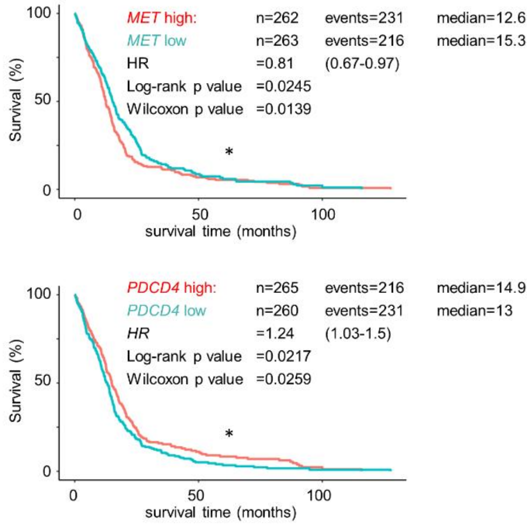
Supplementary Figure 5 related to Figure 5.

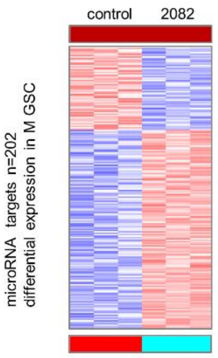
ASO-mediated knockdown of *circ2082* mitigates tumorigenicity of GSCs *in vitro* and *in vivo*.

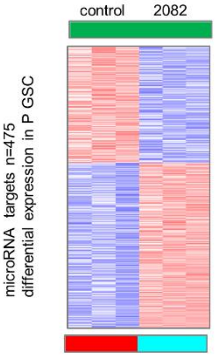
a: Representative micrographs of GSCs (n=3 per subtype) transfected with ASO control or *circ2082* (scale bars: 500µm (**left**)), and box scatter plot of mean ±SD of sphere frequency, and volume (**right**) are shown (lines identify matching pairs, the p-value is indicated).
b: Representative images of brains with M GSC-originated tumors are shown (**left**). Relative quantification of tumor volume 10 days post-implantation is shown (**right**). Data are shown as mean ± SD (matching pairs identified by lines; p^{-}value is indicated).
c: Representative images of sections of M GSC-originated tumors are shown. GFP-positive M GSCs were transfected with Cy5-labeled ASO control or *circ2082*, scale bars: 2mm (**top**), 250µm (**bottom**).

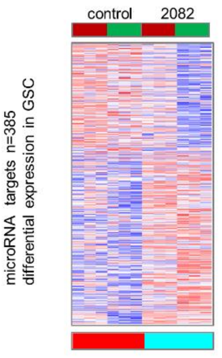

a
GLI2 high: n=265 events=220 median=13.8
GLI2 low n=260 events=227 median=14.4
HR =0.86 (0.71-1.04)
Log-rank p value =0.113
Wilcoxon p value =0.1512
Survival (%)
100
50
0
survival time (months)
0
50
100
JARID2 high: n=262 events=222 median=14.2
JARID2 low n=263 events=225 median=14
HR =1.11 (0.92-1.33)
Log-rank p value =0.286
Wilcoxon p value =0.6782
Survival (%)
survival time (months)


b
CCNG1 high: n=262 events=225 median=14.3
CCNG1 low n=263 events=222 median=13.8
HR =1.03 (0.85-1.24)
Log-rank p value =0.7805
Wilcoxon p value =0.7374
Survival (%)
100
50
0
survival time (months)
0
50
100
SART3 high: n=261 events=214 median=14.5
SART3 low n=264 events=233 median=13.3
HR =1.12 (0.93-1.35)
Log-rank p value =0.2314
Wilcoxon p value =0.4265
Survival (%)
survival time (months)


c
MET high: n=262 events=231 median=12.6
MET low n=263 events=216 median=15.3
HR =0.81 (0.67-0.97)
Log-rank p value =0.0245
Wilcoxon p value =0.0139
*
Survival (%)
100
50
0
survival time (months)
0
50
100
PDCD4 high: n=265 events=216 median=14.9
PDCD4 low n=260 events=231 median=13
HR =1.24 (1.03-1.5)
Log-rank p value =0.0217
Wilcoxon p value =0.0259
*
Survival (%)
survival time (months)


d
control
2082
microRNA targets n=202
differential expression in M GSC


e
control
2082
microRNA targets n=475
differential expression in P GSC


f
control
2082
microRNA targets n=385
differential expression in GSC

Supplementary Figure 6 related to Figure 6.
The expression of *circ2082*/microRNAome-controlled genes predict outcome of glioblastoma patients in subtype-independent fashion.

a-c: Kaplan-Meier curves for survival analysis of *GLI2*, *JARID*, *CCNG1*, *SART3*, *MET*, *PDCD4* in TCGA dataset of glioblastoma patients, the p-value is indicated.
d-f: Heatmap with a supervised clustering for all GSCs (n=6 (**d**), M GSC, n=3 (**e**) and P GSC, n=3 (**f**)) transfected with ASO control or *circ2082* based on the most variable microRNA targets (n=202; n= 475; n=385 out of total 1697, respectively, p-value < 0.05) is shown. GSC's subtype identity is shown as a bar above the heatmap (red – mesenchymal, green – proneural).

Supplementary Table 1 related to Figure 1b, 1c, 4d-e, 4f.
MicroRNA expression dataset (based on NanoString)

Supplementary Table 2 related to Figure 2d, 3e.
Protein identification dataset (based on mass spectroscopy)

Supplementary Table 3 related to Figure 6a.
MicroRNA/mRNA target expression correlation dataset (based on TCGA)

Supplementary Table 4. Reagents and Resources Used.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
DICER (D38E7) Rabbit	Cell Signaling Technology	mAb #5362
DICER Rabbit	Thermo Fisher	PA5-23088
TRBP2 (D7C8K) Rabbit	Cell Signaling Technology	mAb #62043
RBM3 Rabbit	Invitrogen	PA5-51976
HISTONE H3 (D1H2) XP® Rabbit	Cell Signaling Technology	mAb #4499
TUBULINα Mouse (clone DM1A)	Sigma-Aldrich	T9026
GFP Mouse IgG2A Clone # 454518	R&D Systems	MAB4240
Secondary Rabbit anti-Mouse monoclonal	Novus Biologicals	MAB0033
Secondary Goat anti-Rabbit polyclonal	Thermo Fisher	32460
Bacterial and Virus Strains		
BL21 Star (DE3) Chemically Competent E. coli*	Thermo Fisher	C601003
Biological Samples		
Patient brain tissue	*45*	N/A
Patient-derived GSCs	*13*	N/A
NPC cells	Millipore Sigma Lonza *46*	SCC007 PT-2599 N/A
Chemicals, Peptides, and Recombinant Proteins		
EGF	Pepro Tech	AF-100-15
FGF	Pepro Tech	100-18C
B27	Gibco	21103049
Neurobasal	Gibco	21103049

TRIzol™ Reagent	TermoFisher	15596018
RNase R	Epicentre	RNR07250
Critical Commercial Assays		
Power Up SYBR Green Master Mix	Thermo Fisher	A25743
TaqMan Fast Advanced Master Mix	Thermo Fisher	4444963
Human v3 miRNA Assay	Nanostring	CSO-MIR3-12
Human custom pre-miR Assay	Nanostring	This paper
Agilent whole human genome microarray	Arraystar	Agilent-026652 4x44K v2
Arraystar Human Circular RNA Array	Arraystar	AS-S-CR-H-V2.0
TaqMan microRNA Assay	Applied Biosystems	4427975
TaqMan Pre-miRNA Assays	Applied Biosystems	4426961
iScript™ cDNA Synthesis Kit	Bio-Rad	1708890
Nuclear/Cytosol Fractionation Kit	BioVision	K266
Lipofectamine 2000	Invitrogen	11668030
Deposited Data		
NanoString	This paper	Supplementary spreadsheets Table 1
Mass spectrometry	This paper	Supplementary spreadsheets Table 2
Raw and analyzed data	This paper	Supplementary spreadsheet Table 3
Genome microarray	This paper	Deposition: GSE146440
Circular RNA Array	This paper	Deposition: GSE146463
Experimental Models: Organisms/Strains		
Mouse: athymic	Envigo	code#069
Oligonucleotides		
siRNA targeting sequence: DICER #1 AATGTGCTATCTGGATCCTAG #2 CTGCTCGAAATCTTACGCAAA #3 AAGGACGGTGTTCTTGGTCAA #4 CTCGCATAGGCATTCCCAGTA #5 ATCGATCCTATGTTCAATCTA	QIAGEN	 SI00300006 SI04753987 SI02645993 SI02645986 SI02645972
siRNA targeting sequence: RBM3 #1 CAGATCCGTGTGGATCATGCA #2 TGGGACGTTTGTAGAACCTGA #3 ACCGACGAGCAGGCACTGGAA #4 CAAGGAATAATTTCTGATCCA	QIAGEN	 SI04297755 SI04296166 SI04261215 SI04146821
siRNA control:	QIAGEN	1027280

QPCR primers: *MALAT1 (linear, convergent)* F: TGATAGCCAAATTGAGACAA R: TTCAGGGTGAGGAAGTAAAA *circ2082 (circular, divergent)* F: GAAGGAAAAAATCCAGCTGA R: GAATAAAATTTGTCTTTCCTGCC *circ2082 (circular, convergent)* F: AGGATTTGAGCGGAAGAACGA R: ACACCAGCAAAATGTACTCAGC *GLI2* F: CTGTGGGTTAGGGATGGACTG R: GTAAAGTGGGTGGACGTTGCA *JARID* F: GACACCAAACCCAATCACCAC R: GTTCAACCTGCCACTGACCTT *CCNG1* F: CCTTCTGTGTTGGCATTGTCTATC R: CAAGCTCTTGCCAGAAGGTCAG *SART3* F: GGAGATTTGGCAGGCATACCTTG R: CTCTTCCACCTCCTGCTTCAGA *MET* F: TAGCCAACCGAGAGACAAGC R: TGTGCTCCCACCACTAATAAAAG *PDCD4* F: TGTAAACCCTGCAGATCCTGA R: TGGAGGATGCTGAAATCCAAT *18S rRNA* F: AACTTTCGATGGTAGTCGCCG R: CCTTGGATGTGGTAGCCGTTT	Invitrogen	N/A
RBM3 cloning primers: F: AAAGAATTCATGTCCTCTGAAGAAGGA, R: AAACTCGAGTCAGTTGTCATAATTGTC	Invitrogen	This paper
Antisense oligo (ASO):		This paper
Control: GCGTATTATAGCCGATTAAC		
circ2082: GTTTCTAAAAATACACCAGC		
Knockdown and in situ visualization:		
Phosphorothioate bond-Cy5	IDT	
RAP assay:		
5’ biotinylated	Invitrogen	
FISH assay:	Thermo Fisher	
CCATTAAAGAGTGTTCGCAGACAAA		
GTTTCTAAAAATACACCAGCAAAATGTAC		
TaqMan microRNA probes:	Life Technologies	Assay ID:
miR-128		002216
miR-124-3p		003188
miR-1		002222
miR-10b-5p		002218
miR-21-5p		000397
miR-31-5p		002279
U6		001973

TaqMan pre-microRNA probes:	QIAGEN	Assay ID:
MIR128-1		MP00000574
MIR128-2		MP00000588
MIR124-1		MP00000371
MIR124-2		MP00000378
MIR124-3		MP00000392
MIR1-1		MP00003990
MIR1-2		MP00004053
MIR10-2		MP00000168
MIR21		MP00001498
MIR31		MP00007889
Recombinant DNA		
pCDH-EF1-copGFP vector	System Biosciences	CD511B-1
pCDH-EF1-copGFP-RBM3	This paper	N/A
Software and Algorithms		
ImageJ	*47*	https://imagej.nih.gov
GlioVis	*48*	http://gliovis.bioinfo.cnio.es/?ref=labworm
GBM-BioDP	*49*	https://gbm-biodp.nci.nih.gov
Betastasis	*50*	https://betastasis.com
Subcell Barcode	*51*	http://www.subcellbarcode.org
Other		
TCGA glioblastoma platforms data set: Affymetrix HT HG U133A Agilent Human miRNA 8x15K Affymetrix Human Exon 1.0 ST	*52*	https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga