B. MATERIALS AND METHODS

B.1. Materials

B.1.1. Chemicals

Chemicals for experiments were purchased from the following companies, unless indicated otherwise: BD Clontech, Gibco, Sigma, Invitek, Invitrogen, Fermentas, Stratagene, Chemicon, BioLabs, Biochrom, Roth, Roche, Merck, BioRad, Fluka, Serva, Promega.

B.1.2. Enzymes, Kits and Transfection Reagents

Advantage RT-for-PCR Kit (Clonthech)

BCA protein assay reagent Kit (Pierce)

Chromatin Immunoprecipitation (ChIP) assay Kit (Upstate)

CombiZyme DNA polymerase Mix (Invitek)

ECL chemiluminiscent reagents (Amersham Biosciences)

Effectene transfection reagent (Qiagen)

Fugene6 transfection reagent (Roche)

Lipofectamine 2000 transfection reagent (Invitrogen)

Mouse Neuron nucleofector Kit (Amaxa)

NucleoBond PC 100/PC 500 (Macherey-Nagel)

Phusion High-Fidelity DNA polymerase (Finnzymes)

Plasmid-Mini Kit (SeqLab)

Prime-a-Gene Labeling System (Promega)

QIAquick Gel Extraction Kit (Qiagen)

QIAquick Nucleotide removal Kit (Qiagen)

QuikChange Site-directed Mutagenesis Kit (Stratagene)

Restriction endonucleases (BioLabs)

Shrimp Alkaline Phosphatase (SAP) (Promega)

T4 DNA Ligase (BioLabs)

T4 Polynucleotide Kinase (Fermentas)

T7 Transcription Kit (Fermentas)

TaqMan 2x Universal PCR Master Mix No AmpErase UNG (Applied Biosystems)

TaqMan Gene Expression Assay (Applied Biosystems)

Topo cloning Kit (Invitrogen)

B.1.3. Equipment

FACS Calibur (BD)

Fluorescent microscope BX 51 (Olympus)

Confocal equipment TCS SL (Leica Microsystems)

PCR Block (MJ Research)

Dounce homogenizer Sonopuls GM70 (Bandelin)

Hybridisation oven MWG (Biotech Instruments)

Scintillation counter LS 6500 (Beckmann)

Centrifuges: Rotina 35 R (Hetich); 5417R, 5804R (Eppendorf); Biofuge pico (Heraeus)

pH Meter 537 (WTW)

Photometer Ultrospec 2000 (Pharmacia Biotech)

Gel electrophoreses and blotting equipment (BioRad)

Power pac 200/300 (BioRad)

Cell incubator (Hereaus Instruments)

Plate reader ELX 800 (Biotech Instruments)

API PRISM TM7500 Sequence Detection Systems (Applied Biosystems)

GSGene linker UV chamber (BioRad)

B.1.4. Software

Leica Confocal Software v. 2.61 Build 1537

Volocity 3.0, Improvision

ImageJ 1.34s

BD CellQuestPro™

FlowJo v4.4.4

MetaMorph 6.2r4

TaqMan 7500 Fast System Software

CorelDRAW 10

Adobe Photoshop 6.0

Microsoft Office 2000

Statistics software (http://www.physics.csbsju.edu/stats/)

miRNA database (http://www.sanger.ac.uk/Software/Rfam/mirna/)

MFOLD program (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi)

TargetScan software (http://genes.mit.edu/targetscan)

miRanda software (http://www.microrna.org/)

RNA22 (http://cbcsrv.watson.ibm.com/rna22.html)

NCBI database (http://www.ncbi.nlm.nih.gov/)

Ensembl genome browser (http://www.ensembl.org/index.html)

BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)

CLUSTALW (http://align.genome.jp/)

JaMBW (http://hometown.aol.com/lucatoldo/myhomepage/JaMBW/index.html?f=fs)

B.1.5. Oligonucleotides

B.1.5.1. PCR Primers

The primers were designed following standard rules, and analyzed in BLAST for specificity.

Table B.1. Cloning primers used for plasmid constructions

Name	Forward Primer (Sequence 5'-3')	Reverse Primer (Sequence 5'-3')	T_a (ºC)	bp
lin-14	GCG GCC GCC CTG CAC TCA CTT TAC C	GCG GCC GCG GAT CCG ACA GAG CAA CAT TCA TC	65	550
lin-41	GCG GCC GCG AAA TCT CAG GAA AAG TC	GCG GCC GCG GAT CCC GCA GTG AAA TTT GCG A	65	505
mlin-41	GCG GCC GCG GAC TTT GGC AAC AAT CGA ATC CTC	CAC AGT ATT CTC AAG AAC ACA AAT AC	62	553
mir-128	GCG GCC GCG TCG ACA AAA GAG ACC AAG TCA CTG TGA TAT AAA AGA G	CTC GAG TCA CAG TGA GTT GGT CTC TTT TAT ATC ACA GTG AC		

Table B.2. Primer sequences used for cDNA amplification

Name	Forward Primer (Sequence 5'-3')	Reverse Primer (Sequence 5'-3')	T_a (ºC)	bp
a6b1	GAC TCT TAA CTG TAG CGT	CTC TCG CTC TTC TTT CCG	60	380/ 510
Ago1	TCG CTT GGT GGC TTT CCG GGC AC	GGG AGA GGG GTT GAG GAC AAT G	69	390
c-Myc	GTC TCC ACT CAC CAG CAC AAC T	GTC TGC TTG AAT GGA CAG GAT G	66	324
Dicer	CTT AGA ATT CCT GGG AGA TGC GA	GCA TCA TCG GAT AGT ACA CCT GC	67	410
eGFP-Thy1	CCG AAG GCT ACG TCC AGG AG	GCG GCC GCT TTA CTT GTA CA	60	461
FMRP	GAG AGG CTA AAG GAA GAA CAG CTG	GTT ATG CAT CAA ATT TCA AGA TC	60	360
Gemin3	GAA ATG TAA TAT CAA CCT TCT TCC	GGA CTT TAA AAG AAG ACA GAC	56	470
Gemin4	GCA GCC AGA GCT GTT GTA ACT G	GGC ATA GTT TTG TTC TGC CCT C	67	430
let-7a-1/f1	TTC ACC CTG GAT GTT CTG TTC A	CAG GGT AAA ATC ACT ACC CCA CA	65	423
mir-99b–mir-125a	CAC CCG TAG AAC CGA CCT TGC G	CAC AGG TTA AAG GGT CTC AGG GA	65	646
mlin-41 3'UTR	GGA CTT TGG CAA CAA TCG AAT CCT C	CAC AGT ATT CTC AAG AAC ACA AAT AC	62	553
MOV10	AGG CTG CTC ACC CTG GAG GTT	ACT CCA GAC TCC GAT CGT ACA GCT	70	373
TNRC6B	AAC TCA AAT TAA GCA GGA CAC	TCA CTT GAT CCA CCT CCT CG	62	395
TRBP2	CCT CCA AAA GCT GGC AAA G	GGT GGA CAG TTC CAC TAG GC	65	371
β-actin	CTA GGC ACC AGG GTG TGA TGG	CGT AGA TGG GCA CAG TGT GGG	65	387

Table B.3. Primer sequences used for *in vitro* RNA transcription

Name	Forward Primer (Sequence 5'-3')	Reverse Primer (Sequence 5'-3')	T_a (ºC)	bp
let-7a-T7	TAA TAC GAC TCA CTA TAG GAG GTA GTA GGT TGT ATA G	GGA AAG ACA GTA GAT TGT A	65	90
let-7e-T7	TAA TAC GAC TCA CTA TAG GAG GTA GGA GGT TGT ATA G	GGA AAG CTA GGA GGC CGT A	62	84
mir-128b-T7	CGT AAT ACG ACT CAC TAT AGG GGG GCC GAT GCA CTG TA	GAA AGA GAC CGG TTC ACT G	62	79
mir-30-T7	GCG TAA TAC GAC TCA CTA TAG GGC GAC TGT AAA CAT CCT C	GCA GCT GCA AAC ATC CG	62	93

Table B.4. Primer sequences used for chromatin immunoprecipitation assay

Name	Forward Primer (Sequence 5'-3')	Reverse Primer (Sequence 5'-3')	T_a (ºC)	bp
CMV promoter	GTA ATC AAT TAC GGG GTC ATT AG	TGC CAA AAC AAA CTC CCA TTG AC	65	456
GFP	CCG AAG GCT ACG TCC AGG AG	GCG GCC GCT TTA CTT GTA CA	60	461
mlin-41 ORF	AAG ATG ACC GCA TTA TGT TCA CGC	CCT CAG AAT TCA CTG CTA CAT	65	772
Oct4 promoter	TGG GTA AGC AAG AAC TGA GGA GTG	TTC AAG GTC CTC TCA CCC CTG CCT	65	215

Table B.5. Primers and FAM-probes used for Real-Time PCR

Name	Forward Primer (Sequence 5'-3')	Reverse Primer (Sequence 5'-3')	FAM-Prove
muGAPDH	CTG CCA CCC AGA AGA CTG TG	CCG TTC AGC TCT GGG ATG AC	6FAM-TGG CCC CXT CTG GAA AGC TGT GGC G--PH
18 S	CGG CTA CCA CAT CCA AGG AA	GCT GGA ATT ACC GCG GCT	6FAM-CGC AAA TTA CCC ACT CCC GAC CC –BHQ1
pri-let-7a	The primers and FAM probe were designed by Applied Biosystems		
pri-let-7e	The primers and FAM probe were designed by Applied Biosystems		

B.1.5.2. Mutagenesis Primers

Table B.6. Primer sequences used for point mutations in the sensor vectors

Name	Forward Primer (Sequence 5'-3')	Reverse Primer (Sequence 5'-3')	T_a (ºC)
lin-41-mutLCS1	CCT TTT ATA CAA CCG TTC TAC AGT CGA CGC GAT GTA AAT ATC	GAT ATT TAC ATC GCG TCG ACT GTA GAA CGG TTG TAT AAA AGG	55
lin-41-mutLCS2	CCT TTT TAT ACA ACC ATT CTG CAG CTG AAC CAT TGA AAC C	GGT TTC AAT GGT TCA GCT GCA GAA TGG TTG TAT AAA AAG G	55
mlin-41-mut LCS2	GTA CAA CAT TGC CTA AGT CCT AGC TAA GCT TAA A	TTT AAG CTT AGC TAG GAC TTA GGC AAT GTT GTA C	55
mlin-41-mutLCS1	GAA GTG ATA ATT TCT ATC TAC GTT AAC TCT TTG CAT TTC CC	GGG AAA TGC AAA GAG TTA ACG TAG ATA GAA ATT ATC ACT TC	55

B.1.5.3. miRNA Northern blot oligonucleotides

Table B.7. DNA oligonucleotide probes used for miRNA Northern blot

Name	Sequence
let-7a	ACT ATA CAA CCT ACT ACC TCA
let-7c	AAC CAT ACA ACC TAC TAC CTC A
let-7e	ACTATACAACCTCCTACCTCA
mir-124b	GCA TTC ACC CGC GTG CCT TAA
mir-125b	TCA CAA GTT AGG GTC TCA GGG A
mir-128a	AAA AGA GAC CGG TTC ACT GTG A
mir-23a	GGA AAT CCC TGG CAA TGT GAT
mir-26a	AGC CTA TCC TGG ATT ACT TGA A
mir-26b	AAC CTA TCC TGA ATT ACT TGA A
mir-29b	ACA CTG ATT TCA AAT GGT GCT A
mir-29c	TAA CCG ATT TCA AAT GGT GCT A
mir-9	TCA TAC AGC TAG ATA ACC AAA GA
mir-294	ACACACAAAAGGGAAGCACTTT
U6	CCA ATT TTA GTA TAT GTG CTG CCG

B.1.5.4. Ant-miRNA oligonucleotides

Table B.8. LNA modified anti-miRNA oligonucleotides (Exiqon, Proligo)

Name	Sequence
anti-let-7a_LNA	AAC TAT ACA ACC TAC TAC CTC A
anti-mir-125b_LNA	TCA CAA GTT AGG GTC TCA GGG A
anti-let-7e_LNA	ACT ATA CAA CCT CCT ACC TCA

B.1.6. Vectors

pCMV-mir30, kindly provided by Dr. Brian Cullen

pd4eGFP-N1 (Clontech)

pDsRed2-N1 (Clontech)

peGFP-N1 (Clontech)

pEYC1, kindly provided by Dr. Frank Slack

pVT333G (lin14[1-3+4-13]_GFP), kindly provided by Dr. Victor Ambros

Topo pCR2.1 (Invitrogen)

pIRESneo-FLAG/HA Ago1, pIRESneo-FLAG/HA Ago2 corrected (Addgene)

pcFLAG 2-17FMRP, kindly provided by Dr. Yue Feng

pmyc-MOV10 (Addgene)

pmyc-TNRC6B (Addgene)

B.1.7. Bacterial strains

Escherichia Coli XL-1 blue cells (Strategene)

Escherichia Coli XL-10 gold cells (laboratory collection)

Topo10 cells (Invitrogen)

B.1.8. Primary cells, cell lines and mouse strains

Primary cortical neurons and astrocytes

Primary hippocampal neurons

ES cells – murine embryonic stem cell line D3, kindly provided by Dr. Rolf Kemler

P19 EC cells – mouse embryonal carcinoma cell line (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)

HEK293 – Human Embryonic Kidney cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)

C1 Mouse Line (for developmental Northern blot) (Charles River Laboratories)

C57Bl/6J Mouse Line (FEM, Berlin)

B.1.9. Media

P19 growth medium (P19GM): α modified form of Eagle's minimal essential medium (αMEM, Sigma) supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco), 2 mM glutamine (Biochrom), 50 U/ml penicillin and 50 µg/ml streptomycin (Biochrom).

P19 neural induction medium (P19IM): αMEM supplemented with 5% heat inactivated FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

ES cell growth medium: Dulbecco's modified Eagle's medium (DMEM, Gibco), containing 15% heat inactivated FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin, 1% nonessential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Gibco) and 1.000 U/ml murine leukemia inhibitory factor (mLIF, ESGRO).

Medium for HEK293 culture: DMEM, containing 10% FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

Medium for primary neuron culture: Neurobasal Medium without L-Glutamine (Gibco) containing B27 supplement (Gibco), 50 U/ml penicillin and 50 µg/ml streptomycin.

Medium for primary astrocyte culture: DMEM, supplemented with 10% FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin.

B.1.10. Antibodies

Table B.9. Antibodies used for flow cytometry, immunocytochemistry or Western blot.

Antibody, clone, company	Immunocytochemistry working dilution	Flow cytometry working dilution	Western blotting working dilution
Primary antibodies:			
anti-FMRP, mouse monoclonal, clone 1C3-1a, Euromedex	1/1000	1/200	1/1000
anti-β-III Tubulin, mouse monoclonal, clone SDL.3D10, Sigma	1/800	1/500	1/1000
anti-S100β, mouse monoclonal, clone SH-B1, Sigma	1/1000	1/1000	-
anti-Nestin, mouse monoclonal, Rat401, provided by Dr. Hockfild S., Developmental Studies Hybridoma Bank, Iowa	1/1000	1/500	-
anti-NeuN, mouse monoclonal, A60, Chemicon	1/500	1/200	-
anti-MAP2, mouse monoclonal, clone HM-2, Sigma	1/1000	1/1000	-
anti-A2B5, mouse monoclonal clone 105, R&D	-	1/200	-
anti-GFAP, mouse monoclonal, clone G-A-5, Sigma	1/1000	1/400	-
anti-Ago1, rabbit polyclonal, Upstate	1/200	-	1/500
anti-Ago2, rabbit polyclonal, Upstate	1/200	-	1/500
anti-GFAP, rabbit polyclonal, DakoCytomation	1/1000	-	1/1000
anti-GFP, rabbit polyclonal, Abcam	1/500	-	1/1000
anti-HA, mouse monoclonal, clone HA-7, Sigma	1/1000	-	-
anti-Flag, mouse monoclonal, clone M2, Sigma	1/2500	-	1/2000
anti-GW182, mouse monoclonal, clone 4B6, Abcam	1/100	-	-
anti-Myc, rabbit polyclonal, Abcam	1/500		
anti-mlin-41 Serum, rabbit, Pineda Antikörper Service, Berlin	1/5000		1/5000
anti-β-actin, mouse monoclonal, clone AC-15, Sigma	-	-	1/5000
Secondary antibodies:			
anti-mouse RPE-conjugated $F(ab')_2$ Immunoglobulins for flow cytometry, Dacko	-	1/40	-
Alexa Fluor 488/568 $F(ab')_2$ anti-mouse IgG, Molecular probes	1/1000	-	-
Alexa Fluor 488/568 $F(ab')_2$ anti-rabbit IgG, Molecular probes	1/1000	-	-
HRP-labeled sheep anti-mouse IgG, Amersham Bioscience	-	-	1/5000
HRP-labeled sheep anti-rabbit IgG, Amersham Bioscience	-	-	1/5000

Table B.10. Antibodies used for ChIP assay

Antibody, clone	Dilution
anti-RNA Polymerase II mouse monoclonal, clone 8WG16, Covance	1/100
anti-acetyl Histone H3 rabbit polyclonal, Upstate	1/3000

B.1.11. Buffers and Solutions

Table B.11. Buffers and Solutions used

Cell culture solutions:	
Trypsin/EDTA solution, 0.25% (Gibco)	
all-trans retinoic acid (RA) (Sigma)	1 mM stock: 50 mg RA, 156 ml 100% Ethanol, 8 ml H_2O
PBS, 10×, pH = 7.4	2 g/L KCl, 2 g/L KH_2PO_4, 80 g/L NaCl, 14.4 g/L $Na_2HPO4{\times}2H_2O$, to 1 L with H_2O
HBSS with/without Ca^{2+}/Mg^{2+} (Biochrom)	
Geneticin, G418 (Gibco)	50 mg/ml
Solutions used for flow cytometry and immunostaining:	
FACS Washing Solution I	1% BSA (Sigma) in 1×PBS
FACS Washing Solution II	1% BSA and 0.15% Saponin (Sigma) in 1×PBS
FACS Blocking Solution	10% normal rabbit serum, 1% BSA, 0.15% Saponin, 1×PBS
Paraformaldehyde (PFA, Sigma)	4% PFA in 1×PBS
Immunocytochemistry Blocking Solution	5% normal goat serum, 1% BSA, 0.1% fish gelatin, 1×PBS
Solutions used for Northern blot:	
Polyacrylamide-Urea Gel, 12%	80 ml: 38.4 g Urea, 18.2 ml H_20, 8 ml 10×TBE, 24 ml 40% Acrylamide/Bis 19:1, 400 µl 10% APS, 40 µl TEMED
TBE, 10×	1 M Tris, 1 M boric acid, 25 mM EDTA, pH=8.0
Northern Hybridization Buffer for miRNA	0.2 M Sodium Phosphate Buffer (pH=7.0), 7% SDS
Northern Hybridization Buffer for mRNA	0.2 M Sodium Phosphate Buffer (pH=7.0), 7% SDS, 1% BSA
SSPE, 20×	3 M NaCl, 0.2 M NaH_2PO_4, 200 mM EDTApH=7.4
SSC, 20×	3 M NaCl, 0.3 M Sodium citrate, pH=7.0
Sodium Phosphate Buffer, 0.2 M, pH=7.0	115.4 ml 1M Na_2HPO_4, 84.6 ml 1M NaH_2PO_4, 800 ml H_2O
MOPS running Buffer, 10×, pH=7.0	0.4 M MOPS, 0.1 M Sodium acetate, 0.01 M EDTA
Solutions used for bacteria culture:	
LB-medium, pH=7.4	10 g NaCl, 10 g Tryptone (Sigma), 5 g yeast extract in 1 L H_2O
SOC-medium	0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose
Lysis Buffers used for cell extracts:	
Lysis Buffer for *in vitro* miRNA processing assay	0.5% Nonidet P (NP-40), 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 10 mM sodium fluorid, 1 mM DTT, 20% glycerol, 1× protease inhibitors cocktail (Roche)
RIPA Lysis Buffer	1% NP-40, 1% sodim deoxycholate, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium gluoride, 20% glycerol, 1mM DTT, 0.2 mM sodium vanadate, 1× protease inhibitors cocktail
Solutions used for Western blot:	
Protease inhibitors cocktail, 10×	0.2 mg/ml Pancreas extract, 0.02 mg/ml Chymotrypsin, 5 µg/ml Therolysin, 0.2 mg/ml Trypsin, 3.3 mg/ml Papain
Stacking gel buffer	0.5 M Tris, pH 6.8
Separation gel buffer	1.5 M Tris, pH 8.8
Stacking gel 4%	3 ml H_2O, 1.25 ml Stacking gel buffer, 540 µl 37.5:1% Acrylamid/Bis (Fluka), 50 µl 10% SDS, 38 µl 10% APS, 13 µl TEMED
Separation gel 7.5%	5.2 ml H_2O, 2.5 ml Separation gel buffer, 2 ml 37.5:1% Acrylamid/Bis (Fluka), 100 µl 10% SDS, 50 µl 10% APS, 50 µl TEMED
Electrophoresis buffer for protein SDS-PAGE, 10×	0.25 M Tris (Base), 1.92 M Glycin, 1% SDS in 1 L H_2O
Western blotting buffer	192 mM Glycin, 25 mM Tris, 20% Methanol, in 1 L H_2O
Laemmli buffer, 4×	250 mM Tris, pH 6.8, 40% Glycerol, 5% SDS, 10% β-mercaptoethanol, 0.005% Bromphenol Blue
PBT	1×PBS, 0.1% Tween 20
Ponceau Solution 10×	0.2% Ponceau S, 3% TCA
Coomassie Solution	0.05% Coomassie-Blue R250, 6% TCA, 7% Acetic acid, 20% Methanol

B.2. Methods

B.2.1. Cell culture methods

All cell types were cultured at 37ºC in a humidified incubator with 5% CO_2.

B.2.1.1. Maintenance and neural induction of P19 EC cells

P19 EC cells are developmentally pluripotent cells derived from a primary teratocarcinoma induced in the C3H/He mouse strain and have a normal male karyotype. These cells can differentiate into virtually all cell types under the appropriate conditions. When treated with retinoic acid (RA), P19 EC cells develop into neuroectoderm and yield cultures comprising neurons and astroglia [146]. The P19 EC cell line is an excellent model system for studying the factors that regulate neural differentiation. A number of studies have established that the RA treated neuron-like cells express many characteristic proteins of normal neurons (MAP2, NeuN, β-III Tubulin).

Maintenance and neural induction of P19 EC cells followed established protocols [146,147]. P19 EC cell cultures were started from a frozen stock by seeding cells into a 100-mm tissue culture dish with 10 ml of P19 growth medium (P19GM). To passage, cells were removed from the culture dish surface with a 0.25% trypsin/EDTA solution (Gibco). Trypsinisation was stopped by adding P19GM. Cells were transferred into a 15-ml conical tube and pelleted in a clinical centrifuge at 1000 rpm for 3 min. They were resuspended in 5 ml of fresh medium and seeded according to Table B.12. For immunostaining, cells were plated on 0.1% gelatin-coated coverslips in 24- or 12-well plates.

Table B.12. Passaging schedule for undifferentiated P19 EC cells

Diameter of the culture dish	Number of cells seeded	Time to next passage
100 mm	1×10^6	48 h
	4×10^5	72 h

For neural induction, 1×10^6 cells were seeded in 10 ml of P19 induction medium (P19IM) in non-adhesive bacteriological Petri dishes to promote multicellular aggregate formation (embryoid bodies, EB). Immediately after seeding, all-trans retinoic acid (RA, Sigma) was added to final concentration 5×10^{-7} M. After 2 days the medium was changed by transferring the suspension of aggregates to a 15-ml conical centrifuge tube, allowing the aggregates to settle for 10 min. The medium was replaced with the new one of the same composition including fresh RA. Culturing was continued for another 2 days.

To plate differentiated P19 EC cells, the EBs were harvested and washed once with 1×PBS and resuspended in 2 ml of 0.25% trypsin/EDTA, to which DNAse (50 µg/ml) had been added to prevent formation of DNA gels. Cells were incubated for 10 min at 37ºC, the tube was agitated every 2 minutes. Trypsinisation was stopped by adding P19GM. Cells were collected by

centrifugation and the pellet was resuspended in P19GM. 6×10^6 cells were plated in 10 ml of P19GM in a 100-mm dish. After 3 days the medium was changed to Neurobasal medium supplemented with B27 (Gibco). Cells were analyzed after 4, 8 and 12 days of neural induction. Differentiation was monitored by flow cytometry using antibodies against Nestin, NeuN, MAP-2, neuron-specific β-III Tubulin, and A2B5, (Figure B.1).

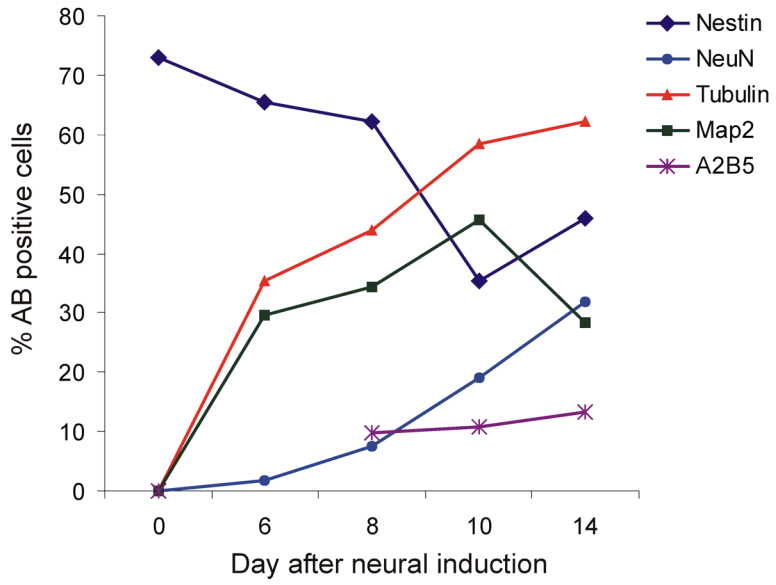

Figure B.1. Monitoring of EC cells differentiation with neuronal and glia markers. Undifferentiated (Day 0) or neural induced P19 EC cells (6, 8, 10, or 14 days after induction) were fixed and stained with antibodies specific for Nestin, NeuN, MAP2, β-III Tubulin, A2B5. Cells were analyzed using flow cytometry.

B.2.1.2. Maintenance and differentiation induction of ES cell line

ES cells are pluripotent, and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. In other words, they can develop into each cell types of the adult body when given sufficient and necessary stimulation for a specific cell type. When given no stimuli for differentiation, ES cells will continue to divide *in vitro* and each daughter cell will remain pluripotent. The D3 ES cell line was obtained from Prof. Rolf Kemler. ES cells were cultured in DMEM containing 15% FBS, 2mM glutamine, 1% nonessential amino acids, 50 U/ml penicillin and 50 µg/ml streptomycin, 0.1 mM β-mercaptoethanol and 1000 U/ml mLIF. mLIF and β-mercaptoethanol are essential to prevent spontaneous differentiation of ES cells. 2.5×10^5 cells were seeded in fresh medium every second day. The same passage procedure was carried out as detailed for P19 EC cells. For immunostaining, cells were plated on 0.1% gelatin-coated coverslips in 24- or 12-well plates.

To induce cardiomyocyte differentiation, hanging drop cultures of 750 cells in 20 µl growth medium without mLIF were incubated on the inner side of a tissue culture dish lid for 3 days to form EBs. EBs were then transferred to a bacterial Petri dish for another 2 days. On Day 5,

individual EBs were transferred to a 24-well tissue culture plate. Differentiation was confirmed by microscopic inspection. In some cases cells were obtained from a collaboration partner (Dr. Andrea Seiler, (ZEBET, BfR) [49]), in which cases differentiation was routinely monitored by flow cytometry at Day 7 using cardiomyocyte markers (anti-α-actinin, anti-sarcomeric myosin heavy chain). The same procedure was followed for neural differentiation with the difference that cells were incubated with 500 nM RA from day 3 to day 5. In a few cases a neural differentiation was achieved by a monolayer induction protocol in defined medium was followed as described in [48]. Differentiation was monitored by flow cytometry using antibodies against MAP-2, neuron-specific β-III Tubulin and GFAP.

B.2.1.3. HEK293 cell culture

HEK293 cells were grown as adherent monolayers in DMEM supplemented with 10% FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Under optimal conditions, HEK293 cells divide every 18-24 h and therefore were normally subcultured twice a week according to the same procedure as P19 EC cells. For immunostaining, HEK293 cells were plated on poly-D-lysine coated coverslips in 24- or 12-well plates.

B.2.1.4. Primary cell culture

Primary neural and astrocyte cultures were prepared by a modified version of a published protocol [148]. Animal sacrifice was conducted under anaesthesia in accordance with the German Law on the Protection of Animals, and all procedures were approved by the local animal committee (Bezirksregierung Köln, AZ, 26.203.2K 35, 35/01). The fetuses were dissected from the uterus, fetal scalps were carefully opened; the cerebellum, midbrain and meninges were removed; the two cerebral hemispheres were washed three times with HBSS without Ca^{2+}/Mg^{2+}, and then incubated for 10 min with Trypsin/EDTA. Trypsinisation was stopped with FBS. The cell suspension was washed twice with HBSS with Ca^{2+}/Mg^{2+}, then cells were resuspended in Neurobasal medium supplemented with B27, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. For neurons, cells were plated at a density of $1\times10^5/cm^2$ on poly-D-lysine-coated culture flasks in Neurobasal medium. Medium was replenished (50%) once a week. For astrocytes, cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Growth medium was replaced three times a week. Microglia cells were removed by agitation prior to harvest. For long term culturing, astrocytes were split and replated after one week. Hippocampal neurons were kindly provided by Nicola Brandt. Cell purity of both astrocyte and neuron cultures was routinely assessed by flow cytometry (see Figure B.2 A and B).

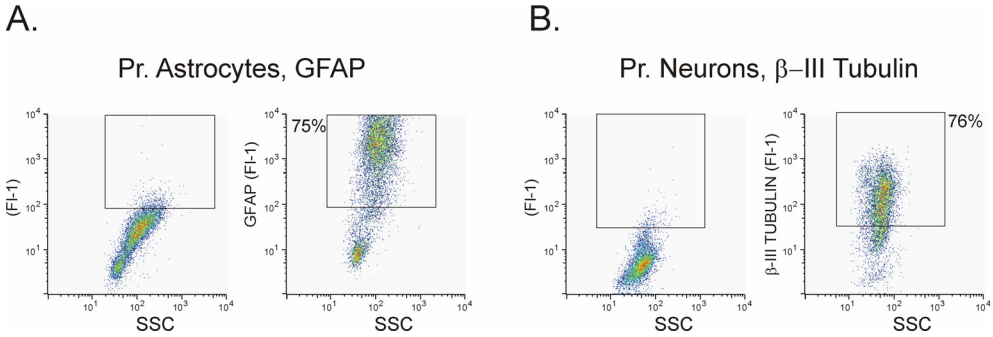

Figure B.2. Intracellular flow cytometry of primary astrocyte and neuronal cultures used for transfection and RNA isolation. Astrocytes were stained with anti-GFAP antibody; Neurons – with anti-β-III Tubulin antibody. **Panel A:** Representative flow cytometry plots of GFAP expression (GFAP Fl-1) versus side scatter (SSC). Gates were established with unstained cells (left plot), the percentage of GFAP positive-gated cells is given. **Panel B**: Flow cytometry plots of β-III Tubulin expression in neurons. As for astrocytes, gates were established with unstained cells, and the percentage of β-III Tubulin positive-gated cells is given.

B.2.2. Transfection of the cells

B.2.2.1. Cell transfection with plasmid DNA

For stable transfection with peGFP-N1 and *let-7*, *mir-125* or *mir-128* sensor constructs, P19 EC cells were transfected using Fugene6 reagent (Roche). From a variety of experimental approaches the following protocol was found to be optimal for these cells: one day before transfection 3×10^5 cells were plated per 60-mm culture dish in 4 ml P19GM. 100 µl of serum-free medium, 6 µl of Fugene6, and 2 µg plasmid DNA were mixed. The reaction was incubated for 15 min at room temperature (RT). Then the mixture was added drop-wise to the cells. For stable transfection, 2 days later 1 mg/ml of geneticin (G418, Gibco) was added to select the transfected cells. After two weeks cells were sorted by flow cytometry. Transfection efficiency varied from 60 to 80%.

For transient transfection with Flag/HA-Ago1, Flag/HA-Ago2, Flag-FMRP, Myc-Mov10 or Myc-TNRC6B, P19 EC cells were transfected in 12-well plates using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. One day before the transfection 8×10^4 cells were plated on a 0.1% gelatin-covered glass in growth medium without antibiotics. On the day of transfection 1 µg of plasmid DNA and 3 µl of Lipofectamine were separately diluted in 100 µl Opti-MEM I medium without serum, and incubated at RT for 5 min. After a 5 minute incubation, DNA was combined with the Lipofectamine mixture and incubated at RT for 20 min. Then the DNA-Lipofectamine complexes were added to each well. After 24-48 hours cells were fixed for immunocytochemistry.

ES cells were transfected with plasmid DNA using Lipofectamine or Fugene6 reagents according to the same procedure as P19 EC cells.

For HEK293, one day before transfection 2×10^5 cells were plated in monolayer on poly-D-lysine covered glass in 12-well plates. Cells were transfected with 1 µg of the appropriate plasmid using Lipofectamine reagent as described for P19 EC cells. HEK293 cells were processed for immunocytochemistry after 24 hours.

Primary neurons were electroporated using the Mouse Neuron Nucleofector Kit (Amaxa) on the day of preparation. 2×10^6 cells were electroporated with 1.5 µg of pDsRed2-N1 control plasmid and 1.5 µg of the plasmid of interest and plated on poly-D-lysine-coated 6- or 12-well plates. Medium was exchanged 2 h after plating to remove cell debris. Cells were processed for immunocytochemistry or for flow cytometry after 72 h. For lipofection, neurons were transfected 7 to 10 days after plating on 18-mm precoated coverslips with Effectene reagent (Qiagen). On the day of transfection 0.5 µg DNA was diluted in DNA-condensation buffer (EC buffer), to a total volume of 75 µl; 5 µl Enhancer were added and mixed by vortexing for 1 s. The mix was incubated at RT for 4-5 min, then 6µl Effectene were added and mixed by pipetting up and down. Samples were incubated for 7-10 min at RT. After incubation, 200 µl of growth medium were added to the tube containing the transfection complex, then the mixture was added drop-wise to the cells. After 48 hours, cells were fixed and analyzed by immunocytochemistry. In some cases cells were transfected using Fugene6 reagent according to the manufacture's instruction. Briefly, neurons were transfected four to seven days after plating on 18 mm cover slips. On the day of transfection, 0,5 µg DNA was mixed with 1,5 µl Fugene6 reagent in 50ml serum-free medium. After 15 min incubation at RT mixture was added drop-wise to the cells.

Primary astrocytes were transfected in six wells with Fugene6 transfection reagent following the guidelines described in the manual. Briefly, 1 µg DNA was mixed with 3 µl Fugene6 reagent in 100 µl medium without antibiotics and serum. The mix was incubated for 15 min at RT and then added drop-wise to the cells. Astrocytes were quantified by flow cytometry 48 h after transfection.

B.2.3. Flow cytometry

In FACS (Fluorescence-Activated Cell Sorter) analysis cells are passed single-file through a laser beam in a continuous flow of a fine stream of cell suspension. Each cell scatters some of the laser light, and also emits fluorescent light excited by the laser. The cytometer typically measures several parameters simultaneously for each cell:

- low angle forward scatter intensity, approximately proportional to cell diameter
- orthogonal (90 degree) scatter intensity, approximately proportional to the quantity of granular structures within the cell
- fluorescence intensities at several wavelengths

For flow cytometry analysis transfected astrocytes were harvested by trypsin/EDTA treatment 48 hours after transfection. 3×10^5 cells were suspended in 0.5 ml FACS Washing Solution I (1×PBS/1% BSA) and assayed in the flow cytometer (FACS CAlibur, BD). Gates were set with control untransfected cells stained with anti-GFAP antibody. The same procedure has been done with primary neurons, but 72 hours after transfection. Gates were set with control untransfected cells stained with anti-β-III Tubulin antibody.

To analyze the eGFP expression in P19 EC cells, transfected with different sensor constructs, cells were harvested at appropriate time points after initiation of neural differentiation by Trypsin/EDTA treatment. 3×10^5 cells were suspended in 0.5 ml FACS Washing Solution I and assayed in the flow cytometer. Gates were set with control untransfected cells. For intracellular antigen detection, 7×10^5 cells were fixed in 2% paraformaldehyde (PFA) solution for 20 min at 4ºC. Fixed cells were washed twice with FACS Washing Solution I and incubated in FACS Blocking Solution (10% normal rabbit serum, 1% BSA, 0.15% saponin in 1×PBS) for 30 min at 4ºC. Primary antibody incubation was carried out in 100 µl of Blocking Solution for 45 min at 4ºC. Controls were incubated only with Blocking Solution. Cells were washed twice with FACS Washing Solution II (1× PBS/1% BSA/0.15% saponin) and incubated with RPE-conjugated secondary antibody in 100 µl Blocking Solution for 15 min at 4ºC. Two washing steps with FACS Washing Solution II were followed by washing with FACS Washing Solution I. After that cells were resuspended in 300 µl FACS Washing Solution I and proceeded to flow cytometry. Gates were set with unstained control P19 EC cells.

For surface antigen detection EC cells were neither fixed nor blocked. Trypsinized cells (3×10^5) were washed once with Washing Solution I, incubated with primary antibodies for 15 min at 4ºC, rinsed again with Washing Solution I, incubated with RPE-conjugated secondary antibody for 10 min at 4ºC, washed twice with Washing Solution I and assayed in a flow cytometer. The antibodies used for flow cytometry are listed in Table B.9.

B.2.4. Immunocytochemistry

For immunocytochemistry, cells (EC, ES, Hek293 cells, primary neurons and astrocytes) were plated on precoated coverslips (gelatin or poly-D-lysine). At appropriate time points cells were fixed in 2% PFA/0.1% Triton X for 20 min at 4ºC, washed twice with ice cold PBS and blocked for 30 min at 4ºC (Blocking Solution: 5% normal goat serum, 1% BSA, 0.1% fish gelatin in 1×PBS). Primary antibodies were diluted in Blocking Solution and incubated with cells for 3 hours at 4ºC. After thrice-repeated washing with ice cold PBS the cells were incubated with secondary antibodies for 1 h at RT. The antibodies used for immunocytochemistry are listed in the Table B.9. Nuclei were visualized by staining with DNA specific dye Hoechst 33342 for 5 min at 4ºC. Then cells

were washed three times with ice cold PBS and fixed on glass slides with Immu-mount (Thermo Shandon). Slides were photographed using an Olympus BX 51 microscope or confocal microscope equipment (Leica TCS SL). Image analysis was performed with the Leica confocal software, MetaMorph, ImageJ or Volocity 3D software packages.

B.2.5. Molecular biology methods

Standard molecular biology methods (such as agarose gel electrophoresis, bacterial culturing, restriction digests, ethanol precipitation, estimation of nucleic acid concentration, etc.) were performed according to "Current protocols in molecular biology" [149] and will not be described in detail.

B.2.5.1. Total RNA isolation

For isolation of RNA from mouse embryos and neonates of timed pairings, brain tissue was dissected, meninges removed, followed by collection in liquid nitrogen. Beginning at postnatal day 2 the cerebellum was dissected off the cortex prior to freezing.

Total RNA was isolated from mouse tissue and cell lines using TRIZOL reagent (Gibco) in accordance with the manufacturer's instructions. Briefly, cells were lysed by adding 1 ml of TRIZOL reagent per 35-mm diameter culture dish. Mouse brain samples were homogenized in 1 ml TRIZOL reagent per 100 mg of tissue. After adding 0.2 ml of chloroform the lysate was shaken by hand for 15 s, incubated at RT for 2-3 min and centrifuged at 12.000 g for 15 min at 4ºC to separate the phases. The upper, aqueous phase containing RNA was collected in a new tube. RNA was precipitated by addition of 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL reagent by centrifugation at 12.000 g for 15 min at 4ºC. Pellets were washed with 70% ethanol and dissolved in RNAse-free water. RNA concentrations were calculated as follows: OD_{260}×40 µg/ml. The integrity of isolated RNA was checked by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.1 µg/ml).

B.2.5.2. Northern blotting

B.2.5.2.1. miRNA Northern blots

Oligonucleotide probes for Northern blots correspond to the miRNA sequences at the miRNA registry (http://www.sanger.ac.uk/Software/Rfam/mirna/index.shtml) and are listed in Table B.7 and Table B.8. The miRNA Northern blot procedure followed a published protocol [4]. Briefly, a 12% acrylamide denaturing (urea) gel was pre-run in 0.5×TBE for 30 min at 300 V. Twenty µg of RNA were mixed with 10 µl of 2× Loading Dye Solution containing formamide (Fermentas) and heated to 80ºC for 3 min. RNA samples were cooled on ice and then run on the urea gel at a

constant 300 V for 3-3.5 hours. The gel was incubated for 5 min in 0.5×TBE plus ethidium bromide, exposed to UV light and photographed to visualize the 5S RNA band as a control for equal loading and RNA integrity. RNA samples were transferred to a nylon membrane (Hybond N+, Amersham Bioscience) by tank electrophoresis at 20-25 V and 4ºC overnight in a BioRad gel blotting apparatus. After transfer, RNA samples were crosslinked with UV light at 120 mjoules/ cm^2 (GSGene linker UV chamber (BioRad)) and baked at 80ºC for 1 h. Ten pmole of oligonucleotide probe were end-labeled with 60 µCi [γ-^{32}P]ATP in a 10 µl end volume using T_4 polynucleotide kinase (Fermentas), and purified using the nucleotide removal kit (Qiagen) according to the manufacturer's protocol. The membranes were prehybridized in Hybridization Buffer (0.2 M sodium phosphate buffer, pH 7.0, 7% SDS) for 30 min at 42ºC. The Hybridization Solution was refreshed and labeled probes added. Hybridization was performed at 42ºC for 18-22 hours. After hybridization, the membranes were washed twice with 2×SSPE, 0.1% SDS, and twice with 0.5×SSPE, 0.1% SDS at 42ºC for 10 min/wash step. The same procedure was followed for LNA probes; hybridization temperature was increased to 55ºC and wash steps were at 65ºC. The membranes were then sealed in plastic bags and exposed to BioMax autoradiographic films (Kodak) at -80ºC. To reprobe membranes, the membrane was stripped by boiling for 5 min in 0.1% SSC, 0.1% SDS, followed by pre-hybridisation as described above.

B.2.5.2.2. mRNA Northern blot

mRNA Northern blots were performed according to the "Current Protocols in Molecular Biology" [149]. The following probes were used for Northern hybridization:

- GFP probe – a ~700 bp fragment from the coding region of the *eGFP* gene
- mlin-41 N-Term probe – a ~700 bp fragment from the N-terminus of the *mlin-41* gene
- lin-41 probe – a ~500 bp fragment from the 3'UTR of the *lin-41* gene
- lin-14 probe – a ~550 bp fragment from the 3'UTR of the *lin-14* gene
- β-actin probe – a ~400 bp fragment from the coding region of the *β-actin* gene

One gram agarose was dissolved in 72 ml water; 10 ml 10×MOPS running buffer and 18 ml 12.3 M formaldegyde were added for a 1% denaturing agarose gel. Ten µg RNA, 1 µg Ethidiumbromide and 2×Loading Dye Solution were mixed, samples were denaturated at 65ºC for 15 min and kept on ice until loading the gel. RNA was separated by denaturing gel electrophoresis in 1×MOPS-running buffer at 80 V for 2.5-3 hours. The gel was rinsed with several changes of RNAse free water to remove formaldehyde, exposed to UV light and photographed to visualize the 28 S (4.7 kb), 18 S (1.87 kb) and 5 S (0.16 kb) rRNA bands as a loading control. The gel was then soaked for 30 min in 0.05 M NaOH, 1.5 M NaCl and neutralized for 20 min by adding 0.5 M Tris.HCl (pH=7.4), 1.5 M NaCl. To improve the transfer efficiency the gel was equilibrated

with 10×SSC for 45 min and blotted overnight using 10×SSC to transfer the RNA onto a nylon membrane (Hybond XL, Amersham Bioscience) by capillary force, as described in "Current Protocols in Molecular Biology"[149]. On the next day the membrane was air-dried and RNA was cross-linked to the membrane using UV-light at 120 mjoules/cm^2. Subsequently, the membrane was hybridized with specific radioactive probes. DNA probes (25ng) were radioactively labeled with 50 µCi [α-^{32}P]dCTP using the Prime-a-Gene Labeling System (Promega). The labeled probes were purified using the nucleotide removal kit (Qiagen). Prehybridization was carried out in 10 ml Hybridization Buffer (0.2 M Sodium Phosphate Buffer, pH=7.0, 7% SDS, 1% BSA) at 65ºC for 1 hour in a hybridization oven. Before hybridization, probes were denatured by boiling for 5 min. The Hybridization Solution was refreshed and denatured probe was then added to the tube. Hybridization was carried out at 65ºC for 16-22 hours. In order to remove the non-specifically bound probe, the following washing steps were carried out in a shaking water bath at 42ºC: 2× 5 min in 2×SSC, 0.1% SDS, 1× 15min 1xSSC, 0.1% SDS, 2× 10 min 0.1×SSC, 0.1% SDS (the last step was curried out at RT). Then the membrane was sealed in plastic bags and exposed to BioMax autoradiographic film (Kodak) at -80ºC. For reprobing, the membrane was stripped by boiling in 0.1×SSC, 0.1% SDS for 5-10 min.

B.2.5.3. cDNA synthesis from total RNA

cDNA was synthesized using the Advantage RT-for-PCR kit (Clontech) with some modifications. Two µg of total RNA (total volume 12.5 µl) were heated together with oligo$(dT)_{18}$ primer at 70ºC for 2 min, and cooled rapidly on ice. Then the following components were added: 4 µl 5× reaction buffer, 1 µl dNTP mix (10 mM each), 0.5 µl Recombinant RNAse inhibitor (20 U), 1 µl DNAse (2 U, RNAse free). The reaction was incubated at 37ºC for 30 min, then heated to 75ºC for 5 min, and placed on ice. After DNAse treatment, 1 µl of MMLV reverse transcriptase and 1 µl recombinant RNAse inhibitor were added to the reaction, and samples were incubated at 42ºC for 1 hour. To stop the cDNA synthesis, the reaction was heated at 94ºC for 5 min. The cDNA was diluted to a final volume of 100 µl by adding 80 µl of RNAse free water and stored at –70ºC. cDNA integrity was monitored using primers specific for β-actin (Table B.2).

B.2.5.4. Polymerase Chain Reaction (PCR)

B.2.5.4.1. Semi-quantitative PCR

DNA was amplified using a PCR block (MJ Research). PCR reactions (50 µl) were performed using either the CombiZyme DNA polymerase mix (Invitek) or the Phusion High-Fidelity DNA polymerase (Finnzymes).

The following components were mixed for a CombiZyme polymerase reaction: 1× OptiPerform Buffer III, 2.5 mM $MgCl_2$, 250 µM dNTPs, 1× OptiZyme Enhancer, 0.5 µM forward primer, 0.5 µM reverse primer, 15-500 ng Template DNA, 2 U CombiZyme DNA Polymerase, H_2O to a final volume of 50 µl.

Phusion polymerase reactions contained: 1× Phusion HF Buffer, 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer, 15-500 ng Template DNA, 1 U Phusion DNA Polymerase, H_2O to a final volume of 50 µl.

PCR profiles used in the work are tabulated in the Table B.13:

Table B.13. Programs for PCR reaction using either CombiZyme Polymerase Mix or Phusion Polymerase.

Steps	CombiZyme Polymerase Mix	Phusion Polymerase
Initial denaturation	95ºC for 2 min	98ºC for 1 min
Denaturation	94ºC for 30-60 s	98ºC for 10 s
Annealing	60-70ºC for 1 min	58-65ºC for 15-30 s
Extension	72ºC for 1 min	72ºC for 30-60 s
Cycles	25-35	25-35
Final extension	72ºC for 5 min	72ºC for 10 min
Storage	4ºC	4ºC

PCR products were analyzed by agarose gel electrophoresis.

B.2.5.4.2. Real-Time PCR

Real-time PCR was used as a quantitative assay to determine the representation of primary miRNA transcripts in RNA from various sources and developmental stages using control primers as standards. cDNA for Real-Time PCR was synthesized with the Advantage RT-for-PCR kit as described above. One µl cDNA was used for each reaction. Both standard housekeeping gene primers and specific miRNA probes were designed with Primer-Express software (Applied Biosystems). The primers and probes for genes of interest were ordered as Custom TaqMan Gene Expression Assays from Applied Biosystems. The PCR mix for GAPDH (Glyceraldehyde-3-phosphate degydrogenase) housekeeping gene controls contained 1µl cDNA template, 10 µl 2× TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems), 0.5 µM of each primers and 0.25 µM probe in a total volume of 20 µl. PCR mix for genes of interest contained 1 µl cDNA template, 10 µl 2× Universal PCR Master Mix No AmpErase UNG, 1µl 20× TaqMan Gene Expression Assays Mix (containing 18 µM primer 1, 18 µM primer 2, 5 µM probe) in a total volume of 20 µl. Standard reactions were performed using an Applied Biosystems PRISM 7500 Sequence Detection System. The following cycle parameters were used: AmpliTaq activation 95ºC for 10 min; PCR: denaturation 95ºC for 15 s and annealing/extension 60ºC for 1 min (repeated 40 times). All experiments were carried out in duplicate. The results were analyzed using TaqMan 7500 software.

B.2.5.5. DNA cloning methods

Gel extraction:

DNA fragments were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

Plasmid linearization and dephosphorylation:

Vectors used for cloning were linearized with the appropriate restriction enzymes and 5' phosphates were removed when necessary using Shrimp Alkaline Phosphatase (SAP, Promega). Both restriction enzymes and SAP were heat-inactivated for 15 min at 65ºC.

Ligation:

Insert restriction fragments were ligated into the linearized and dephosphorylated vectors using T_4 DNA Ligase (BioLabs) in 20 µl reaction volume overnight at 16ºC. T_4 DNA Ligase was inactivated by 65ºC for 15 min and the vectors were transformed into competent *E. coli* cells.

E. coli transformation:

XL-10 gold or Topo10 chemically competent *E. coli* cells were thawed on ice, 200-400 ng of plasmid DNA were added to 50 µl cells and incubated on ice for 30 min. The heat shock step was carried out at 42ºC for 30 s, 250 µl SOC-Medium was added and the cells were incubated at 37ºC for 1 h on a shaker. 100 µl from transformed bacterial cells were plated onto LB-plates containing ampicillin (100 µg/ml) or kanamycin (30 µg/ml) to select transformed bacteria. Plates were incubated at 37ºC overnight. Individual colonies were picked and grown in LB-medium with appropriate antibiotics for plasmid DNA preparation.

Extraction of plasmid DNA:

Small-scale preparations of plasmid DNA were performed in 3 ml of LB-medium by Plasmid-Mini Kit (SeqLab). Medium culture (25 ml) or large culture (100-200 ml) preparations were carried out using Plasmid-Midi and Plasmid-Maxi kits (NucleoBond Macherey-Nagel) respectively, according to the manufacturer's protocols.

Restriction enzyme digestion:

To check the presence and the orientation of the insert of interest, plasmid DNA was digested with the appropriate restriction endonucleases (BioMol) at 37ºC for 1-2 hours in 10-30 µl reaction volume. The product size was controlled by agarose gel electrophoresis using 100-bp DNA Ladder, extended (Roth).

B.2.5.6. Plasmid constructs cloned for this work

For the *let-7* sensor construct, l*et-7* binding sites from *lin-41* 3'UTR were amplified from the plasmid pEYC1 [24] using *lin-41* primers from the Table B.1. The primers and *let-7* binding sites are presented in Scheme B.1.

C. elegans abnormal cell LINeage family member (*lin-41*) mRNA,
3' UTR (NM_001025828)

ACACTTTCTTCTTGCTCTTTACCCATTTCAGATTGTTTTTTAAAATAATCTTTTGATCCC
TTGATCCTTCTTGCATCTAGAGTACAGTTCAACTTGTACTTTCTTTCTTTTTCCCCCACT
CCTAGTCGATGATCGGCCCCATCAAATCGGGCGCAAAGCACACACATTCCAGAAATGTTC
CCTTTTTTCCCCCCGAGTATTGTCCTTTTCCCCTTTTCTACACAGGTTAATTAAGAGTTA
TGTATTTCATATAGTATTTGTGTAATGTTTATTTTCCTGTGCTTCTACCCGGTAGAACAT
CCAAAAAGCTCCCCCGTCCCCCCAAATCCATGTTCCATCTGTATATCCCAGTTTTTTGTC
TGTTTTATCTTCTCTCGAGCGCTTCAGCCAAATCCCCTTCCCCTCGTAATTATCCTTGTT
TTTCATACATTTATGCAAGTTTTCTCTCACCATTTTAATGGTTTCCCATCCATTCATATG
GCTCCGCCCCTTCCCTGTGCACTTTTTATCTGAAAATTCTTTGATATTTAGAGAAATTTG
AGAATATTTCGATGAGATTCATGTAGGTTTTTCCAAAAAATCGAACGAATTTTGTCGGAA
TATTTGAAATCTCAGGAAAAGTCTAAAGAATTAAAACACCCACAATAGCACCTCTTTTCC
TCAAATTGCACCAACTCAAGTATACCTTTTATACAACCGTTCTACACTCAACGCGATGTA
AATATCGCAATCCCTTTTTATACAACCATTCTGCCTCTGAACCATTGAAACCTTCTCCCG
TACTCCCACCAATAGATTATTGCACTTTTCTGAGAGTTTTTCTGTGTTGGAATCATAATT
TTCTAAACTGATTCGCATAATTTCCAACACTGAAAAACTTTCTCAACACCTCTGGTGACT
ATTTTCTTTTCCGGTGTTAATTGTCCCAATTGCCTAATGTCCCCAGTGTTCATTTAAGCT
CCCCATTTATTTTTATTTCACTGTCTTGGTTTTTGTGCCCTAGCGCTAAATATTGTTTTA
TTTTAATGCATGCTTCCTGCACGCCCCTCCCCCTTCTTGCGCACCCAATTTTACAACAAT
TTGTAATTTAAATTCGCAAATTTCACTGCGAAATTTGAAATATATTGCTGATAACATAAT
TGG

```
let-7       UGAG-GUAGUA-GGUUGUAUAGUU
                |||| |||| | ||||||||||
lin-41      ACUCACAUCUUGCCAACAUAUU--
LCS#1


let-7       UGAGGUAGUA-GGUUGUAUAGUU
                 ||||| || | ||||||||||
lin-41      -CUCCGUCUUACCAACAUAUU--
LCS#2
```

Scheme B.1. 3'UTR of *C. elegans lin-41* mRNA. The cloning primers are underlined; the *let-7* binding sites are shaded. *let-7* complementary sites 1 and 2 (LCS 1 and 2) are marked in dark grey, another four putative binding sites in light grey, LCS 5 and 6 overlap.

The forward primer contained a NotI restriction site, the reverse primer – NotI and BamHI restriction sites. The resulting 3'UTR PCR Fragment (505 bp) was cloned into the pCR2.1 TOPO Vector using the TOPO-kit (Invitrogen), followed by excision with NotI. The NotI fragment was gel-purified and inserted into the NotI site of peGFP-N1 or pd4eGFP-N1 (Clontech) situated within the 3'UTR of the *eGFP* mRNA. The fragment was inserted in both sense and antisense orientation to generate peGFP-lin-41 S, peGFP-lin-41 AS, pd4eGFP-lin-41 S, and pd4eGDP–lin-41 AS. The insert orientation was verified with BamHI digestion.

The same strategy was used to generate the *mir-125* sensor containing the *lin-4* response elements from the *lin-14* 3'UTR. The PCR template was the plasmid pVT333G(lin14[1-3+4-3]_GFP), the resulting plasmids were named peGFP-lin-14 S,

peGFP-lin-14 AS. The cloning primer sequences are presented in the Table B.1. The 3' UTR sequence with *mir-125* binding sites is presented in Scheme B.2.

C. elegans abnormal cell LINeage family member (*lin-14*) mRNA, 3′ UTR

```
AATGCCAATTTTTCGAGTCATCCTTCGGGCAATGTTCATTACACTTTCTCTCTGTTGTACTTGAGCATGTTTCAA
TTTCAATCACAAATGCCTTTTTTTGGAGAGAATTGAAGGCAAAACCAAACTAAATTGAGTTTGGTGGAAATTGAAA
TTGCAACATGCTCAATTCATTTTGTTTTTTTCTTTTAACTATATGGATGCCACGCTGGATTGACTCTTCCGTACA
CTCACGCTCATTCCAAATTATCCCCATCTGACCCCGGACTGTTTTATCCAAACCTATACTGATGTATATTGTATG
CGGCATGTTATTTTTTCATAACCACAAGCATTTAGCGCTTTCGGCTTCAGATTTCTAATCGCTGTTTCTTTACTG
CTTTGCCCTTTTTTCTAACTTCTGAATTTCGTTATTATGCAACAATTCTACCTCAATTTTTTTGTATCTTTCCCC
AATTCTAGTTGACATTATTGGTTTTGATTGTTTTCCTAGCTCTGAAATCCCACAACTAGGCGCCACTGAATTGTA
TCTTTCATTCCCACTTTCGTTTTTCGCACATTGTAGTATCTCTCAATTAGGAAACACTTGAAAACCTCTATTGCT
TAGGATTGATGAGAGATATTGCTTTTTCCTGCACTCACTTTACCTTTGTCTCACCTCAAAAATTGCTCTCAGGAA
CATTCAAAACTCAGGAATTTGTCACCTTGGTCTCTCATCATATATCTTACCTCTTGTCACACCCCCCATCCCCAG
TCTCAAGTTCATTTCTTACTTTGTAACTCCGTTTAGTGCGCCCAATTCTCGTCATTTTGATTACACTCTCTTTTA
ATCCAACTCAGGGAACCAATTTTTTTTCTCATTGAACTCAGGAATTTCTTCTACCTCAGGGAACCTACCTCATCC
ACTTTTCAGTTGTTTGGGGCCAAATATCTATATCCAAAGTAGTAGTCTACAATTTAGTATTTTATTATTACCTCC
CGCCGTTTTAGCTTTTAATGTTAAAATCAGGAACTTTTGAAAATGATCTTCACCTCATTCAGAAGCAAAAATCAG
GCATTTTCCAAAGATTTTGAAAACACATAAACCTCCTTCCAAGTCAAAACTCACAACCAACTCAGGGACCTTTTT
CTTACTTCTGTATCACAAAAATGATTATATTTCTGATGAATGTTGCTCTGTCATAAATCAATTTATTTCTTTTGA
ACCGAAAGCCGAAATGTGTTTTTCCTATTTGTCGTCATTGTTCTCATCCACCCCAACTTCCTCTCATTATTGAAG
TTTCTCATGTATTCTACCTCATTTATTATTGTTTTTTTTTGTTCAAAATGTCAATGTGACAAAACAAATTGCCCG
TAATGTCTGAAACTCTGTTCTTTCCTATGTAACTGTATACGAAAAATTCCCCCGTTAATTTTTAATATGTATTCT
GTGTCCCATCTTTGCCCCAGTTTATTTTTTGTACCCCTGTGAGCGTTCAAATTACCTCAGCATCCAACAATCTTT
CCATCTTAACATCCATCCCATTGTACCTCTGAATCTTGCTTCGCTTACCTCGTAACAAATATATTTTTATCGGCT
TAAAACCTAATAAATCATTTACCAG
```

```
mir-125b        AGUGUUCAAUCCCAGAGUCCCU
                |||| ||  ||   | |||||||
lin-14          UCAAAAAUU--GCUCUCAGGA-

mir-125b        AGUGUUCAAUCCCAGAGUCCCU
                ||||  |||||    |  ||||
lin-14          UUA-AUGUUAAAAU--CAGGA-

mir-125b        AGUGUUCAAUCCCAGAGUCCCU
                ||||||   |     |||||||
lin-14          UCACAACCAA----CUCAGGGA
```

Scheme B.2. 3'UTR of *C. elegans lin-14* mRNA. The primers used for cloning are underlined, *mir-125* binding sites are marked in grey.

For the *mlin-41* sensor, a fragment containing several potential *mir-125/let-7* binding sites was amplified from an undifferentiated EC cell cDNA library using mlin-41 primers presented in the Table B.1, the fragment is described in Scheme C.2. (see Section C.4.3). The forward and reverse primers contained NotI restriction sites. EcoRI digestion has been done to control insert orientation. peGFP-mlin-41 S and peGFP-mlin-41 AS constructs were generated using the strategy described above for the *let-7* sensor construct.

For the *mir-128* sensor, the following partially complementary oligonucleotides were annealed and extended with T7 DNA polymerase:
5' GCGGCCGCGTCGACAAAAGAGACCAAGTCACTGTGATATAAAAGAG 3' and
5' CTCGAGTCACAGTGAGTTGGTCTCTTTTATATCACAGTGAC 3'. *mir-128* binding sites

are underlined, mismatches are indicated by a dotted line. A plasmid (pCR2.1 TOPO) containing tandem insertions of this sequence was generated using restriction sites in the oligonucleotides (SalI and XhoI). A NotI fragment containing the *mir-128* response element was inserted into peGFP-N1 as described above.

All constructs were verified by sequencing (SMB, Institut für Genetik, Berlin).

B.2.5.7. Site-directed mutagenesis

The QuikChange site-directed mutagenesis kit (Stratagene) was used to insert point mutations into miRNA binding sites in the *let-7* or *mlin-41* sensor plasmids. Complementary oligonucleotides (see Table B.6) for both DNA strands, containing the desired mutations, were annealed to the denatured target plasmid and extended to nicked circular strands by the PhuUltra High-Fidelity DNA polymerase. The parental methylated and hemimethylated DNA was digested using the restriction enzyme DpnI to select for newly synthesized DNA. Digestion products were transformed into XL1-blue supercompetent *E. coli* cells, where the DNA nicks were repaired. Plasmids were prepared from the bacterial clones by Midi DNA preparation kit, and the mutations verified by sequencing.

B.2.5.8. In vitro pre-miRNA transcription

About seventy base pair templates for *in vitro* transcription were amplified using specific primer pairs from Table B.3. The *pre-mir30* DNA template was amplified from the pCMV-mir30 plasmid (see Section B.1.6), *pre-let7e* – from a genomic mouse DNA fragment containing the pre-*let7e* subcloned in Topo pCR2.1 vector, *pre-let7a* was amplified by nested PCR from mouse genomic DNA, *pre-mir128* DNA template was generated from a genomic mouse DNA fragment containing the *mir-128b* gene subcloned in Topo pCR2.1 vector (laboratory collection). The PCR products were purified from a 2.5% agarose gel using the QIAquick Gel Extraction kit and 1 μg of each template was transcribed by T7 RNA polymerase (T7 Transcription kit, Fermentas) in the presence of [α-^{32}P]UTP (25 μCi at concentration of 10 μCi/μl). The reactions were incubated at 37ºC. After 2 hours pre-miRNAs were purified by phenol/chloroform extraction and precipitated with 100% isopropanol in the presence of 0.3 M ammonium acetate. Pellets were dissolved in RNAse free water at 20.000 cpm/μl. RNAs were stored at -80ºC.

In vitro transcribed pre-miRNAs were used for the *in vitro* miRNA processing assay (B.2.6.2).

B.2.5.9. mRNA degradation assay

Actinomycin D (Calbiochem) inhibits DNA-primed RNA polymerase by complexing with DNA via deoxyguanosine residues. Actinomycin D was used to block new mRNA synthesis, in order to analyze the turnover and stability of the existing mRNA pool. Undifferentiated or neural differentiated P19 EC cells stably transfected with peGFP-lin-14 S or peGFP-lin-14 AS plasmids; peGFP-lin-41 S or peGFP-lin-41 mutant plasmids; and peGFP-mlin-41 sensor or peGFP-mlin-41 mutant constructs were treated with Actinomycin D (10 µg/ml). After 0, 0.5, 1, 2, 4, or 8 hours of treatment total RNA was isolated (B.2.5.1), reverse transcribed (B.2.5.3) and cDNAs were analyzed by PCR (B.2.5.4) with specific primers (eGFP-Thy1, c-Myc, β-actin; see Table B.2). *GFP, mlin-41, β-actin,* and *c-Myc* mRNA expression levels were also analyzed by Northern blot (B.2.5.2.2).

B.2.6. Biochemical methods

B.2.6.1. Protein electrophoresis and Western blots

Protein extracts were prepared from P19 EC cells or primary neurons and astrocytes. Cells were resuspended in RIPA Lysis Buffer or Lysis Buffer used for *in vitro* miRNA processing (Table B.11) in the presence of a protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation at 14.000 rpm, 4ºC for 20 min. Supernatants were snap-frozen in liquid nitrogen and stored at -80ºC. The total protein concentration in the extracts was measured by the BCA protein assay kit (Pierce) according to the manufacturer's description. Briefly, BSA (25 µg/ml – 2000 µg/ml) was used as a standard. The measurement was performed in 96-well plates in the ratio of sample to working reagent 1:8. After a 30 min incubation at 37ºC, the absorbance was measured at 562 nm on a plate reader (Biotech Instruments).

Twenty to forty µg of protein extracts in Laemmli sample buffer were denaturated at 95ºC for 10 min, separated by SDS-PAGE (for gel and buffer recipes see Table B.11) and transferred to an Immobilon P membrane (Millipore) in Western blotting buffer at 10 V for 20 min. The transfer efficiency was determined by Ponceau Red staining for 5 min at RT.

To block non-specific binding to the membranes, they were incubated overnight at 4ºC in Blocking Solution (3% BSA in PBT). The primary antibodies were diluted in Blocking Solution according to the Table B.9 and incubated with membranes for 2 hours at RT. After 3 15 min washing steps with PBT, horseradish peroxidase conjugated secondary anti-mouse or anti-rabbit antibodies (Amersham Biosciences) were applied for 1 hour. The membranes were washed as above. For visualization of immuno-reactive bands chemiluminiscent detecting ECL reagents (Amersham Biosciences) were used as recommended by the manufacturer. The membranes were then exposed to ECL Hyperfilm (Amersham Biosciences). To reprobe membranes, the old antibody

complexes were stripped in 0.2 M glycine, 0.1% SDS, 1% Tween20, pH 2.2 for 3 hours at RT prior to blocking.

B.2.6.2. In vitro miRNA processing assay

pre-miRNA templates were transcribed *in vitro* in the presence of [α-^{32}P]UTP (B.2.5.8). Cytoplasmic extracts for the *in vitro* reaction were prepared from undifferentiated or neural differentiated P19 EC cells, ES cells, primary neurons and astrocytes in Lysis Buffer (0.5% Np-40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 10 mM sodium fluoride, 1 mM DTT, 20% glycerol and 1× protease inhibitors cocktail (Roche)) [89]. Cell pellets were resuspended in Lysis Buffer. Extracts were cleared by centrifugation at 14.000 rpm for 20 min at 4ºC. Supernatants were collected in fresh tubes and immediately frozen in liquid nitrogen. The quantitation of proteins in the extracts was performed using the BCA protein assay reagent kit (B.2.6.1).

For *in vitro* miRNA processing assay 10 µg of protein extracts of interest were incubated for 90 min at 37ºC with 20.000 cpm pre-miRNA in 75 mM NaCl, 20 mM Tris-HCl, pH 7.5, 3 mM $MgCl_2$ and 0.1 U/µl RNAse inhibitor. As a control pre-miRNA was digested with recombinant Dicer (0.1 U/reaction) according to manufacturer's recommendations (Ambion). Reaction products were resolved in 12% denaturing electrophoresis gel (gel recipe see in the Table B.11). Subsequently, gels were stained with ethidium bromide to visualize size standards (100 nt RNA marker and 22 nt siRNA duplex), vacuum dried and visualized by autoradiography.

B.2.6.3. Chromatin Immunoprecipitation assay (ChIP assay)

In order to analyze CMV promoter activity of eGFP vectors carrying miRNA binding sites, chromatin was precipitated either with antibody specific for active chromatin (anti-acetyl-histone H3 antibody, Upstate) or with anti-RNA polymerase II antibody (8WG16, Covance). The assay was performed using the Chromatin Immunoprecipitation assay kit (Upstate) according to the manufacturer's protocol. Briefly, 1×10^6 undifferentiated or neural differentiated P19 EC cells were used per sample. Histones were cross-linked to DNA by adding formaldehyde directly to the culture medium to a final concentration of 1% and incubating for 10 min at 37ºC. After washing two times with ice cold PBS, cells were scraped into conical tubes and were pelleted for 5 min at 2.000 rpm at 4ºC. Cell pellets were resuspended in 200 µl of SDS Lysis Buffer with protease inhibitors and incubated for 10 min on ice. Then the lysates were sonicated 4 times for 10 s at 20% of power and 10% of pulses (Dounce homogenizer Sonopuls GM70, Bandelin) in order to shear DNA to lengths between 200 and 1000 bp. After sonication samples were centrifuged for 10 min at 13.000 rpm at 4ºC, supernatants were transferred to a new 2-ml tube and 10-fold diluted with ChIP dilution buffer containing protease inhibitors. At this step 20 µl of cell supernatants were kept as an input control sample. To reduce non-specific background, samples were pre-cleared with 75 µl of Protein A

Agarose slurry (50%) containing salmon sperm DNA for 30 min at 4ºC with agitation. Immunoprecipitation with antibodies was carried out overnight at 4ºC with rotation. For a negative control a no-antibody immunoprecipitation was done. On the next day 60 µl of Protein A Agarose slurry (50%) containing salmon sperm DNA was added to collect the antibody/histone complexes and samples were incubated for 1 hour at 4ºC with rotation. The protein A agarose/antibody/histone complexes were washed for 4 min on a rotation platform at 4ºC with 1 ml of each of the buffers listed in the order as given below:

One wash with low salt wash buffer;

Two washes with high salt wash buffer;

Two washes with LiCl wash buffer;

Two washes with TE Buffer;

All buffers were supplied with the kit.

Histone complexes were eluated from the antibody in 500 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$). Agarose was pelleted and 20 µl 5M NaCl was added to eluates, the histone-DNA crosslinks were reversed by heating at 65ºC for 4 hours. Next, eluates were treated with Proteinase K at 45ºC for 1 hour; DNA was extracted with phenol/chloroform and precipitated with 100% ethanol. Pellets were resuspended in 15 µl water and PCR with primers specific for CMV Promoter sequences was performed. As a control, the differentially regulated Oct4 promoter was amplified in parallel.