

Supplementary Information

MicroRNA 130 Family Regulates the Hypoxia Response Signal through the P-body Protein DDX6

Ken Saito, Eisaku Kondo, Masayuki Matsushita

FIGURE LEGENDS

Supplementary Figure S1. Predicted miRNAs in the HIF-1 α 3'UTR. (A) 3'UTR seed matches to human HIF-1 α were identified using the partially overlapping predictions by miRBase (<http://microrna.sanger.ac.uk/targets/v2/>). Underlines show the miRNA target sites. The binding of miRNAs to the target sites (underlines) are shown. (B) miR-130a/b target sites in mouse and human HIF-1 α are shown. Bold type shows residues of the seed sequence.

Supplementary Figure S2. 3'UTR seed matches to human HIF-1 β were identified using the partially overlapping predictions by TargetScan (<http://www.targetscan.org/>). Underlines show the miRNA target sites. The binding of miRNAs to target sites (underlines) are shown.

Supplementary Figure S3. Effects of miR-130a and 130b. (A) HEK 293 cells were transfected with 0.1 to 100 nM of pre-miR-130a and 130b for 48 h, and then cells were exposed to hypoxia for 8 h. Expression levels of HIF-1 β were detected by western blots. Pre-miR negative control (Pre-miR N.C.) was 100 nM. Left lane (control) shows untransfected cells. HIF-1 β expression levels were calculated by the relative expression of actin. The ratio to the control is shown. (B) HEK293 cells were transfected with pre-miRNAs and the mHIF-1 α 3'UTR reporter gene. Renilla luciferase expression was standardized to firefly luciferase. Results are presented as mean \pm S.D. (n=3, control vs 130a: p=0.056, control vs 130b: p=0.028). (C) In the transient transfection of pre-miR-130a/130b, cells were exposed to hypoxia for 8 h and HIF-1 α mRNA levels were analyzed by qRT-PCR. The values were corrected by β -actin mRNA. Results are presented as means \pm S.D. (n=3).

Supplementary Figure S4. Knock-down of genes predicted to be miR-130 targets. (A) siRNAs for miR-130 target genes were transfected into HEK 293 cells. After 48 h, cells were exposed to hypoxia for 8 h. HIF-1 α expression was detected by western blotting. (B) Knock-down efficiency was examined by qRT-PCR. The ratio to the control siRNA is shown.

Supplementary Figure S5. DDX6 localization in HEK293 cells. (A) Cells were treated with normoxia and hypoxia for 48 h and were stained with anti-DDX6 and anti-DCP1A antibodies. The localization of endogenous proteins was examined by fluorescence

microscopy. Nuclei were stained with DAPI. Scale bar = 10 μ m. **(B)** HEK293 cells were exposed to normoxia and hypoxia for 8, 24, and 48 h. Endogenous DDX6 expression was detected by western blotting.

Supplementary Figure S6. Tissue distribution of pri-miR-130a. **(A)** *In situ* hybridization experiment on cryo-sections of E18.5 mouse embryos using antisense probes that recognize pri-miR-130a (left) or sense probes of pri-miR-130a (right). **(B)** Pri-miR-130a was expressed in the cortex (top panel, left), cerebellum (middle panel, left), and kidneys (bottom panel, left). Granule cells in the cortex and cerebellum are indicated by arrowheads. Renal tubes in kidneys are indicated by asterisks. Scale bar = 1 mm. Squares represent areas shown as higher magnifications. Scale bar = 100 μ m.

Supplementary Figure S7. Mature miR-130 levels in hypoxia. HeLa and NIH-3T3 cells were cultured under normoxia (N, solid bars) or hypoxia (H, open bars) for 72 h. Endogenous mature miR-130a and 130b levels were detected by qRT-PCR. RNA levels for miR-130a and 130b were normalized by 5S rRNA. The ratio to normoxia is the mean of three independent experiments \pm S.D. (P=0.1).

Supplementary Figure S8. Localization of DDX6 in neuronal cells. **(A)** After exposure to normoxia (left panel) and hypoxia (middle panel) for 48 h, cells were stained with DDX6 antibody or Dcp1a antibody as a P-bodies marker. The right panels (reoxygenation) show the localization of DDX6 and Dcp1a under normoxia for 12 h after hypoxia for 48 h. Nuclei were stained with DAPI. Scale bar = 10 μ m. **(B)** After exposure to normoxia (solid bar) and hypoxia (open bar) for 48 h, DDX6 mRNA levels in neuronal cells were measured by qRT-PCR. The values were corrected by β -actin mRNA. Results are presented as means \pm S.D. (n=3).

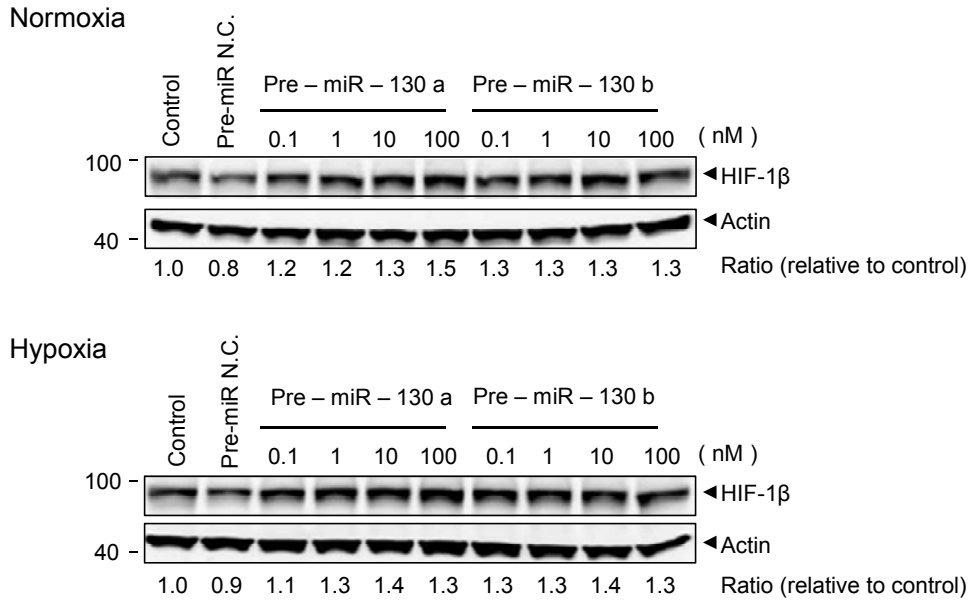
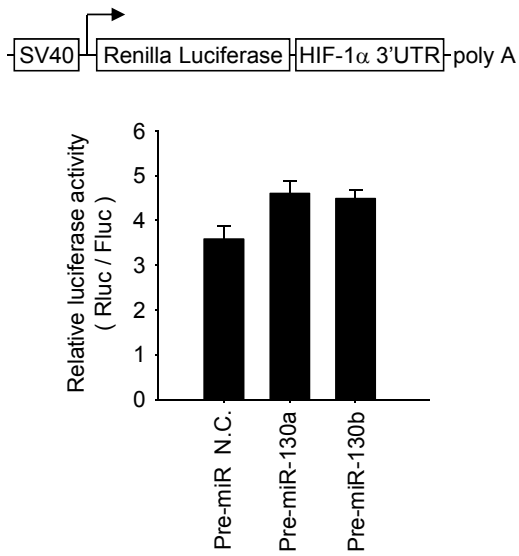
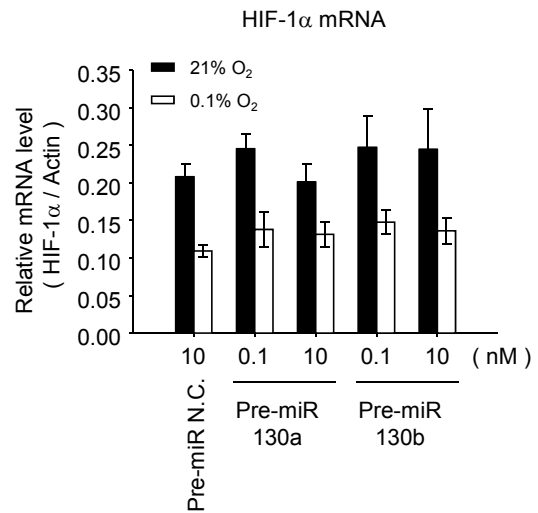
Supplementary Figure S9. Localization of DDX6 under oxidative stress and hypoxia. Neuronal cells were treated with or without 0.5 mM arsenite for 15 min as an oxidative stressor, and then cells were stained with DDX6 antibody and TIAR antibody as stress granule markers. Neuronal cells were also stained with DDX6 antibody and TIAR antibody under hypoxia and reoxygenation conditions. DDX6 foci and TIAR foci are indicated by arrowheads. Nuclei were stained with DAPI. Scale bar = 10 μ m.

actattgg

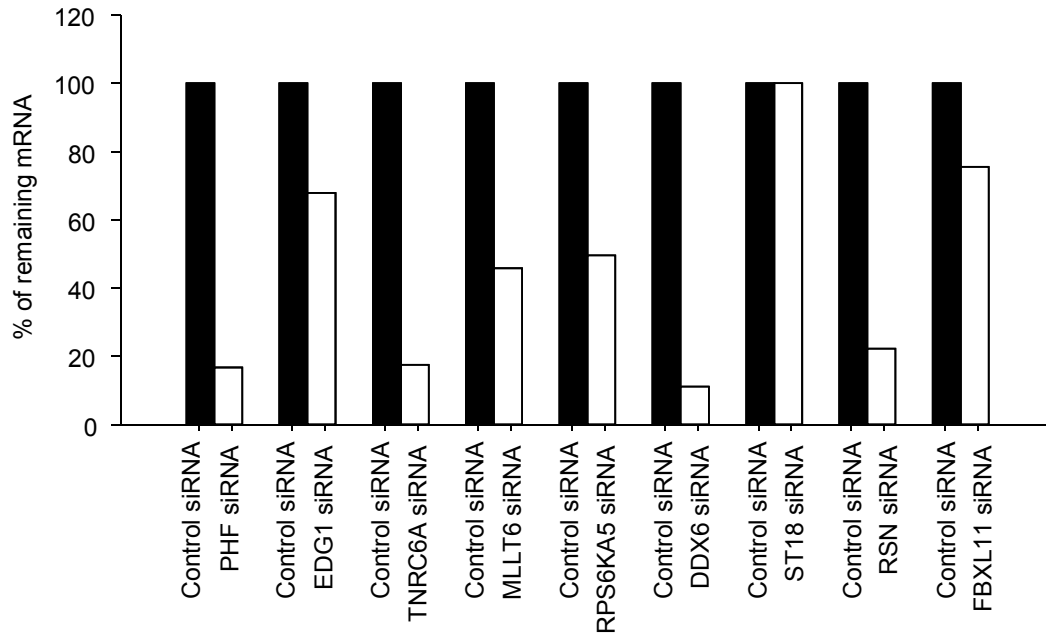
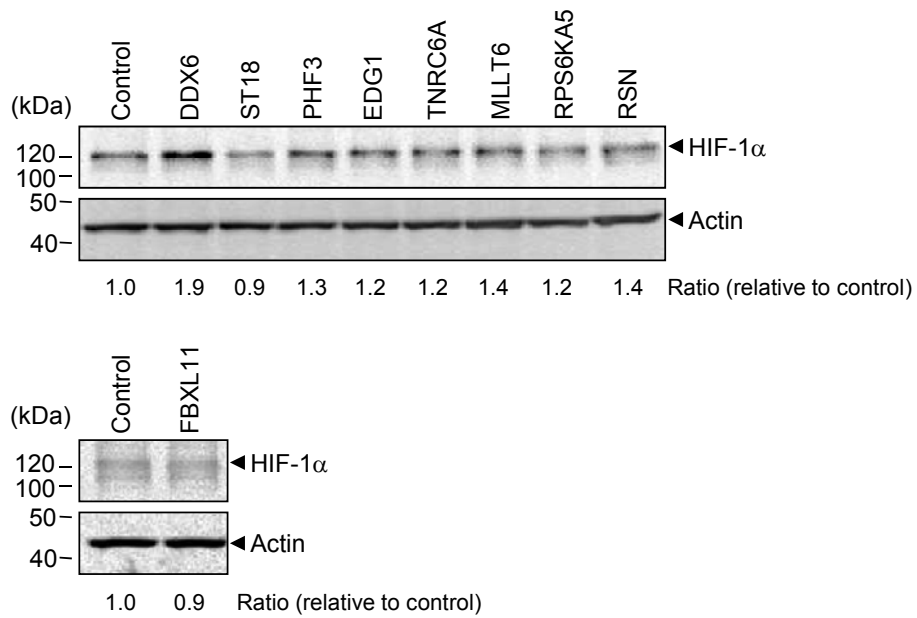
2581 ggtgaggata aggggtgggg gagaaaaaat cactgtttgt ttttaaaaag caaatctttc
2641 tgtaaacaga ataaaagttc ctctcccttc ccttccctca cccctgacat gtaccccctt
2701 tcccttctgg ctgttcccct gctctgttgc ctctctaagg taacatttat agaagaaatg
2761 gaatgaatct ccaaggcttt taggactgtc tgaaaatttg aggctgggtg aagttaaaac
2821 acctttcctt atgtctcctg acctgaaatt gtatagtgtt gatttgtgct gagatcaaga
2881 ggcaggttag aagaacctga catccactgt ttgccttggga tagtatggct tgtttttgga
2941 aagaaattct gaagagagtg gaggagagga gaaatgtcct catatttgag gaccatgaaa
3001 cattgtaggt atatatgggg cttagcaag tttgagcata ggctcttttt gctgcctgtg
3061 agcagtcctt ctggaaagaa acatgtgagt aagtgagaga gagtgtgtgt gtatgtgtgt
3121 gtgtgtgtgt gtgcgcacac atgcttctgt atttcactct ttctccctat tagggagtta
3181 tgcaaaaattt gtccccgatt ttacctttgt ctttctgtgt acttttcaaa gagtcctaag
3241 gagttaaatc ttccaggtat tttccactta gtattgcagc caaagaatat ttaaataaac
3301 gtctttgctg cgcttgcatc catgccagc caatatacaa ctgtaaagca aatatagaaa
3361 gtcggctgtt gatacgattg tctgttatcg aacacattca gtgataaagc tgggttactg
3421 ctgcttttgg tgctctcacc ttatctggaa gatctgcaaa cattaccta ataggctggc
3481 aagataaaca ctttctggaa cccgagactt ggccataaag ataatgctgc atttttctgt
3541 cagaatcaca tatgatgtgt gttctgtaga ² ggttatttct gcatggaaac tcaacttctt
3601 ggattagccg tcccagtgaa aatcctcatt gttggagtgt aaaccaaata cgaagccctc
3661 ttgcaaagta gcctctttca tcccatactc aaaataccca gtttagcaag caactgagat
3721 ttaagtctct ctggccctaa gaggtttttc ctctttgctc cctccaatct tgagattggg
3781 ttttgcttta gagtgcaagt atcataattc cgtatgatag atggggcctg gacacccatc
3841 tcaacagqgt cacttggtaa ttaacaatag ccatataaat gcgatacag gttactacce
3901 tcacccttta ccttctcag ⁴ gtaacagtcg tagataccag cttttttttt ttttttttta
3961 aattgqcttt ggccaqtagc taaagtgcaa gactgaatta atgagaagat atatt⁵aatg
4021 tagtcataggg gactgagga gcaagggtgg ccttgaagag gccaaaggaa tgtccatttg
4081 ctgagtttcc cttccttatg tctccagtct ggtgccaggt agtggagtaa aaaaggagac
4141 agtttatttt tttattctat gtgcacactt acagtataca tatatattta taccacaatt
4201 tacgaaacca aaaagttgag tttccaatgg aacccttgtt tttta⁶ataat cgacttttta
4261 aatgtgatca ggactataat attgtacagt tattataggg cttttgggga aggggaggat
4321 agcgagaaga tgctctgggg gttttgtttt tgcttttctc tcagggtttt atttttgact
4381 gttttgtttt cttgttggcc atttctgtat tgctggcctc tgtgctaagc tttacaqtagg
4441 caaaaataat gacatgtagc aaagat⁸ttt aaacaaaata ttttttctt ttgtaaaatt
4501 tcttgtgttg tgtgatcttg ⁸ attg⁸cggtt tatcattcct ttccagttca taacaacag
4561 gcaccacaaa ccagaggaat ctatagt⁸tta agctccagac atacaaacat aaggcacatt
4621 gtgtctttaa tttcaggaat cagaaatcat aggttctga tcacattgca cgcctcccc
4681 ctcaactgtc ctctgatcc tgacacattc tgagtaacat cagcaggaat gctctgacca
4741 tgaggtgggg gttttggggg gggcggtg⁸cc tgggttcttg ggagagaggg gaagagtcgg
4801 gacttga⁸aaa cacttagggc acatctggat gccttcccc agtatgtcct tttctggatt
4861 aaaatgagtg aaatt⁸taac tgttcaaaaa aaaaaaaaaaaa aaa

No.	Predicted miRNA	No.	Predicted miRNA	No.	Predicted miRNA
1	hsa-miR-29a/29c/29b	4	hsa-miR-135	7	hsa-miR-129/129-5p
2	hsa-miR-103/107	5	hsa-miR-193	8	hsa-miR-221/222
3	hsa-miR-10a/10b	6	hsa-miR-23ab		

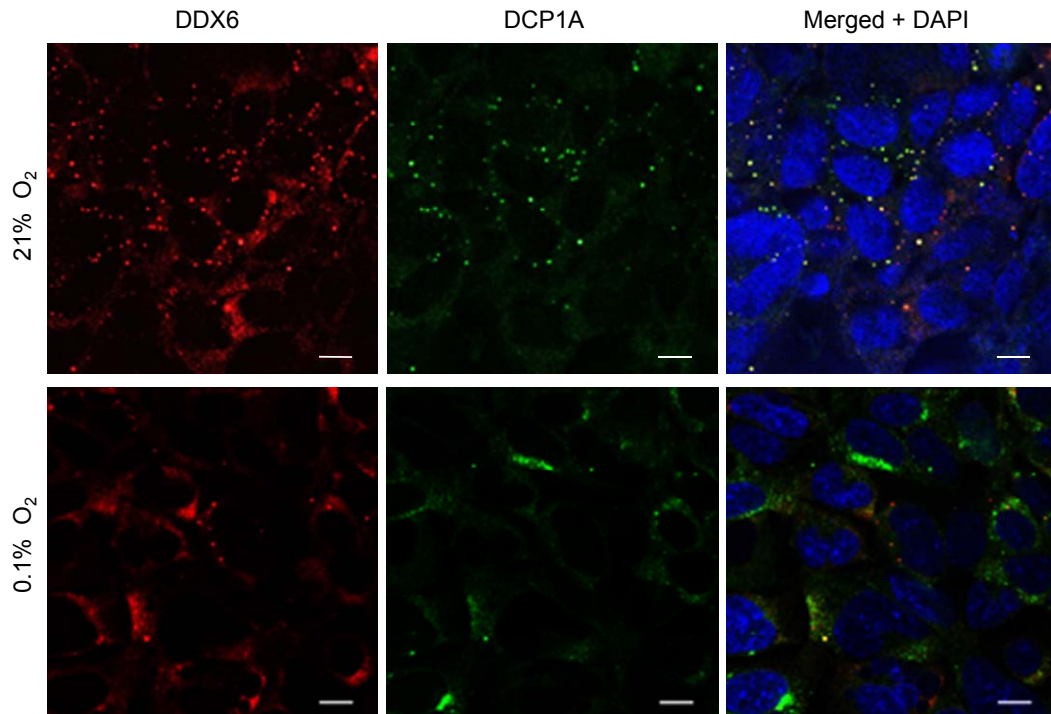
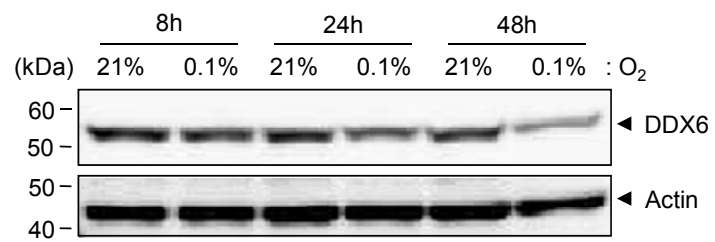
Supplementary Figure S2

A**B****C**

Supplementary Figure S3

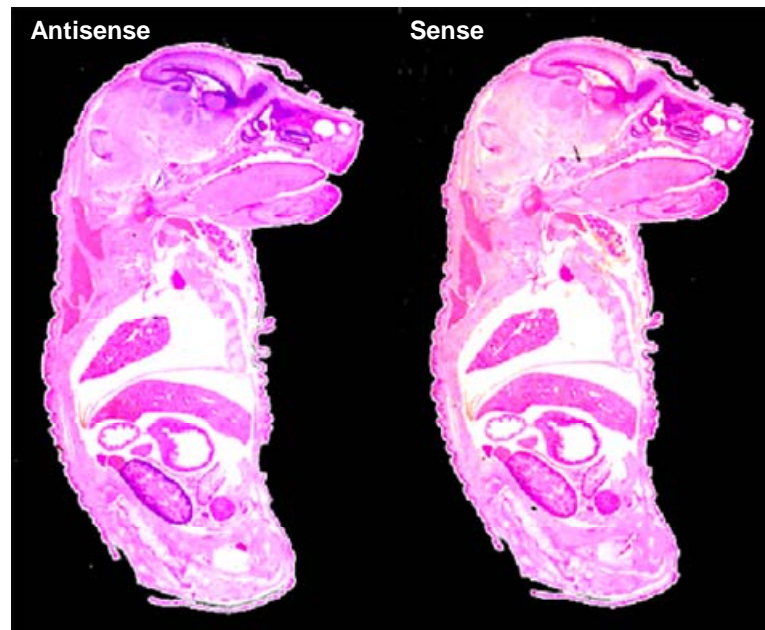
A**B**

Supplementary Figure S4

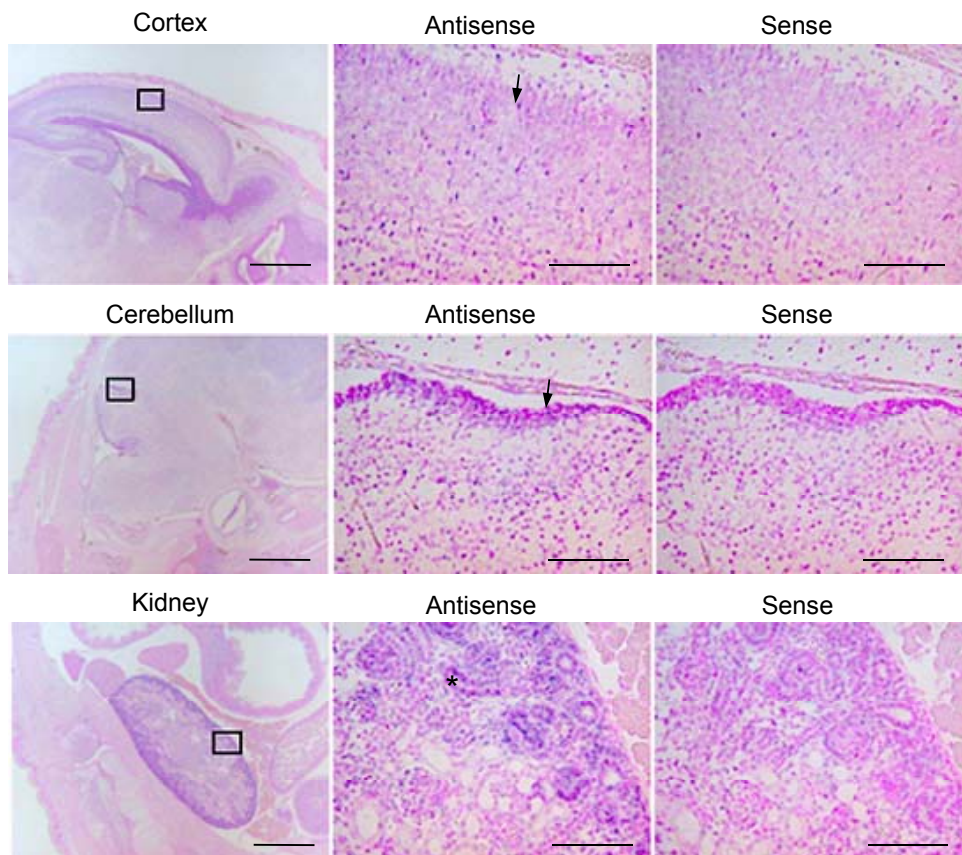
A**B**

Supplementary Figure S5

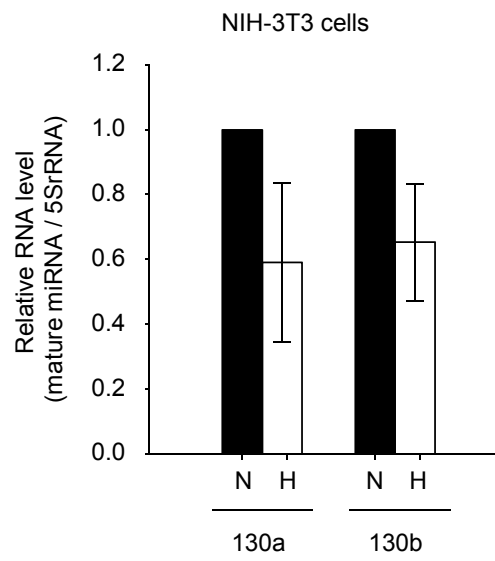
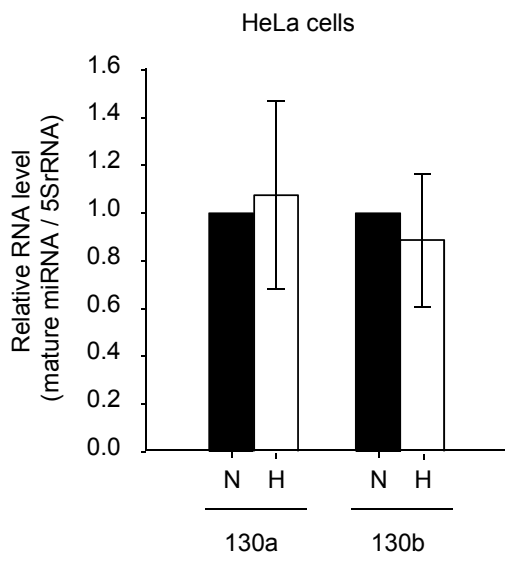
A



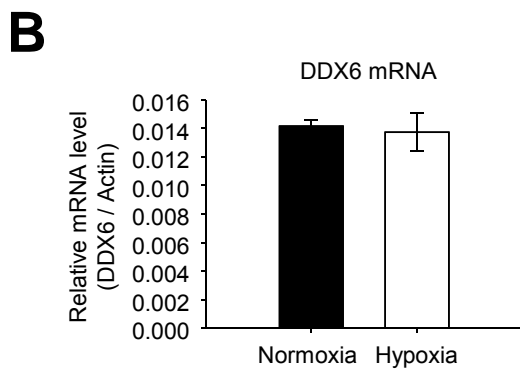
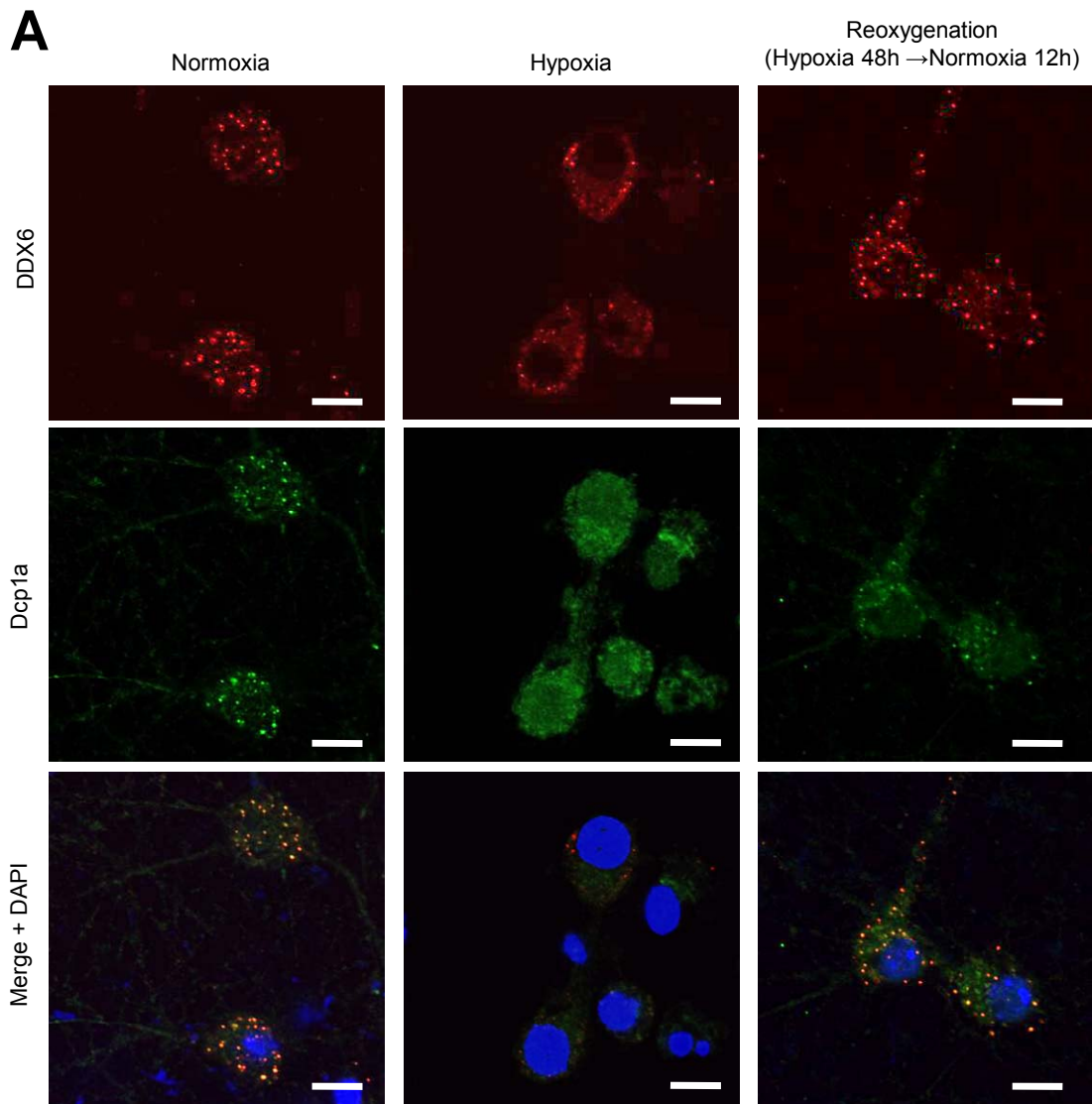
B



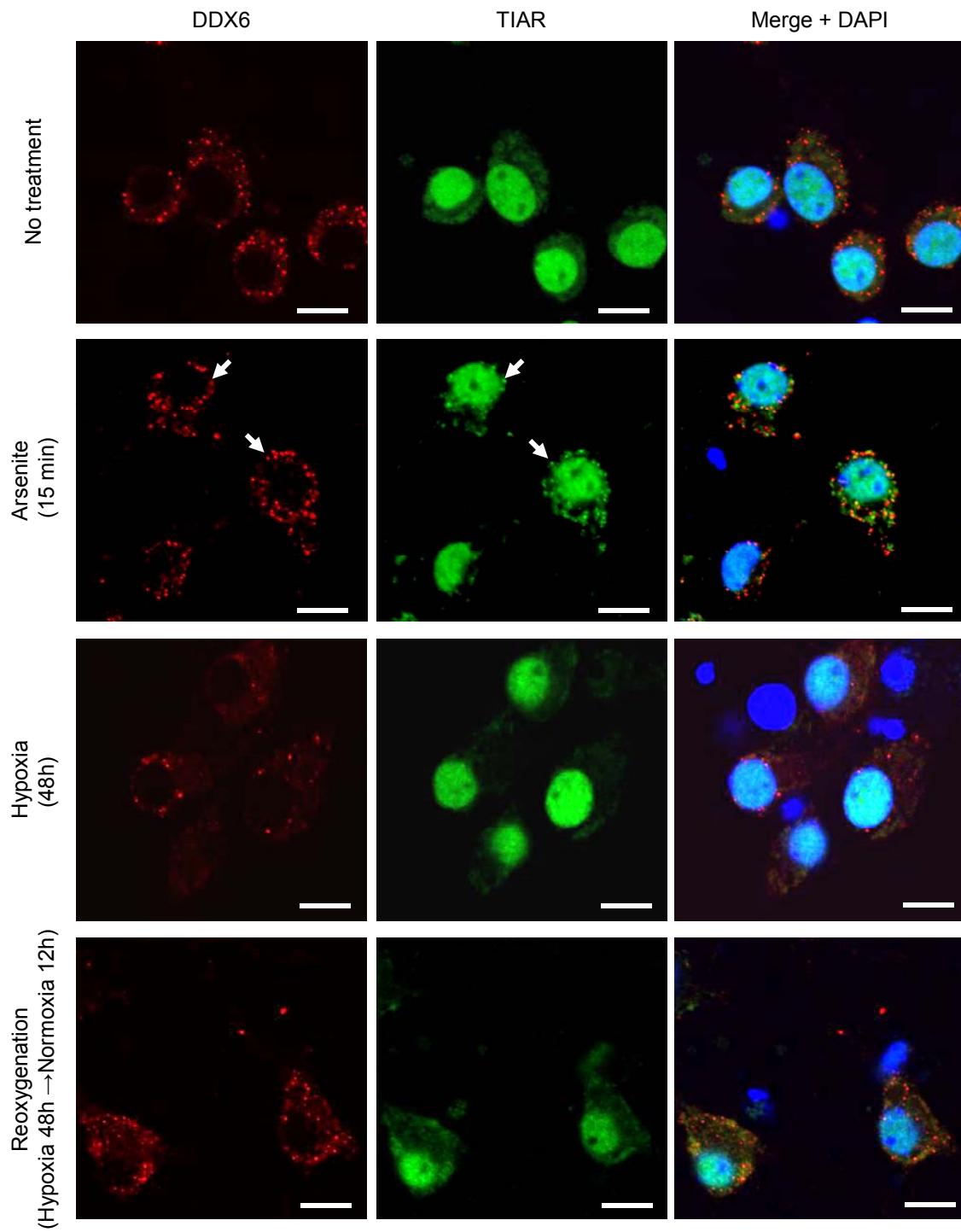
Supplementary Figure S6



Supplementary Figure S7



Supplementary Figure S8



Supplementary Figure S9

Supplementary Table S1. Prediction of miR-130a/b Target Genes in the Top 50

Database	Target Scan	PICTAR	miRANDA		miRbase	
	miR-130/301	miR-130/301	miR-130a	miR-130b	miR-130a	miR-130b
1	MIER1	RPS6KA5,variant1	EPB41L5	<u>DDX6</u>	c18orf1	RACGAP1
2	SLAIN1	MI-ER1	<u>DDX6</u>	<u>MLLT6</u>	RACGAP1	<u>PHF3</u>
3	FLJ45187	<u>FBXL11</u>	<u>RPS6KA5</u>	ACVR1	<u>PHF3</u>	MET
4	MYBL1	FLJ45187	<u>MLLT6</u>	CDK11	MET	<u>TNRC6A</u>
5	PAN3	EIF2C4	CDK11	<u>MEOX2</u>	<u>TNRC6A</u>	Jade1
6	<u>DDX6</u>	FLJ30046	TGFBR2	<u>RSN</u>	MARCH2	MARCH2
7	<u>CLIP1</u>	EIF2C1	POU3F2	SDC4	<u>EDG1</u>	<u>EDG1</u>
8	ENPP5	WDR47	<u>MEOX2</u>	EPS15	LMTK2	LMTK2
9	TSC1	ZNF238,variant 1	ACVR1	WNT1	FAM43A	FAM43A
10	ESR1	ZNF238,variant 2	<u>FBXL11</u>	LRIG1	<u>MEOX2</u>	<u>MEOX2</u>
11	LDLR	CDK11	NRD1	ABR	<u>MLLT6</u>	<u>MLLT6</u>
12	GDA	FLJ31818	EIF2C1	MTMR4	COX7A2L	MLLT10
13	FLJ20366	CFL2,variant 1	DNCL12	JARID2	<u>ST18</u>	COX7A2L
14	RAB5A	CFL2,variant 2	EPS15	JARID2	FRZB	<u>ST18</u>
15	GJA1	SYNJ1,variant 1	ZFPM2	TGFBR2	PLAA	PLAA
16	TRIM2	SFRS2IP	COL19A1	<u>ST18</u>	HAT1	NFIL3
17	PIGA	FBXO28	C21orf107	TSC1	RTCD1	HAT1
18	EREG	WDR20,variant 1	HOXA5	ACSL4	SACM1L	RTCD1
19	MECP2	DLL1	TGOLN2	<u>FBXL11</u>	RAB34	SACM1L
20	ADCY1	ARL10C	TXNIP	POU3F	CHST1	RAB34
21	FLJ31818	DKFZp566C0424	MLL	C10orf6	TMEM159	CHST1
22	GOLT1B	ZFYVE9,variant 3	CLN2	NRP1	CTSK	TMEM159
23	CDC2L6	ZFYVE9,variant 1	INHBB	ELAVL2	CEBPE	CTSK
24	ZNF217	<u>MEOX2</u>	ROBO1	TRPS1	ABHD3	ABHD3
25	ACSL4	ZFPM2	APG16L	EIF2C1	ATP6V1B2	ARL6IP
26	RNF38	<u>ST18</u>	<u>PHF3</u>	ADCY1	ARL6IP	KCTD13
27	PHF20	HABP4	C11orf15	ZFPM2	KCTD13	NEUROG1
28	CHST1	C9orf54	LRP8	MLL	UBE2D1	UBE2D1
29	ACVR1	USP6	<u>TNRC6</u>	RAB14	INOC1	INOC1
30	RAP2C	USP32	COX7A2L	SIAT8C	<u>RPS6KA5</u>	<u>RPS6KA5</u>
31	FBXO28	LOC220594	CD69	RBBP8	XAB1	RXFP2
32	WDR20	PLCB1,variant 1	<u>EDG1</u>	ARHGAP21	NUP107	NUP107
33	<u>ST18</u>	ROBO1,variant 2	TARDBP	CD69	SNAPAP	SNAPAP

(Continue Supplementary Table S1)

Rank No	Database	Target Scan	PICTAR	miRANDBA		miRbase	
	miR-130/301	miR-130/301	miR-130a	miR-130b	miR-130a	miR-130b	
34	TSHZ1	ROBO1,variant 1	NHLH2	TOMM34	NPFFR2	NPFFR2	
35	WDR47	SDFR1,variant β	RNF38	SATB2	OSTF1	OSTF1	
36	BRWD1	SDFR1,variant α	C14orf31	<u>TNRC6</u>	TEX261	PSAP	
37	EIF2C4	FLJ20366	GRM6	MYST2	PSAP	ST8SIA3	
38	CPEB1	GMRP-1	C14orf31	CFLAR	ST8SIA3	KIAA0196	
39	ZNF3	PLCB1,variant2	<u>RSN</u>	SNX27	SESTD1	SESTD1	
40	TBL1XR1	MTF1	SATB2	MTF1	RALGPS2	RALGPS2	
41	<u>FBXL11</u>	CGI-141	ESR1	HAS3	RAB9B	RAB9B	
42	ZFYVE9	ACVR1	BAHD1	ULK2	GADD45B	GADD45B	
43	DNAJC16	FLJ11011,variant1	PXK	LMTK2	OTUD4	SUGT1	
44	ZNF238	FLJ11011,variant2	OR1K1	NEUROG1	SGCB	LY6D	
45	TGFBR2	SMAD5,variant 1	QKI	E2F7	SUGT1	TRPC4	
46	SH3D19	SMAD5,variant 2	RAB34	PHF12	LY6D	FIP1L1	
47	DICER1	SMAD	ACBD3	C11orf8	CYP2U1	RWDD2	
48	FAM73A	FLJ11011,variant3	QKI	RPA2	TRPC4	ACVR1	
49	AAK1	SIRT7	PRKAA1	CSK	FIP1L1	NP_612361.1	
50	ERBB2IP	CDC14A,variant1	PPM1F	TGOLN2	RWDD2	GOLT1B	

Target genes of miR-130a and 130b were identified by TargetScan Release 3.0 (<http://www.targetscan.org/>), PICTAR (<http://pictar.mdc-berlin.de/>), miRanda Release 2005 (<http://www.microrna.org/>) and miRbase (<http://microrna.sanger.ac.uk/>). Under lines were shown as the commonly observed genes and highly ranked genes in four databases.

Supplementary Table S2. Target sequences of siRNAs

siRNA	Sequence (5' to 3')
DDX6	CCGAAATGGCTTATGCCGCAA
ST18	AAGACAAATCTTTAACAATAA
PHF3	ATGCATTATCTTCAACCTCAA
EDG1	CTCGGTCTCTGACTACGTCAA
TNRC6A	AAGAGCTTAACTCATCTTTAA
MLLT6	CAGGCTGTCTCAACAGCCTTA
RPS6KA5	AAGCCAGTCATTCGAGATGAA
RSN6	AACGATGAATTACGTCTGAAA
FBXL11	CCAAATAAGTTTCGCTATC

Supplementary Table S3. Oligonucleotide sequence

Cloning		Primer sequence (5' to 3')
DDX6/pcDNA3	Forward	AAGCTTCGCCACCATGAGCACGGCCAGAACAGAGAACCCTGTT
DDX6/pFLAG	Forward	CTCGAGCTATGAGCACGGCCAGAACAGAGAAC
	Reverse	GGATCCTTAAGGTTTCTCATCTTCTACAGGCTC
DDX6 3'UTR	Forward	GTCGACCAAGCATGCTTTGACAAATTACAA
	Reverse	GCGGCCGCGGCATTGCGTTCATCTCTTTACAGAAGAAC
HIF-1 α 3'UTR	Forward	CTCGAGGCGTTTCCTAATCTCATTCT
	Reverse	GCGGCCGCTGATATAACAAAACAGTACAGTTAGTGTTA
HIF-1 α IRES	Forward	CTCGAGCGCGAGGACTGTCCTCGCCGC
	Reverse	TGGATCCCGGGCGAATCGGTGCCCGCGTTG
DDX6 3'UTRmutation	Forward	CTTGTCTGAACGGTGTGCTGACTGAACATTAGCTGAACGAACTGC
DDX6 3'UTRmutation	Forward	TGTTCCACTTGTCTGAACGGTGTGCTGACTG
qRT-PCR		Primer sequence (5' to 3')
human Actin	Forward	CCTCATGAAGATCCTCACCGA
	Reverse	TTGCCAATGGTGTGATGACCTGG
5S rRNA	Forward	CATACCACCCTGAACGCGCCC
	Reverse	CCTACAGCACCCGGTATTCCC
VEGF	Forward	CCCTGATGAGATCGAGTACAT
	Reverse	CGGCTTGTCACATCTGCAAGT
Pri miR-130a	Forward	TCACTATTAGGTACAGAGTAG
	Reverse	CCTCAAGCAGCATTACCATCA
Pre miR-130a	Forward	GAGCTCTTTTACATTGTGCT
	Reverse	GATGCCCTTTTAAACATTGCAC
Mature miR-130a	Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCCCT
	Forward	GCCGCCAGTGCAATGTTAAA
	Reverse	GTGCAGGGTCCGAGGT
Mature miR-130b	Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCCCT TTCAT
	Forward	GCCGCCAGTGCAATGATGAA
	Reverse	GTGCAGGGTCCGAGGT
lin-4	Stem loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAC
	Forward	GCCCTCCCTGAGACCTCAA
	Reverse	GTGCAGGGTCCGAGGT

(Continue Supplementary Table S3)

Synthetic lin-4		UCCCUGAGACCUCAAGUGUGA
RT-PCR for mRNP		Primer sequence (5' to 3')
human HIF-1 α	Forward	ATGAGCCAGAAGAAGACTTTT
	Reverse	GTGGTTGAGAATTCTTGGTG
human GAPDH	Forward	AGCCACATCGCTCAGACAC
	Reverse	GCCCAATACGACCAAATCC
mouse HIF-1 α IRES	Forward	CTCGAGCGCGAGGACTGTCCTCGCCGC
	Reverse	TGGATCCCGGGCGAATCGGTGCCCGCGTTG
mouse HIF-1 α CDS	Forward	ATGAGCCGGAAGAAGACTTTTG
	Reverse	GTGGCTGGGAGTTCTTCGTA
mouse Actin	Forward	CCTCATGAAGATCCTGACCGA
	Reverse	TTGCCAATAGTGATGACCTGG

Supplementary Table S4. Northern and in situ probes

Probe	Sequence (5' to 3')
mature miR-130a LNA	ATgCCcTTTtAaCAtTGcACTg
mature miR-130a 2MM LNA	ATgCCaTTTtAaCAtTGtACTg
mature miR-130b LNA	ATGcCCtTTcAtCAtTGcACTg
Pri miR-130a sense	CACCACCACAACACAACCAGTTATTTCTTTGAGAAGTGTCAAATGATGGGA CTCCACAGGGCGGTCTCACTTGGCTCTGCACCACCACCCCTCAAGAAAAAG GTGATCTTTGCTGGGAAGGAAATGAGGACGAGGGACGAGAGGAAGGCCGTG ACGTGAGCTGAGTGTGGCCAGGGACTGGGAGAAAGGGTGAGGAGGCCGGC CGGCATGCCTTTGCTGCTGGCCGGAGCTCTTTTCACATTGTGCTACTGTCTAA CGTGTACCGAGCAGTGCAATGTTAAAAGGGCATCGGCCTTGTAGTACTACCCA GTGCCGGCAGCCTCCTCAGCATCACTGCATTTTCTCCACCTGAGCACCAGTC AGCTACTCTGCTGGCAATTTGGATATATGGAGCCCTCCCTTAGATGAGGAAGCTA AGGCTGTAGAGCTCCTAAGTTAACTGCTCTATCCTGAGCCCAACAGGAAGGCAC CCATTTTTGTAGACCTGTCCAGAGAGATTTGAACTGAGGGGGTGAATGGT
Pri-miR-130a antisense	Complement sequence of Pri miR-130a sense
DIG labeled mouse HIF-1 α sense probe	CCTCAAGAAACGACCACTGCTAAGGCATCAGCATAACAGTGGCACTCACAGTCGGA CAGCCTCACCAGACAGAGCAGGAAAGAGAGTCATAGAACAGACAGACAAAGCTCA TCCAAGGAGCCTTAACCTGTCTGCCACTTTGAATCAAAGAAATACTGTTCTGAG GAAGAATTAAACCCAAAGACAATAGCTTCGCAGAATGCTCAGAGGAAGCGAAAAA TGGAACATGATGGCTCCCTTTTTCAAGCAGCAGGAATTGGAACATTATTGCAGCA ACCAGGTGACTGTGCACCTACTATGTCACCTTCTGGAAACGAGTGAAAGGATTC ATATCTAGTGAACAGAATGGAACGGAGCAAAAGACTATTATTTTAATACCCTCCG ATTTAGCATGCAGACTGCTGGGGCAGTCAATGGATGAGAGTGGATTACCACAGCT GACCAGTTACGATTGTGAAGTTAATGCTCCCATACAAGGCAGCAG
DIG labeled mouse HIF-1 α antisense probe	Complement sequence of HIF-1 α sense

Locked nucleic acids (LNA) were indicated by lower case in mature miR-130a, miR-130a 2MM and 130b LNA sequence.