MicroRNA-125b transforms myeloid cell lines by repressing multiple mRNA

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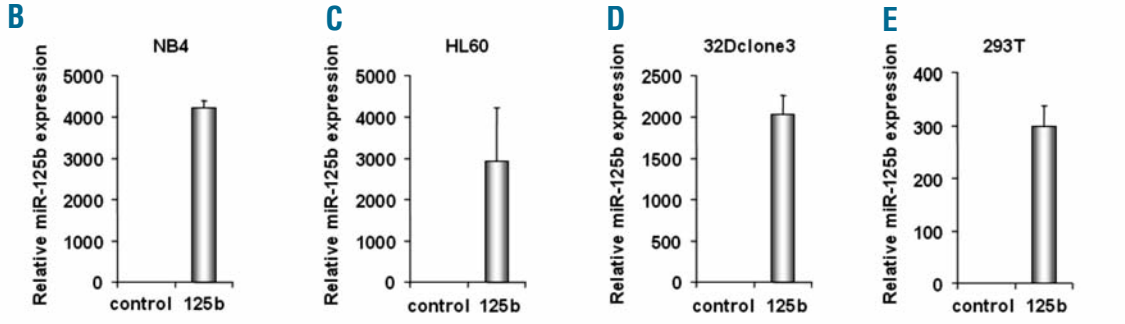
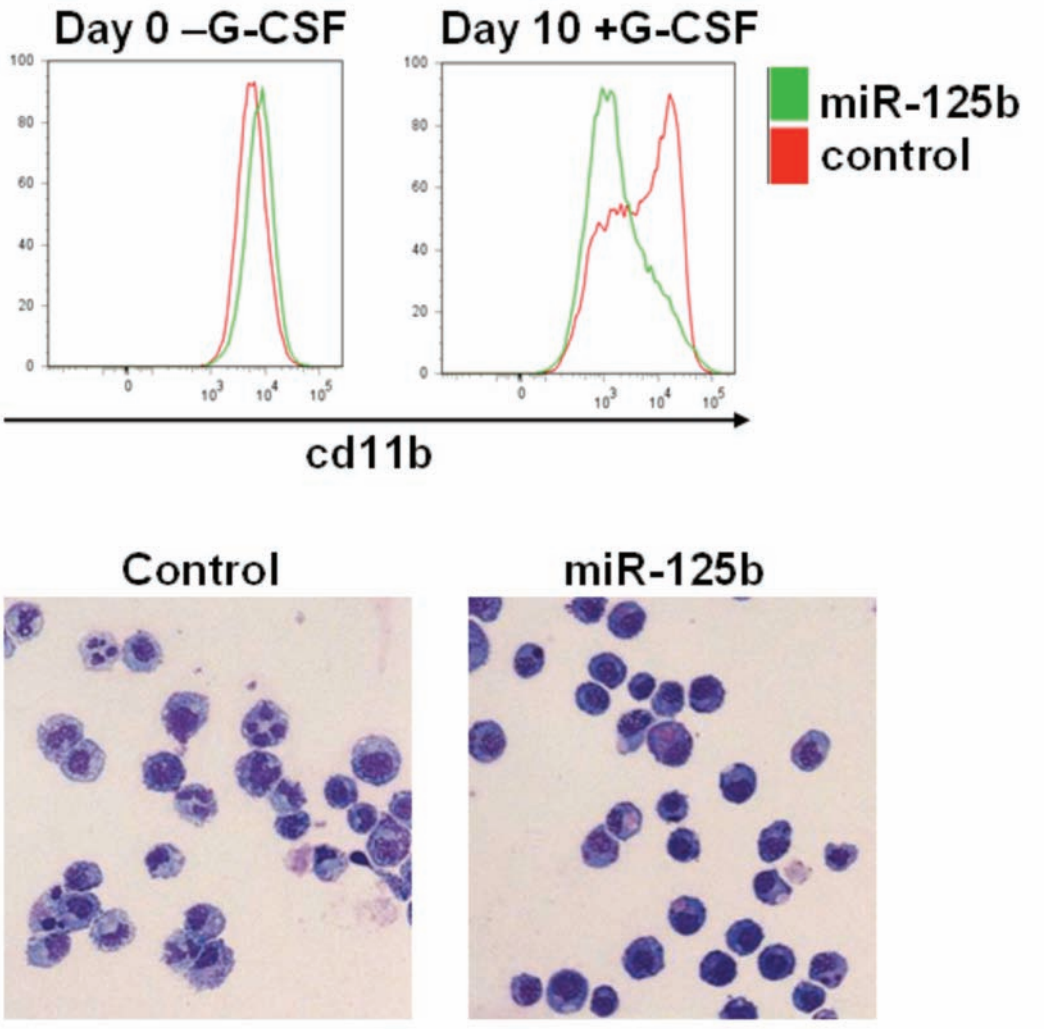
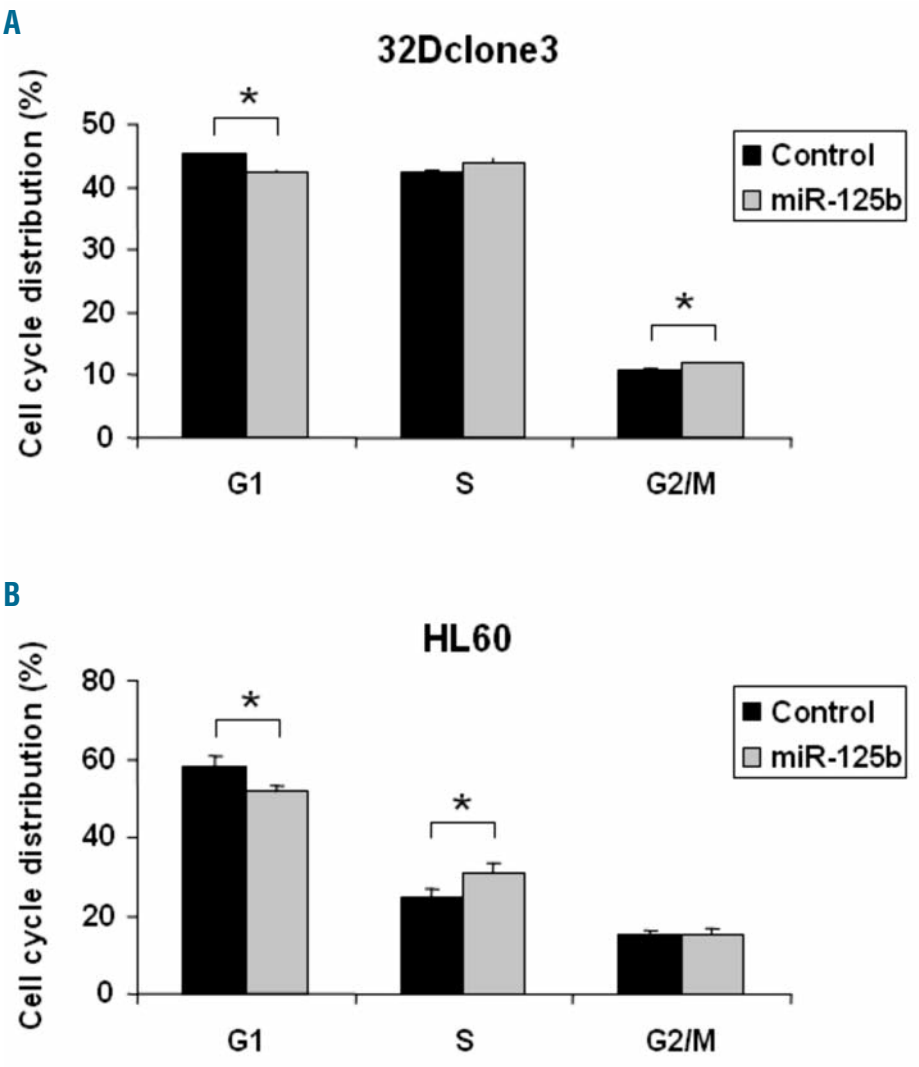
Myeloid disorders	t(2;11)(p21;q23) AML and MDS	t(15;17)(q22;q21) APL	t(15;17)(q22;q21) APL	t(9;22)(q34;q11) CML	Trisomy 21 AMKL	AML1 amplification AML
miR-125b overexpression	6 to 90 fold	average 20 fold	average 760 fold	4 to 70 fold	average 26.4 fold	450 fold
References	Bousquet *et al.* (3)	Jongen-Lavrencic *et al.* (23)	Zhang *et al.* (6)	Enomoto *et al.* (7)	Klusmann *et al.* (4)	Bousquet *et al.* (3)



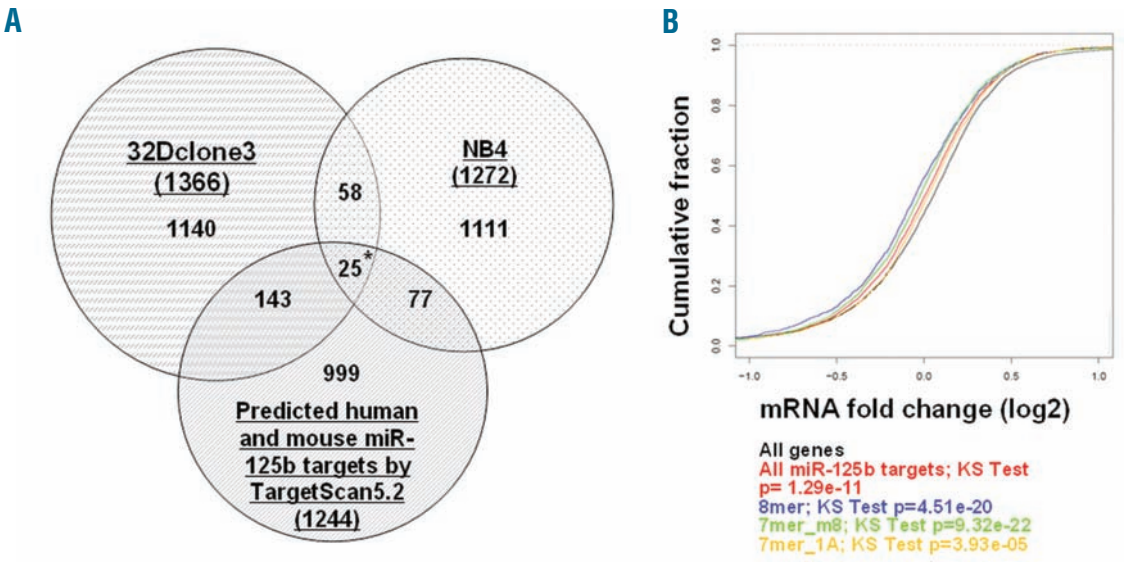
Online Supplementary Figure S1. (A) miR-125b expression levels. The table summarizes the different levels of miR-125b overexpression found in patients with myeloid leukemia reported in the literature. Of note, miR-125b level was evaluated on total bone marrow samples. AML, acute myeloid leukemia; MDS, myelodysplasia; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; AMKL, acute megakaryoblastic leukemia. (B) Relative miR-125b expression in NB4 cells transiently transfected with miR-125b mimic compared to NB4 cells transiently transfected with control mimic evaluated by quantitative reverse transcriptase-PCR. (C) Relative miR-125b expression in miR-125b infected HL60 cells compared to control cells evaluated by quantitative reverse transcriptase-PCR. (D) Relative miR-125b expression in miR-125b infected 32Dclone3 cells compared to control cells evaluated by quantitative reverse transcriptase-PCR. (E) Relative miR-125b expression in 293T cells transiently transfected with miR-125b mimic compared to 293T cells transiently transfected with control mimic evaluated by quantitative reverse transcriptase-PCR.



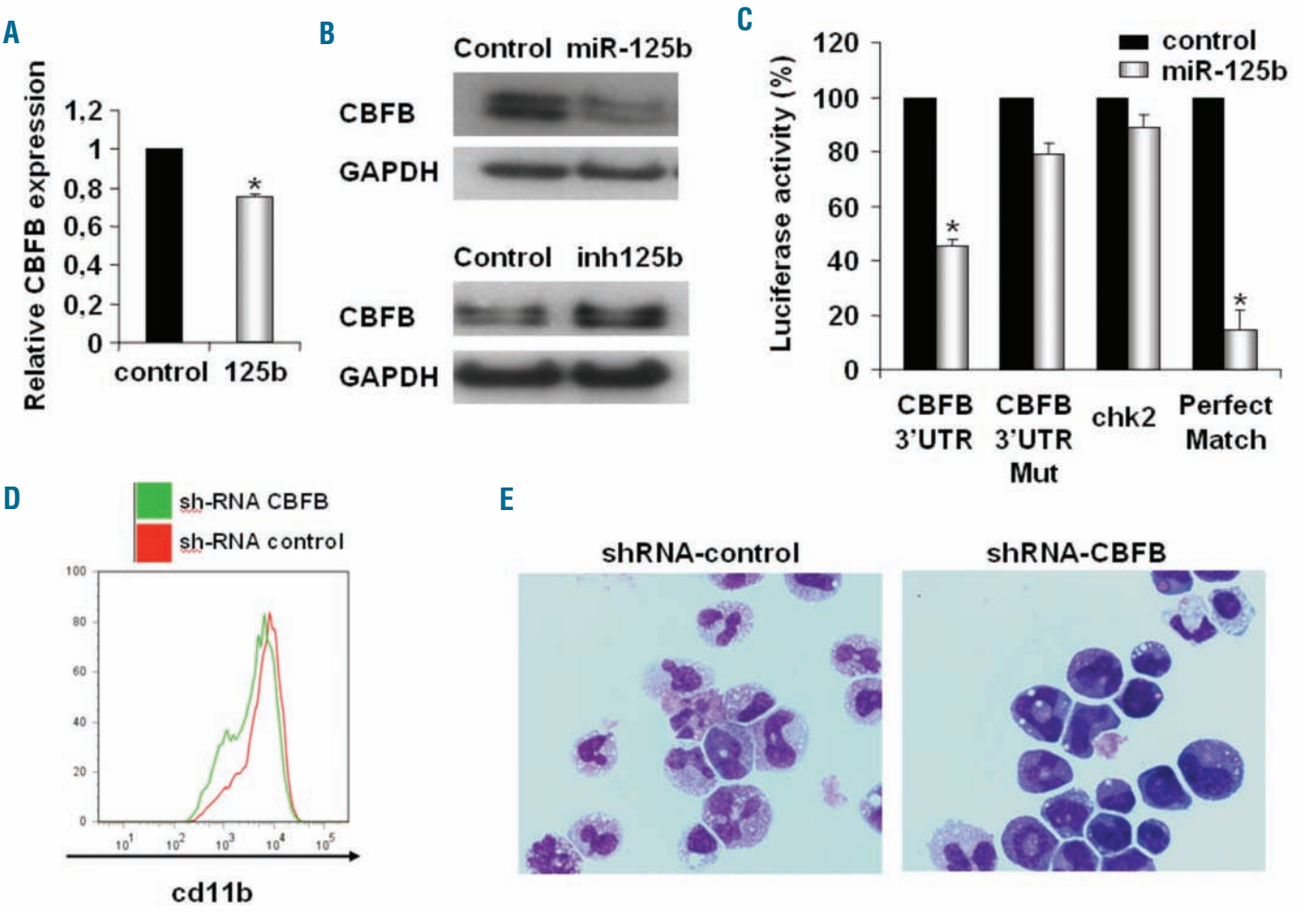
Online Supplementary Figure S2. miR-125b blocks granulocytic differentiation of mouse 32Dclone3 cell line (A). 32Dclone3 cells were stably infected with XZ vector (control) or XZ-miR-125b vector (miR-125b) and sorted for GFP$^+$ cells. These cells were then induced towards granulocytic differentiation by adding granulocyte-colony stimulating factor and differentiation was assessed on day 7 post-induction by flow cytometry after staining with the myeloid marker CD11b. (B). Morphological analysis of May Grünwald Giemsa stained cells. A representative experiment is shown.



Online Supplementary Figure S3. miR-125b over-expression enhances proliferation. (A) Cell cycle analysis of miR-125b infected 32Dclone3 cells compared to control cells. The histogram represents the mean of the percent of cells in G1, S or G2/M phases from three independent experiments. *P<0.05 (B) Cell cycle analysis of miR-125b-infected HL60 cells compared to control cells. The histogram represents the mean of the percent of cells in G1, S or G2/M phases from three independent experiments. *P<0.05.



Online Supplementary Figure S4. Global down-regulation of miR-125b predicted targets. (A) Venn diagram showing the overlap between the genes down-regulated in 32Dclone3 and NB4 cells over-expressing miR-125b and predicted miR-125b targets. Predicted miR-125b targets were obtained from TargetScan5.2 software for both species. A total of 2396 genes, in mice and in 2964 in humans, irrespective of site conservation, are putative miR-125b targets; 1244 genes are predicted targets in both mice and humans. For each cell line, genes down-regulated more than 1.15-fold in duplicate RNA-sequencing experiments were selected. For 32Dclone3 cells infected with XZ-miR-125b, 1366 genes out of 10417 expressed genes were down-regulated more than 1.15-fold compared to control cells infected with XZ in duplicate RNA-sequencing experiments. For NB4 cells transiently transfected with miR-125b mimics, 1272 genes out of 10772 expressed genes were down-regulated more than 1.15-fold compared to control cells transiently transfected with control mimics. Eighty-three genes were down-egulated in both 32Dclone3 and NB4 cells over-expressing miR-125b. Among these, 25 genes are predicted miR-125b targets. Hypergeometric analysis showed that there is an enrichment in predicted miR-125b targets among the genes down-regulated in both cell lines (*P=5.10^{-6}). (B) Global down-regulation of miR-125b targets in 32Dclone3 cells over-expressing miR-125b. mRNA from 32Dclone3 cells infected with XZ-miR-125b or XZ (control) virus was analyzed by mRNA sequencing.The relative expression of each gene, calculated as a $\log_2$ ratio is shown on the x asis. The cumulative fraction (y axis) was plotted as a function of the relative expression (x axis). Levels of mRNA bearing different predicted miR-125b binding sites (8-mer, 7mer-m8, and 7mer-1A; for definitions, see *http://www.targetscans. org*) as defined by TargetScan were compared to mRNA that did not bear seed matches (black line). All of the colored 'target' curves are shifted to the left with P values <0.05 as determined by the one-sided Kolmogorov–Smirnov test, indicating a preferential down-regulation of predicted miR-125b targets in the presence of miR-125b.



Online Supplementary Figure S5. *CBFB* is a miR-125b target in murine cells (A) Quantitative reverse transcriptase-PCR of *CBFB* mRNA in 32Dclone3 over-expressing miR-125b compared to 32Dclone3 control cells. **P*<0.0005. (B) Western blot showing the down-regulation of CBFB protein in miR-125b overexpressing cells compared to control cells (upper panel). *CBFB* is increased in 32Dclone3 cells transiently transfected with an inhibitor of miR-125b (lower panel). (C) Repression of luciferase activity due to the binding of miR-125b to the 3'UTR of *CBFB*. The 3'UTR of *CBFB* containing the predicted binding site for miR-125b was cloned following the *renilla* luciferase open reading frame in the psicheck2 vector. *CBFB* 3'UTR mut corresponds to the same construct with an internal mutation in the binding site for miR-125b. chek2 is the empty vector and it serves as a negative control. The perfect match construct is the positive control that contains only the miR-125b binding site. Each construct was co-transfected in 293T cells with miR-125b or control mimics and luciferase activity was assessed 2 days after transfection. Renilla activity was normalized to the firefly internal psicheck control. The figure shows the relative luciferase activity normalized to transfections with control mimics. Three independent experiments were performed. **P*<0.0005. (D) 32Dclone3 cells were transiently transfected with constructs coding for sh-RNA against *CBFB* or control sh-RNA and selected with antibiotic for 2 weeks. Differentiation ability was assessed by flow cytometry analysis 7 days after induction of differentiation with granulocyte colony-stimulating factor. (E) Corresponding May Grünwald Giemsa staining showing a partial blockage of differentiation in cells expressing an sh-RNA against *CBFB*. Results of a representative experiment of three are shown.

Online Supplementary Table S1. Primers.

Primer Names	Sequence 5'-3'
Q-PCR	
Cbfb F (mouse)	TTAGAGAGAGAAGCAGGCAAGG
Cbfb R (mouse)	GTGCTAATGCATCTTCCTGCTG
Abtb1 F (mouse)	ATCTGAGCTGCGGGGTGACC
Abtb1 R (mouse)	TGCGGCCACAGAAGAAAGCCT
Bak1 F (mouse)	CTGGACAAGGACCAGGTCCC
Bak1 R (mouse)	TAGCTTCGAAAGACCTCCTCTG
Plk3 F (mouse)	GCATCAGCGCGAGAAGATCCT
Plk3 R (mouse)	TTTCGGCTACAGAGCTCCAGG
Prkra F (mouse)	GACAGCGGGACCTTCAGTTTG
Prkra R (mouse)	CGTGTACTTGCACATCGGATCT
Ppp1ca F (mouse)	CATCTGCAGAGCACATCAGGTT
Ppp1ca R (mouse)	CATCATGGCACCAGCATTGTCA
Ppp2ca F (mouse)	ACCAGCTGGTGATGGAGGGAT
Ppp2ca R (mouse)	TTGCAGCTTGGTTACCACAACG
Tp53inp1 F (mouse)	AACTCAAGTGGTCCCAGAATGG
Tp53inp1 R (mouse)	GGGCGAAAACTCTTGGGTTGTT
Gapdh F (mouse)	AATGTGTCCGTCGTGGATCT
Gapdh R (mouse)	GGTCCTCAGTGTAGCCCAAG
Hprt F (mouse)	TCAGTCAACGGGGGACATAAA
Hprt R (mouse)	GGGGCTGTACTGCTTAACCAG
BAK1 F (human)	GTACTTCACCAAGATTGCCACC
BAK1 R (human)	CATGCTGGTAGACGTGATGGG
PLK3 F (human)	GCATCAGCGCGAGAAGATCCT
PLK3 R (human)	TTCCAGATGTGGGCCAGGGA
PRKRA F (human)	ACCTGCACAGGTGAAGGTACAA
PRKRA R (human)	CATTAAGGGGTCAGGAACTGCAA
PPP1CA F (human)	GTGCCAGCATCAACCGCATCT
PPP1CA R (human)	GCAGGCAGTTGAAGCAGTCAG
PPP2CA F (human)	GGTGCCATGACCGGAATGTAG
PPP2CA R (human)	GAGGTGCTGGGTCAAACTGCA
TP53INP1 F (human)	CCACCCGTGGGACTGATGAAT
TP53INP1 R (human)	GAGCAGCAAGAGCTGCAACATA
MLN51 F (human)	TAATCCCAGTTACCCTTATGCTCCA
MLN51 R (human)	GTTATAGTAGGTCACTCCTCCATATACCTGT
ACTIN F (human)	TCCCTGGAGAAGAGCTACGA
ACTIN R (human)	AGGAAGGAAGGGTGGAAGAG
Cloning	
CBFB 3'UTR F	taaCTCGAGCTGATAGTCACCCTATCCCCTTTA
CBFB 3'UTR R	ataGCGGCCGCCCGGAATCAATGGGAGAAATGCAT
ABTB1 3'UTR F	taaCTCGAGCACGCAGCAAGATGGCATTACAAT
ABTB1 3'UTR R	ataGCGGCCGCTGTATGCGGAAATAGCCGAAGCAA
Mutagenesis	
CBFBmut F	GCCACAGACTGGCTTCTGTTTTATTCAtcGATTTTTTTTTTAATCAGTCAG
CBFBmut R	CTGACTGATTAAAAAAAAAATCgaTGAATAAAACAGAAGCCAGTCTGTGGC
ABTB1mutF	GATTTGGGGCAGGCACCACTCAtcGAAAACTGGGGTACGG
ABTB1mutR	CCGTACCCCAGTTTTCgaTGAGTGGTGCCTGCCCCAAATC

Online Supplementary Table S2. Twenty-five predicted genes (Targetscan5.2 human and mouse) commonly down-regulated more than 1.15-fold in 32Dclone3 and NB4 over-expressing miR-125b as determined by RNA sequencing.

Gene name		32Dclone3 fold change (control *vs.* miR-125b)	NB4 fold change (control *vs.*) miR-125b	Predicted binding site (Targetscan 5.2)
ABTB1	ankyrin repeat and BTB (POZ) domain containing 1	3.89	1.47	1 concerved 8mer
ACOX3		1.42	1.18	1 non-conserved 7mer-m8
AP4E1	adaptor-related protein complex 4, epsilon 1 subunit	1.47	1.5	1 non-conserved 7mer-m8
ARRDC4	arrestin domain containing 4	1.97	1.66	1 non-conserved 7mer-1A
BTG2	BTG family, member 2	1.94	1.72	1 non-conserved 7mer-m8
CBFB	core-binding factor, beta subunit	1.33	1.65	1 non-conserved 7mer-1A
CCNJ	cyclin J	1.92	1.47	1 conserved 8mer
CDC42SE1	CDC42 small effector 1	2.02	1.4	1 conserved 8mer 1 conserved 7mer-m8
CYB561D1	cytochrome b-561 domain containing 1	2.15	1.35	1 non-conserved 8mer 1 non-conserved 7mer-m8
DUSP5	dual specificity phosphatase 5	1.74	1.77	1 non-conserved 7mer-1A
DUSP6	dual specificity phosphatase 6	1.69	1.41	1 conserved 7mer-1A
EAF1	ELL associated factor 1	1.50	1.20	1 conserved 8 mer 1 non-conserved 7mer-m8
FAM116A	family with sequence similarity 116, member A	1.79	1.38	1 conserved 7mer-m8 1 non-conserved 7mer-m8
JHDM1D	jumonji C domain containing histone demethylase 1 homolog D (S. cerevisiae)	1.9	1.52	1 non conserved 7mer-1A
LACTB	lactamase, beta	1.79	1.76	1 conserved 8mer
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	1.46	1.44	1 conserved 8mer
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	3.01	2.51	1 conserved 8mer
PCTP	phosphatidylcholine transfer protein	2.63	2.88	1 conserved 7mer-m8
PTPRJ	protein tyrosine phosphatase, receptor type, J	2.1	1.38	1 non-conserved 7mer-1A
SGPL1	Sphingosine-1-phosphate lyase 1	1.30	1.30	1 conserved 8mer 1 non-conserved 8mer
SH2B3	SH2B adaptor protein 3	1.62	1.23	1 conserved 7mer-1A
TMEM123	transmembrane protein 123	2.33	1.34	1 conserved 7mer-m8
USP12	Ubiquitin specific peptidase 12	1.65	1.93	1 non-conserved 7mer-m8
VPS4B	Vacuolar protein sorting 4 homolog B	1.75	1.27	1 conserved 8mer 1 non-conserved 7mer-1A
ZSCAN29	Zinc finger and SCAN domain containing 29	1.46	1.28	1 conserved 8mer