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Supplemental Information

miR-125b Upregulates miR-34a and Sequentially Activates Stress Adaption and Cell Death Mechanisms in Multiple Myeloma

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Table S1. Mutational analysis of MM cells using Ion Reporter Browser.

A

Genes	Locus	Genotype	Type	Location	Amino acid change	Coding	Exon	p-value	Variant effect	Allele Source
MET	chr7:116411990	C/T	SNV	exonic	p.Thr1010Ile	c.3029C>T	14	0.00001	missense	Hotspot
BRAF	chr7:140453132	T/A	SNV	exonic	p.Lys601Asn	c.1803A>T	15	0.00001	missense	Hotspot
TP53	chr17:7578448	G/G	INDEL	exonic	p.Ala161fs	c.481delG	5	0.00001	frameshift deletion	Novel
TP53	chr17:7578449	T/T	SNV	exonic	p.Ala161Thr	c.481G>A	5	0.00001	missense	Hotspot

B

Genes	Locus	Genotype	Type	Location	Amino acid change	Coding	Exon	p-value	Variant effect	Allele Source
CSDE1	chr1:115258747	CC/TC	SNV	Exonic, downstream	p.Gly12Asp	c.35G>A	2	0.00001	missense	Hotspot
PTEN	chr10:89685268	A/C	SNV	splicesite_5			3	0.00001		Hotspot
TP53	chr17:7578368	CACCATCGCTATCTGAGCAGCGCTCATGGTGGGGGCAGCGCCTCACA/CACCATCGCTATCTGAGCAGCGCTCATGGTGGGGGCAGCCCTCACA	INDEL	splicesite_3			5	0.00001		Novel
TP53	chr17:7578407	GC/CC	SNV	exonic	p.Arg175Gly	c.523C>G	5	0.00001	missense	Hotspot
TP53	chr17:7579470	CGG/CGC	SNV	exonic	p.Pro72Arg	c.215C>G	4	0.00001	missense	Novel

C

Genes	Locus	Genotype	Type	Location	Amino acid change	Coding	Exon	p-value	Variant effect	Allele Source
ERBB4	chr2:212589802	A/C	SNV	exonic	p.Phe247Cys	c.740T>G	6	0.00001	missense	Novel
PIK3CA	chr3:178936091	G/A	SNV	exonic	p.Glu545Lys	c.1633G>A	10	0.00001	missense	Hotspot
EGFR	chr7:55242482	CATCTCCGAAAGCCAACAAGGAAAT/TATCTCCGAAAGCCAACAAGGAAAT	SNV	exonic	p.Thr751Ile	c.2252C>T	19	0.00001	missense	Hotspot
KRAS	chr12:25398284		SNV	exonic	p.Gly12Ala	c.35G>C	2	0.00001	missense	Hotspot
TP53	chr17:7577085		SNV	exonic	p.Glu285Lys	c.853G>A	8	0.00001	missense	Hotspot

A: U266 cell line; B: SKMM-1 cell line; C: RPMI 8226 cell line; SNV: single nucleotide variants; INDEL: deletion; Variant effect : effect of the variant on the coding sequence; Hotspot: variant overlaps a hotspot file; Novel: not known variant.

Table S2. TP53 variants analysis.

	cDNA description	Protein description	Exon number	Effect	TA Class
U266	c.481G>A	p.A161T	5-exon	missense	Partially functional
U266	c.481delG	p.A161fs	5-exon	Frameshift	NA
SKMM-1	c.215C>G	p.P72R	4-exon	missense	NA
SKMM-1	c.523C>G	p.R175G	5-exon	missense	Non-functional
RPMI 8226	c.853G>A	p.E285K	8-exon	missense	Non-functional

TA Class: transactivation activities; NA: Not Available

Supplementary Figure legends

Figure S1. Evaluation of the transfection efficiency. Flow cytometric analysis of transfection efficiency in MM cell lines (A) as fluorescence intensity. B) Analysis, by qPCR, of miR-125b expression levels in MM cell lines transfected with miR125b. Values are mean ± standard deviation (SD), n = 3.

Figure S2. Cell viability modulation after miR-125b mimic and its modified analogs transfection. (A-C) MTS assay of MM cell lines transfected with 100 nM of miR-125b, miR-NC, miR-125b-Omet (Omet), miR-125b-LNA (LNA) or miR-125b-2'F (F). MM cell lines U266 (A), SKMM-1 (B) and RPMI 8226 (C) were analyzed after 24h and 48h from transfection. CTR = untreated cells used as control. Each experiment was repeated at least three times and the data are shown as a mean ± standard deviation (SD).

Figure S3. Evaluation of the transfection efficiency. Analysis, by qPCR, of miR-125b expression levels in U266 cells 48h after transfection with miR125b or its modified analogues. Values are mean ± standard deviation (SD), n = 3.

Figure S4. Densitometric analysis of Western Blot from Figure 3C/D. U266 cells transfected with 100 nM of miR-125b, miR-NC, miR-125b-Omet (Omet), miR-125b-LNA (LNA) or miR-125b-2'F (F) were collected for Western blotting analysis after 24h and 48 h from transfection. Densitometric analysis of IL6-R (A), cRaf (B), Akt (C), Bcl-2 (D), Mcl1 (E), STAT3 (F), pSTAT3 (G), EIF5A (H), 4EBP1 (I), p4EBP1 (L) and p53 (M) bands was performed with ImageJ analysis tool. Each column reflects the relative amount of the protein as a ratio of each protein band relative to the corresponding loading control. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ compared to miR-NC group.

Figure S5. Apoptosis evaluation by FACS analysis. U266 cell line was transfected with miR-125b, miR-NC or miR-125b-Omet at a final concentration of 100 nM for 24h (A) and 48h (B). After double staining with PI and Annessin V, as described in “Materials and Methods”, cells were analyzed by flow cytometric analysis. The data are representative of three different experiments that have always yielded similar results. Stacked bar histogram represents the percentage of cells in the four quadrants.

Figure S6. Flow cytometric analysis of autophagy. U266 cell line was transfected with miR-125b, miR-NC, miR-125b-Omet (Omet), miR-125b-LNA (LNA) or miR-125b-2'F (F) at a final concentration of 100 nM for 48h. After monodansylcadaverine (MDC) staining, cells were analysed on the BD Accuri C6™ flow cytometer. B and D) Replicates of the histograms reported in Fig. 4C of the main manuscript. A, C and E) Representative gating strategy of the three replicates.

Figure S7. Flow cytometric analysis of autophagy. U266 cell line was transfected with miR-125b, miR-NC, miR-125b-Omet (Omet), miR-125b-LNA (LNA) or miR-125b-2'F (F) at a final concentration of 100 nM for 72h. After monodansylcadaverine (MDC) staining cells were analysed on the BD Accuri C6™ flow cytometer. The histogram shows the relative intensity fluorescence intensity (MFI) with respect to control sample. The results are shown as means ± standard deviation (SD) for three independent experiments.

Figure S8. Analysis of senescence. Senescence-associated β-galactosidase assay. U266 cells were transfected with 100 nM of miR-125b, miR-NC, miR-125b-Omet (Omet), miR-125b-LNA (LNA) or miR-125b-2'F (F). After 72h from transfection the cells were incubated with staining solution as described in “Materials and Methods” and counted with a light microscope. The histogram shows the percent of blue cells. The results are shown as means ± standard deviation (SD) for three independent experiments.

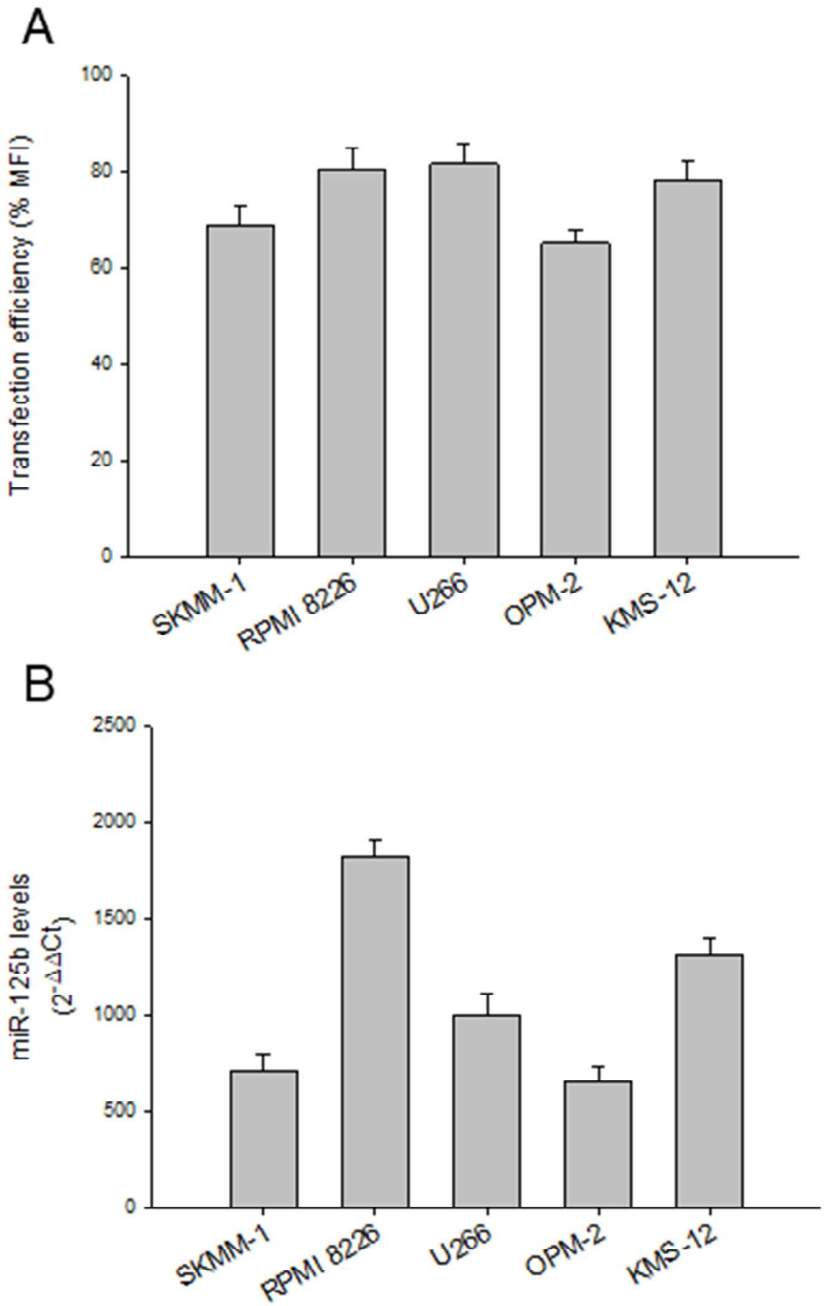
A
Transfection efficiency (% MFI)
100
80
60
40
20
0
SKMM-1
RPMI 8226
U266
OPM-2
KMS-12
B
miR-125b levels
(2^-ΔΔCt)
2500
2000
1500
1000
500
0
SKMM-1
RPMI 8226
U266
OPM-2
KMS-12

Figure S1

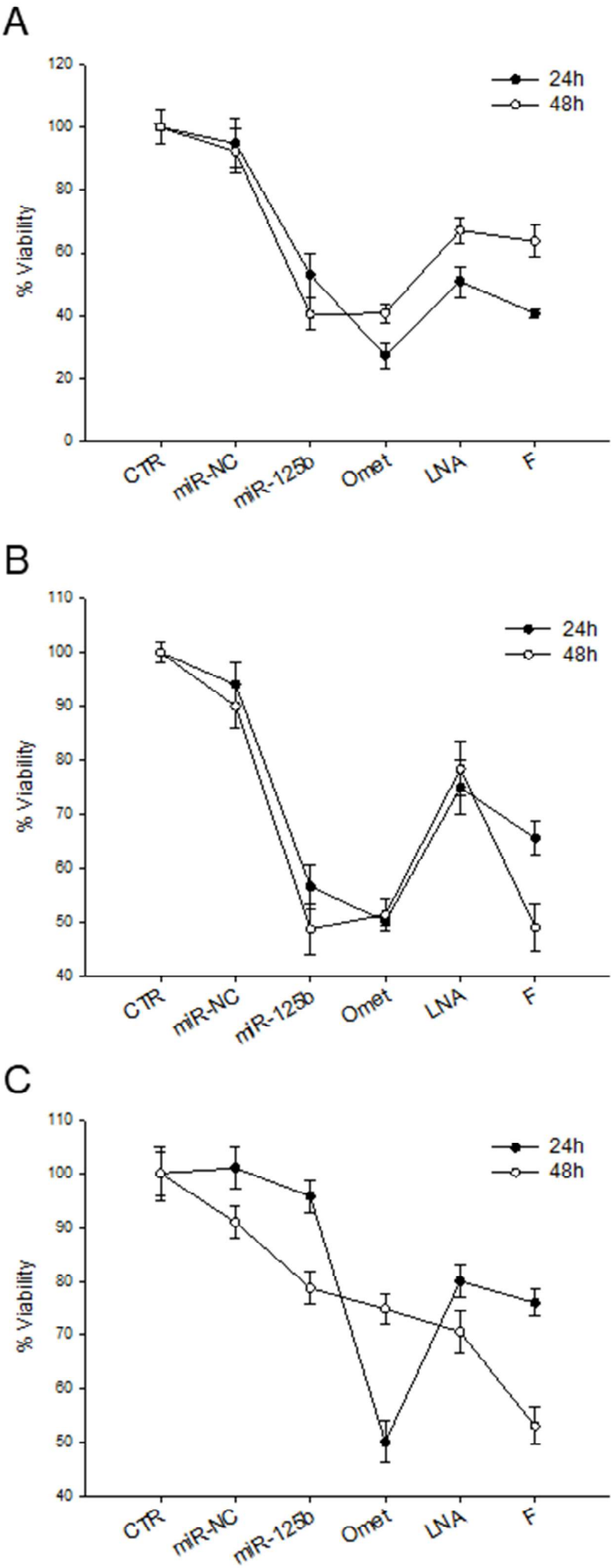
A
120
100
80
60
40
20
0
% Viability
24h
48h
CTR
miR-NC
miR-125b
Omet
LNA
F
B
110
100
90
80
70
60
50
40
% Viability
24h
48h
CTR
miR-NC
miR-125b
Omet
LNA
F
C
110
100
90
80
70
60
50
40
% Viability
24h
48h
CTR
miR-NC
miR-125b
Omet
LNA
F

Figure S2

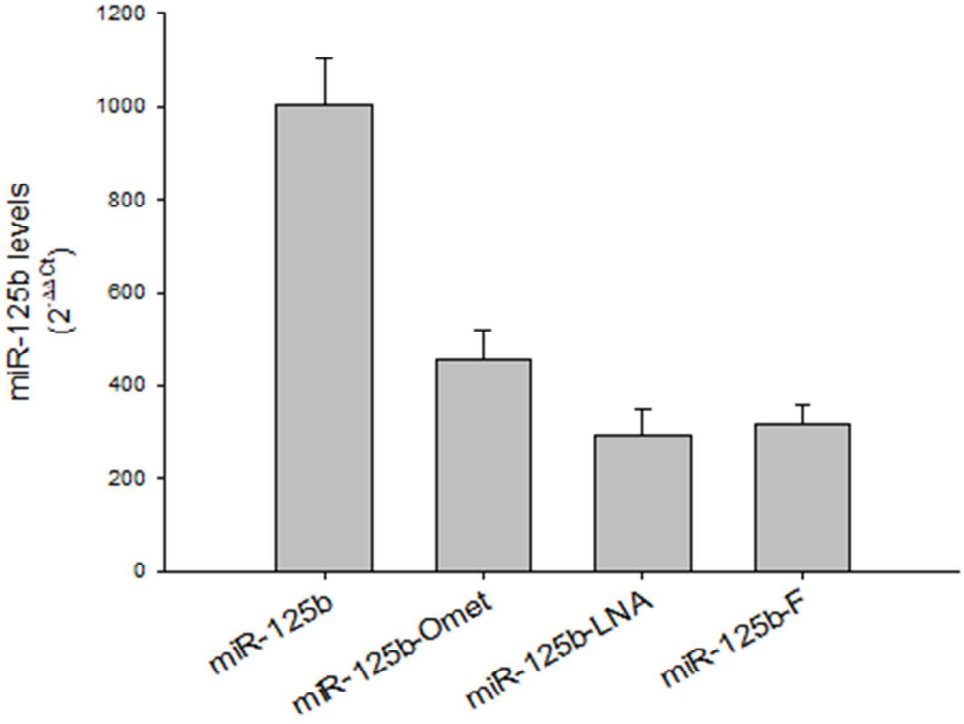
miR-125b levels
(2-ΔΔCt)
1200
1000
800
600
400
200
0
miR-125b
miR-125b-Omet
miR-125b-LNA
miR-125b-F

Figure S3

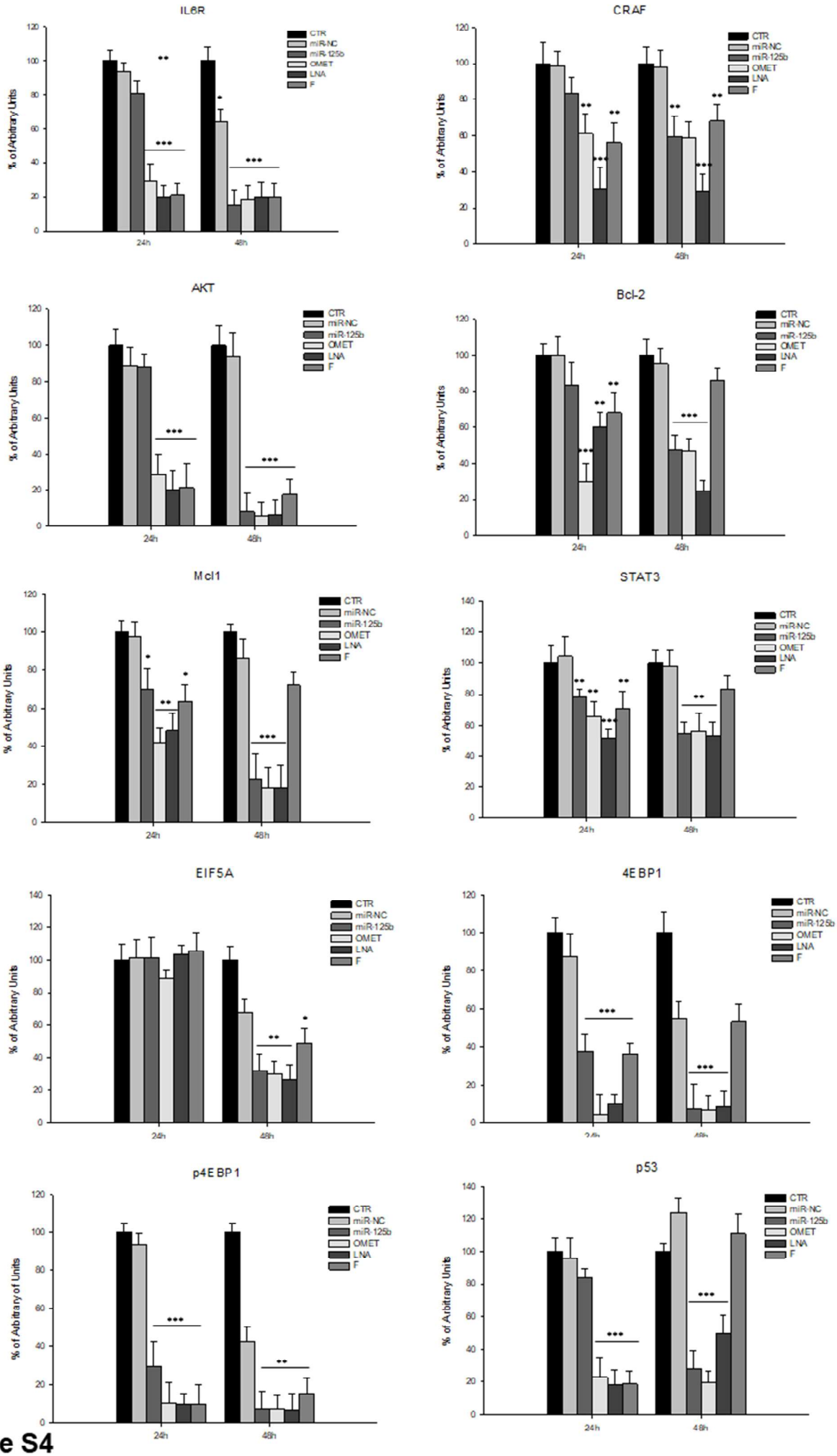
IL6R
CRAF
AKT
Bcl-2
Mcl1
STAT3
EIF5A
4EBP1
p4EBP1
p53
% of Arbitrary Units
CTR
miR-NC
miR-125b
OMET
LNA
F
24h
48h

Figure S4

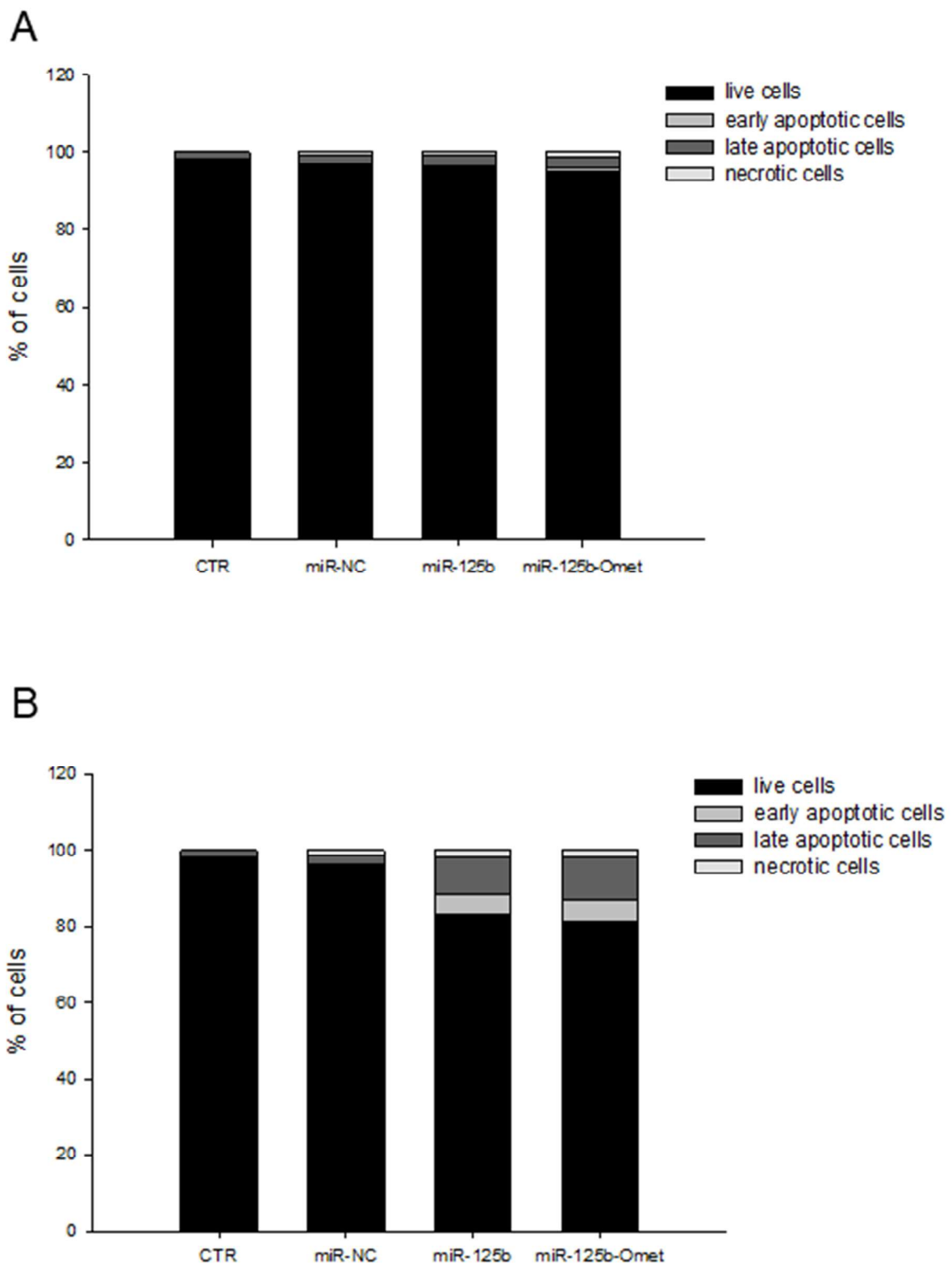
A
120
100
80
60
40
20
0
% of cells
live cells
early apoptotic cells
late apoptotic cells
necrotic cells
CTR
miR-NC
miR-125b
miR-125b-Omet
B
120
100
80
60
40
20
0
% of cells
live cells
early apoptotic cells
late apoptotic cells
necrotic cells
CTR
miR-NC
miR-125b
miR-125b-Omet

Figure S5

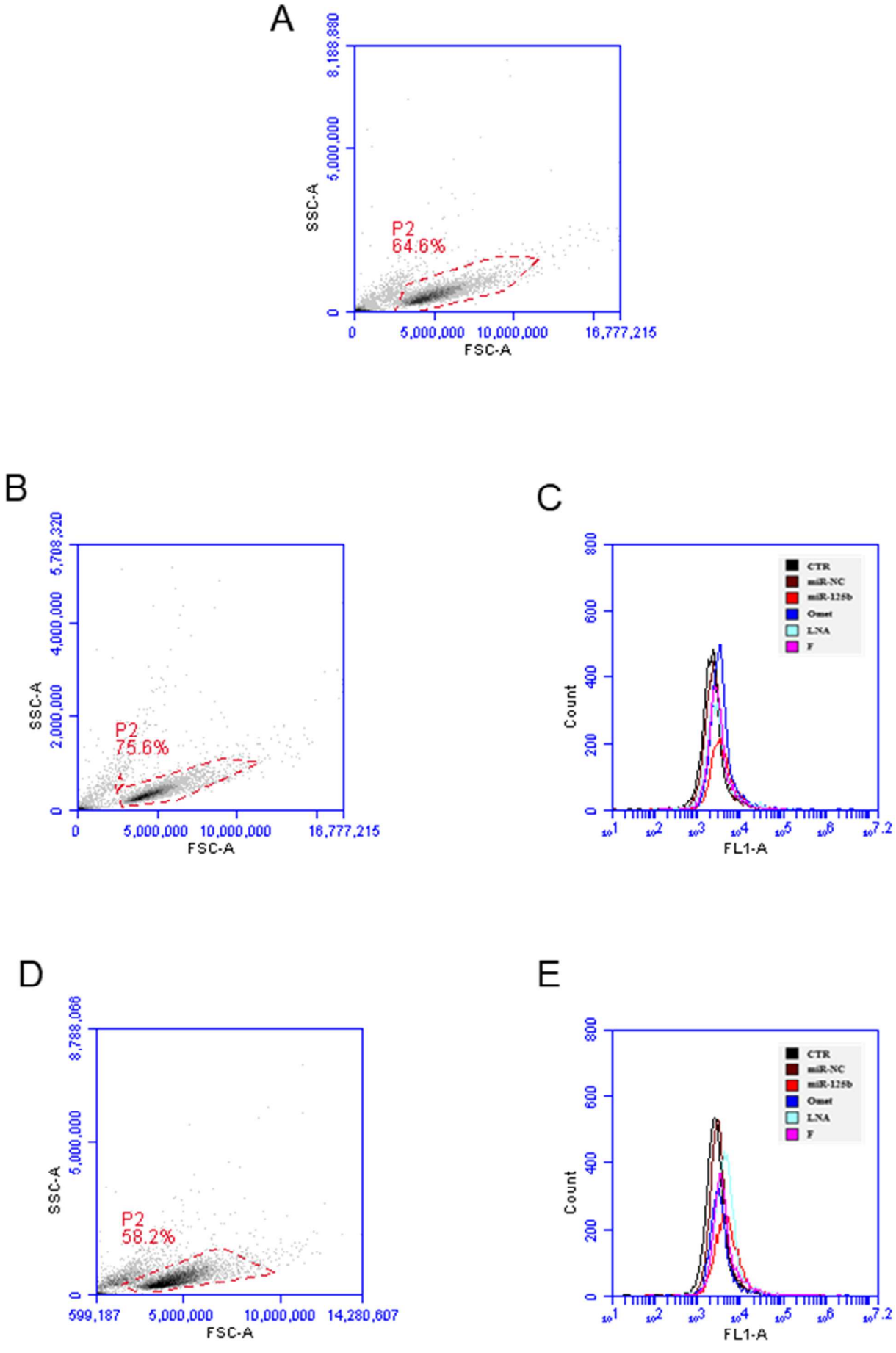
A
SSC-A
8,188,880
5,000,000
0
P2
64.6%
0
5,000,000
10,000,000
16,777,215
FSC-A
B
SSC-A
5,708,320
4,000,000
2,000,000
0
P2
75.6%
0
5,000,000
10,000,000
16,777,215
FSC-A
C
Count
800
600
400
200
0
CTR
miR-NC
miR-125b
Onset
LNA
F
FL1-A
D
SSC-A
8,788,066
5,000,000
0
P2
58.2%
599,187
5,000,000
10,000,000
14,280,607
FSC-A
E
Count
800
600
400
200
0
CTR
miR-NC
miR-125b
Onset
LNA
F
FL1-A

Figure S6

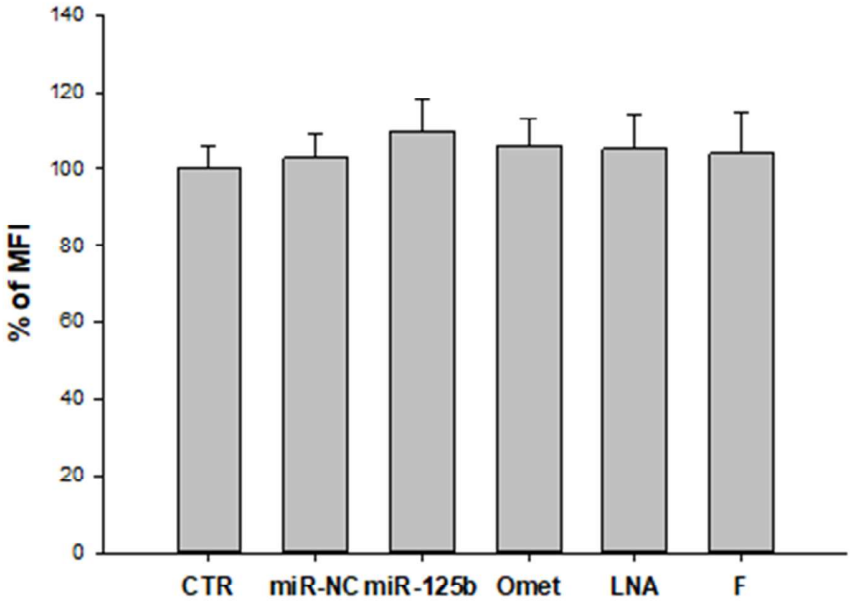

Figure S7

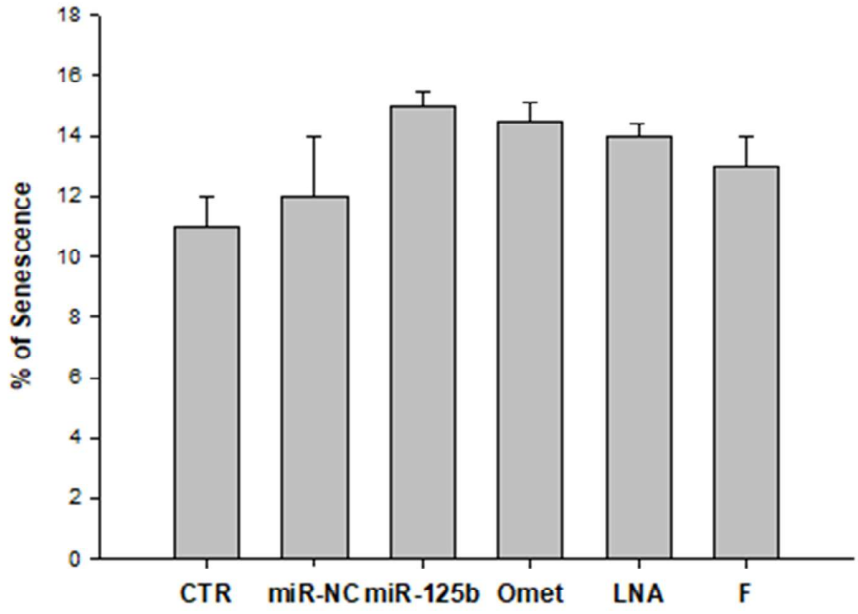
% of Senescence
0
2
4
6
8
10
12
14
16
18
CTR
miR-NC
miR-125b
Omet
LNA
F

Figure S8