Elucidating the role of microRNA-18a in propelling a hybrid epithelial-mesenchymal phenotype and driving malignant progression in ER-negative breast cancer

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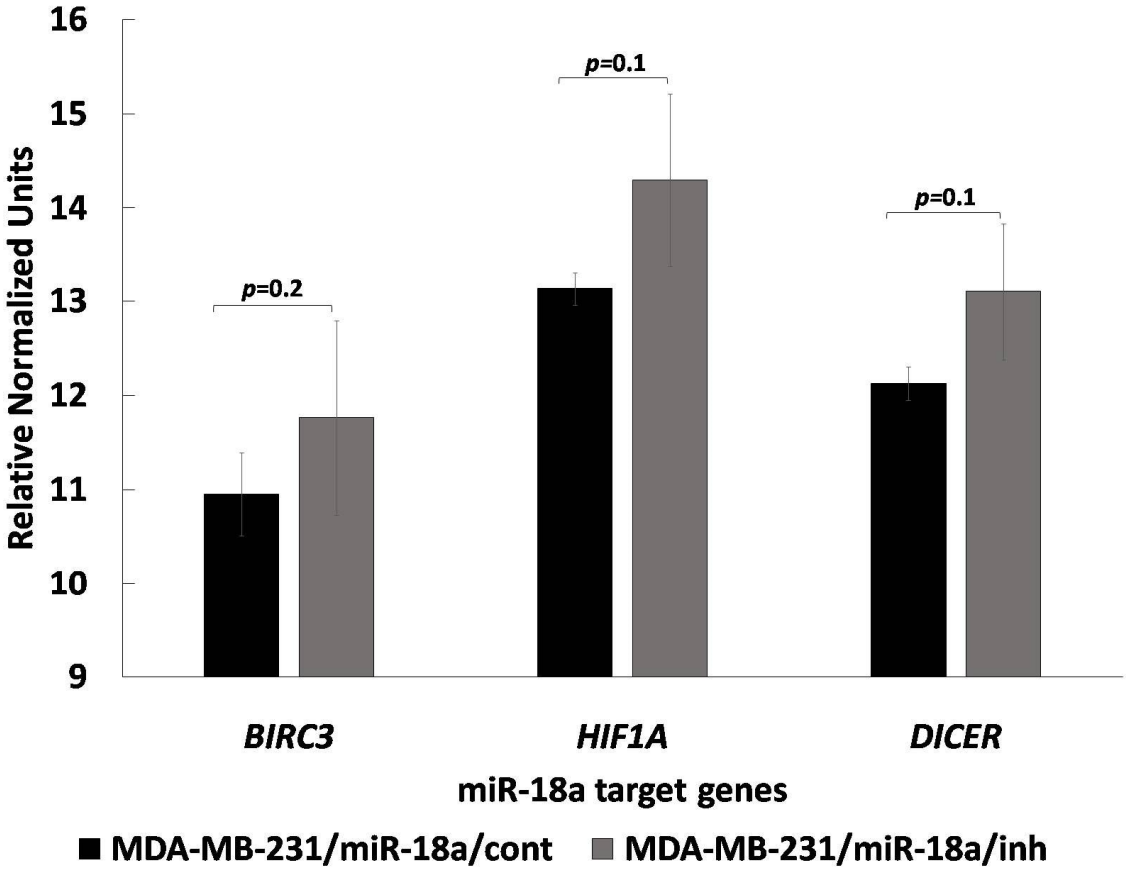
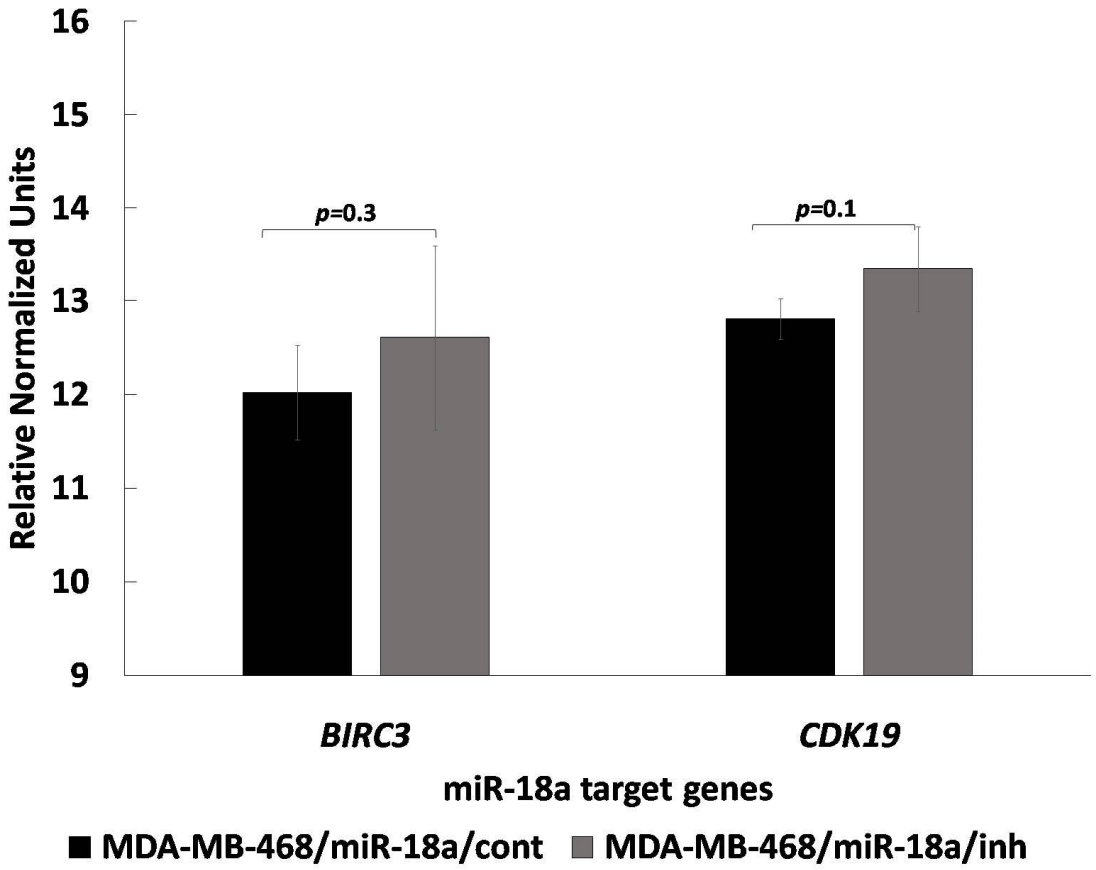
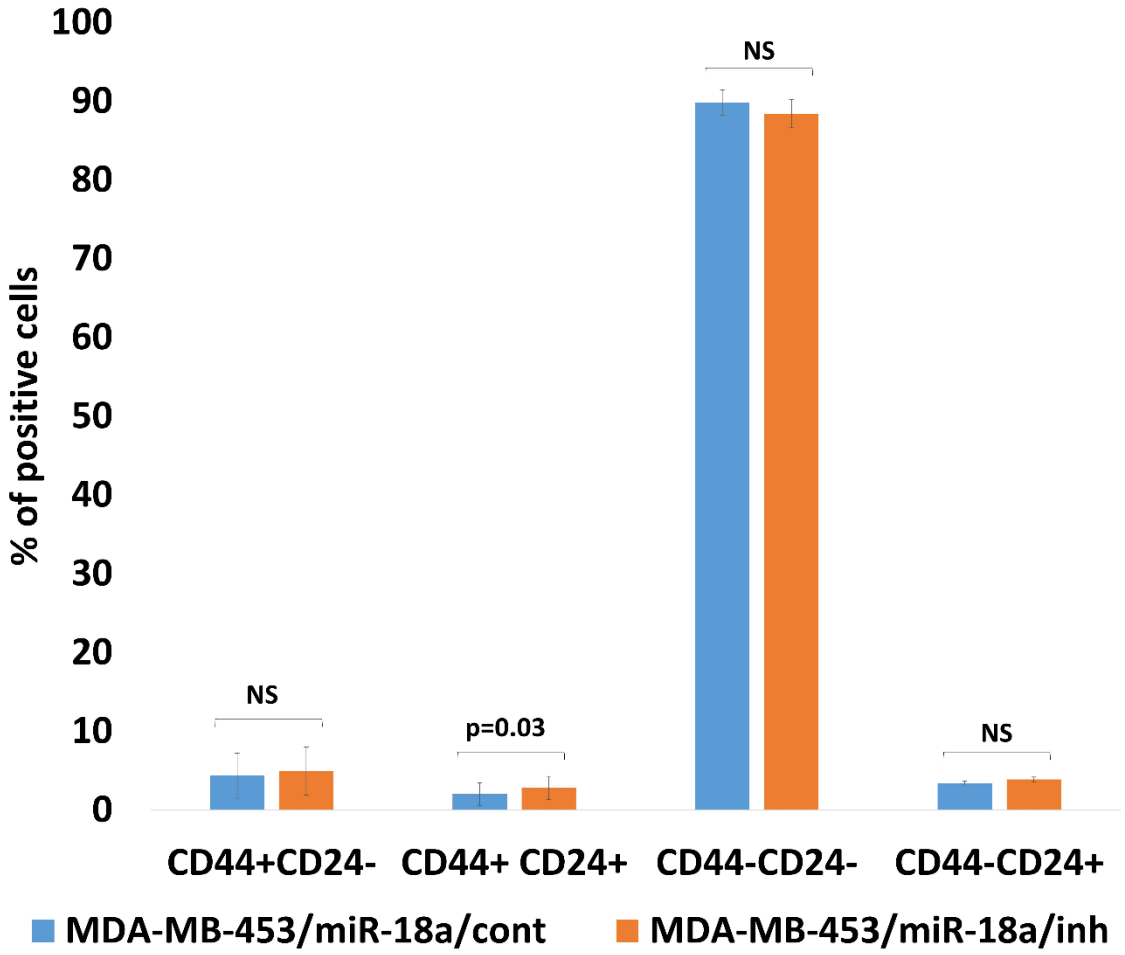
Supplementary Figure S1: Expression level of *BIRC3*, *HIF1A*, *DICER* and *CDK19* after miR-18a inhibition as measured by q-PCR

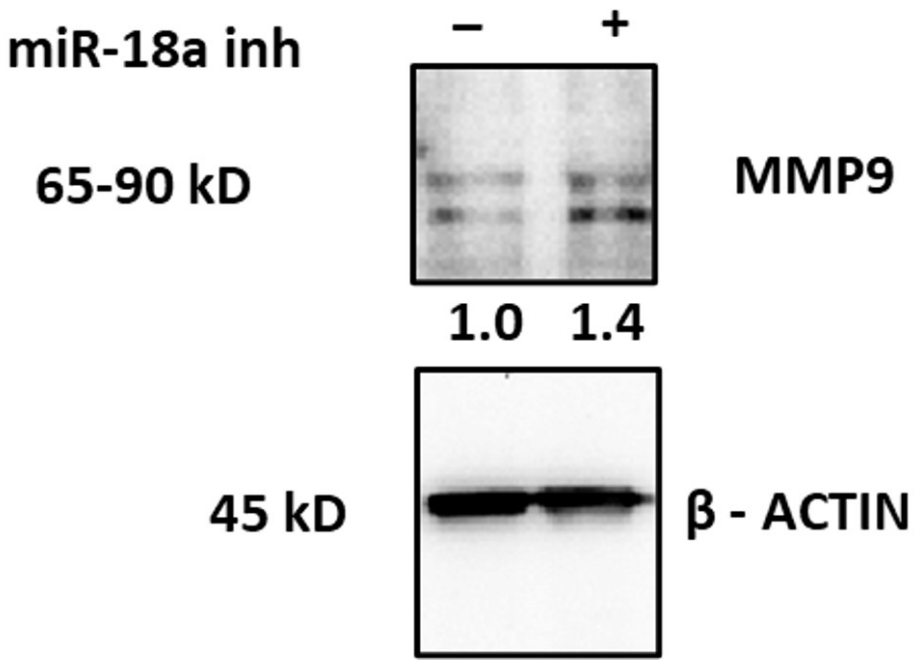



16
15
14
13
12
11
10
9
Relative Normalized Units
p=0.3
p=0.1
BIRC3
CDK19
miR-18a target genes
MDA-MB-468/miR-18a/cont
MDA-MB-468/miR-18a/inh

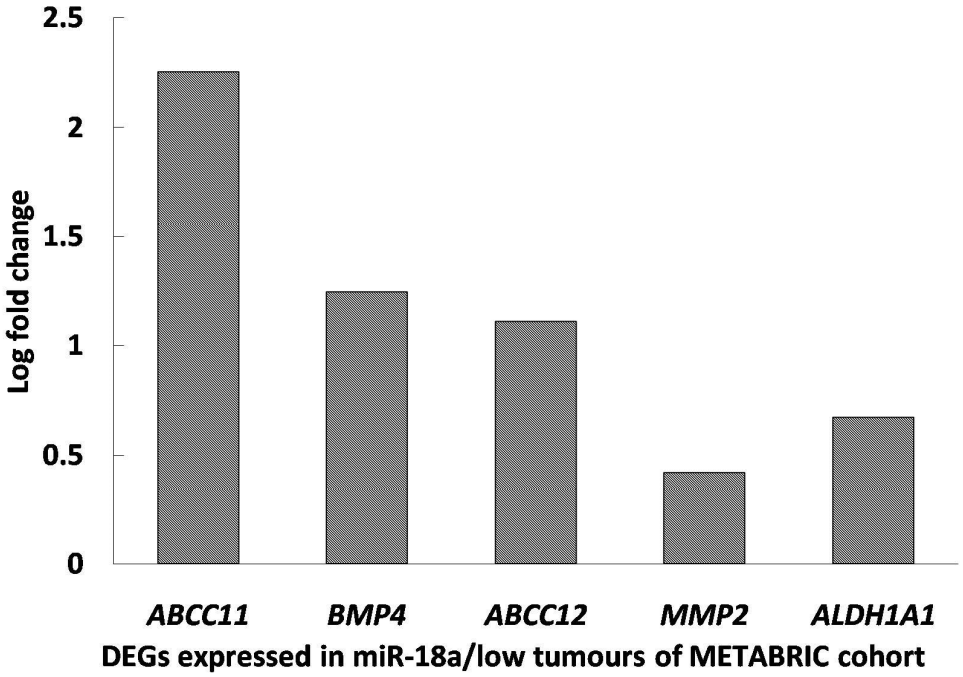
Supplementary Figure S2: Quantitative assessment of CD44 and CD24 expression in MDA-MB-453/miR-18a/cont and MDA-MB-453/miR-18a/inh as assessed by flow cytometry (cumulative result from 3 independent trials).

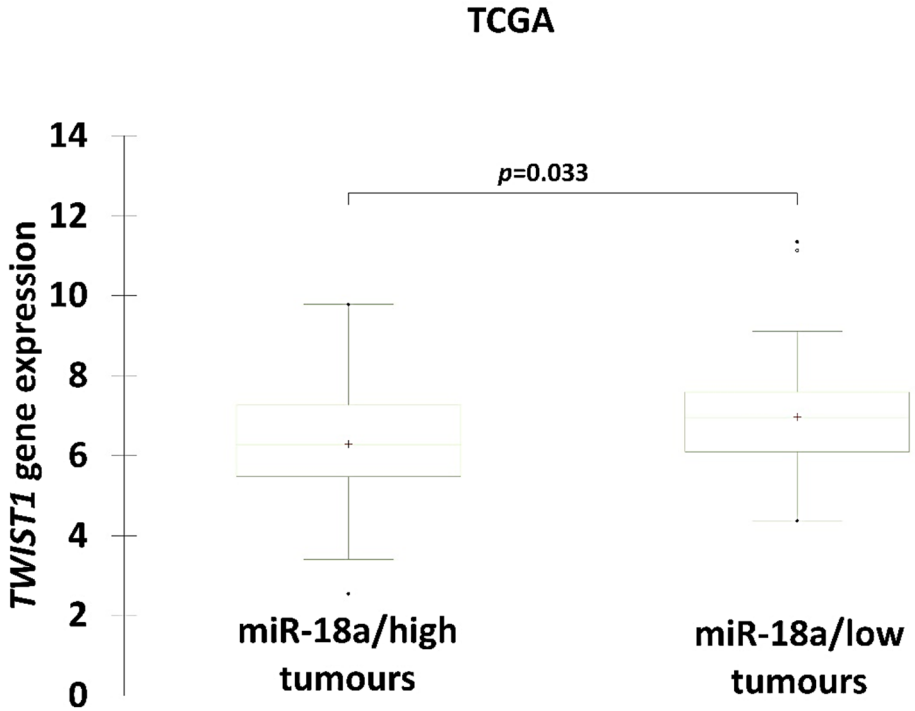


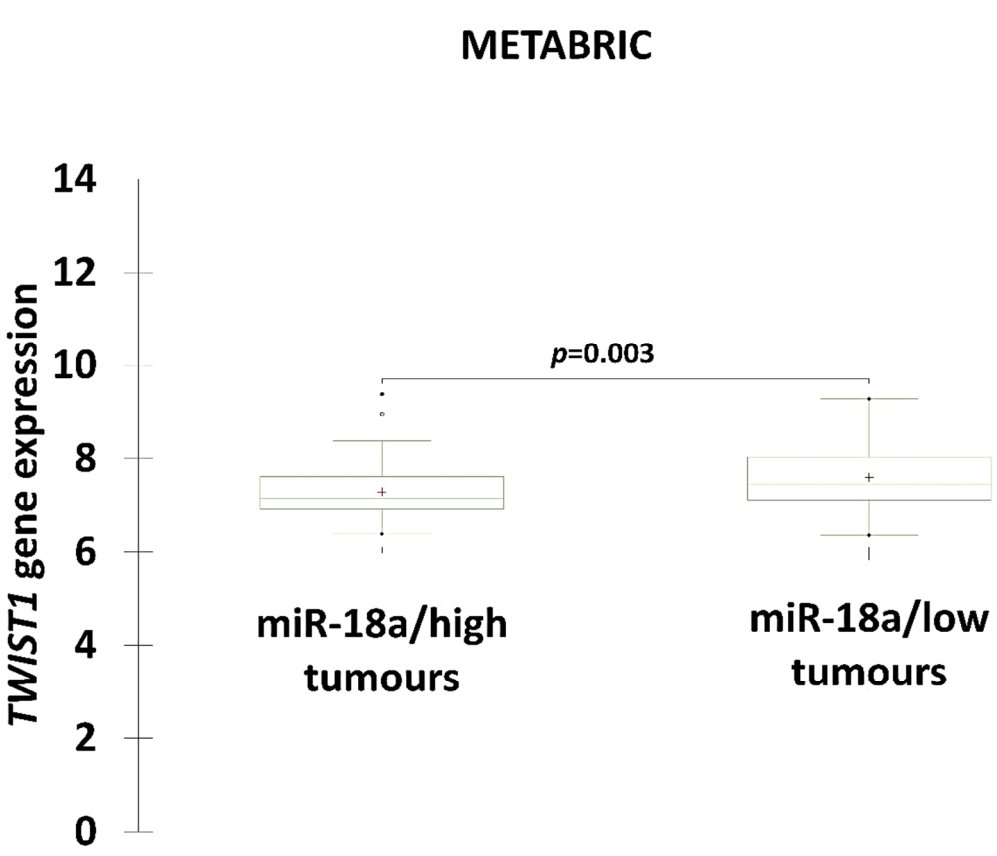
Supplementary Figure S3: Change in protein expression levels of MMP9 levels in MDA-MB-453/miR-18a/cont and MDA-MB-453/miR-18a/inh cells.



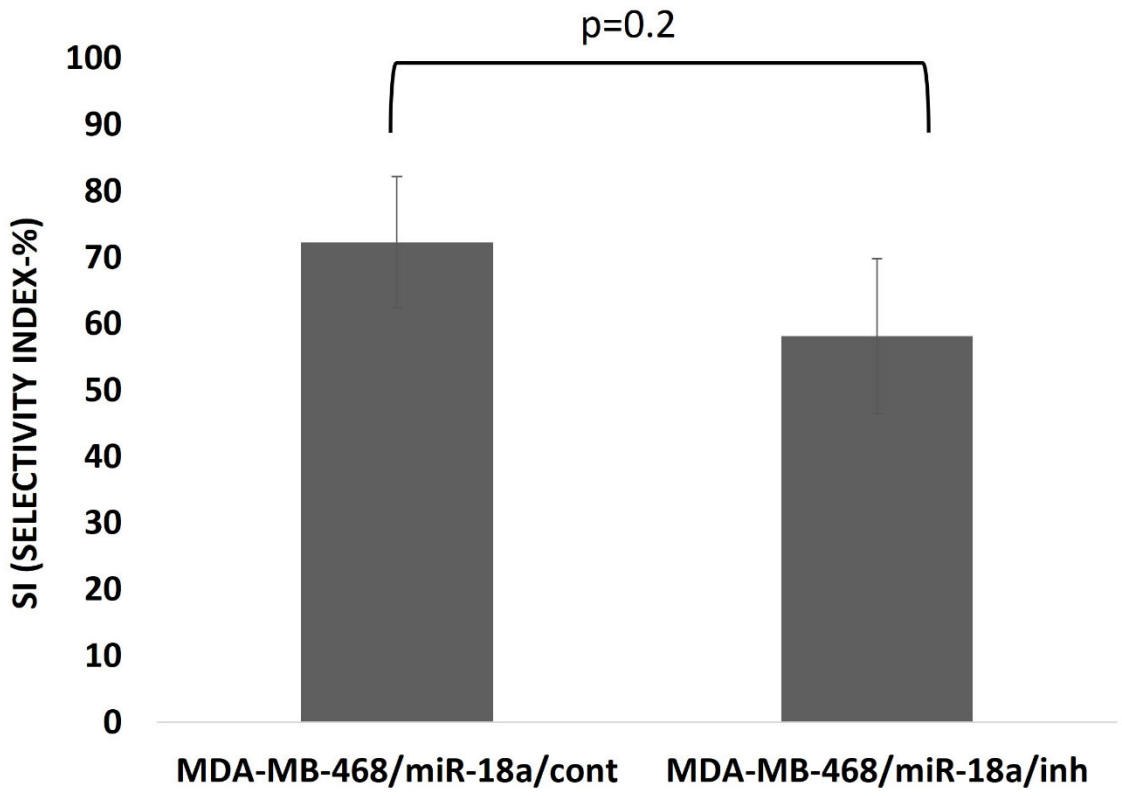
Supplementary Figure S4: DEGs associated with EMT and drug resistance in miR-18a/low, ER-negative tumours of METABRIC



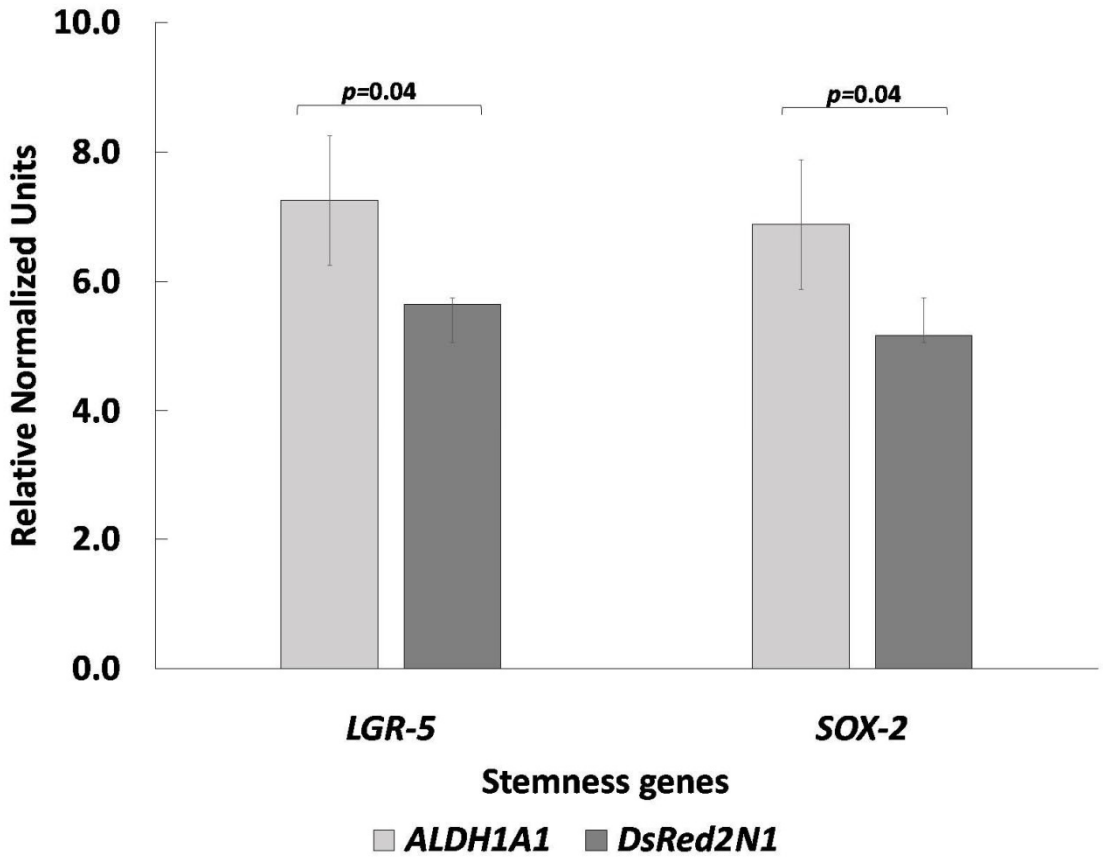



METABRIC
TWIST1 gene expression
14
12
10
8
6
4
2
0
p=0.003
miR-18a/high tumours
miR-18a/low tumours

Supplementary Figure S5a: The SI index in percentage calculated for MDA-MB-468/miR-18a/inh when compared to MDA-MB-468/miR-18a/cont (calculated with reference to MCF10A).



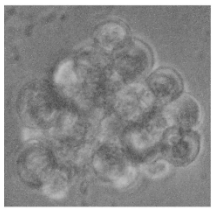
Supplementary Figure S5b: Expression level of stemness genes in ALDH1A1-DsRed2N1 cells vs DsRed2N1 cells.



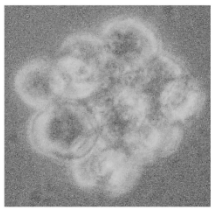
Supplementary Figure S6: Representative images of spheres obtained from MDA-MB-453/miR-18a/cont and MDA-MB-453/miR-18a/inh cells and table and graph depicting clonogenicity (stem cell frequency) calculated from extreme limiting dilution assay performed on MDA-MB-453/miR-18a/cont and MDA-MB-453/miR-18a/inh groups across the same initial seeding dose range of 1-1000 cells.

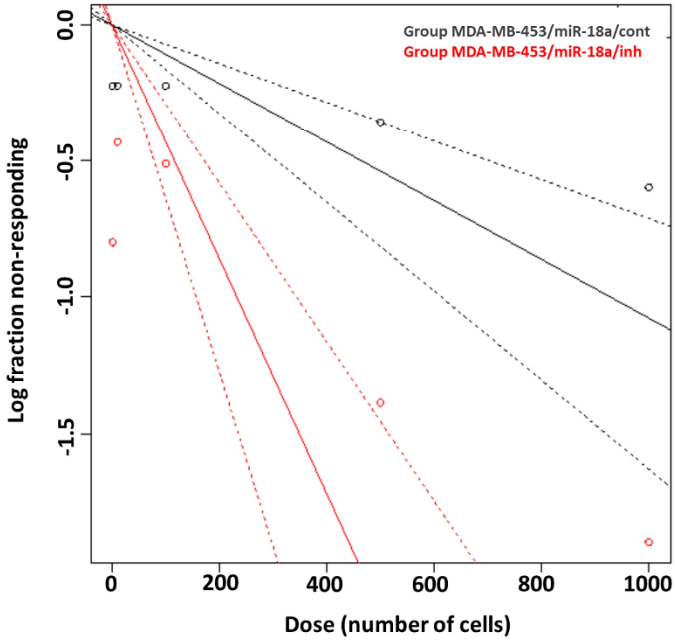
Cell number	MDA-MB-453 /miR/18a/control	MDA-MB-453 /miR/18a/inhibitor
1000	9	17
500	6	15
100	4	8
1	4	11
Stem cell frequency	1/929	1/233
p value = 9.69e-09		

MDA-MB-453/miR-18a/control

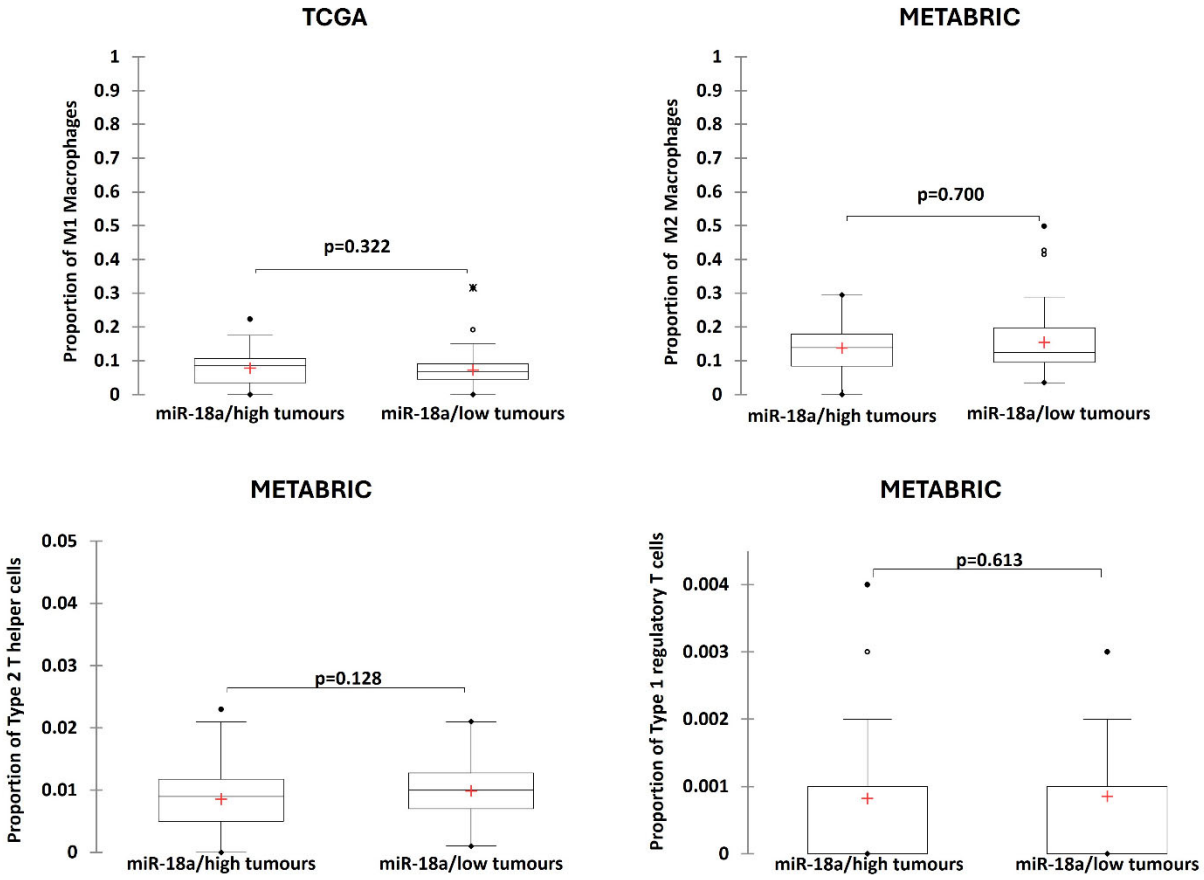


MDA-MB-453/miR-18a/inhibitor

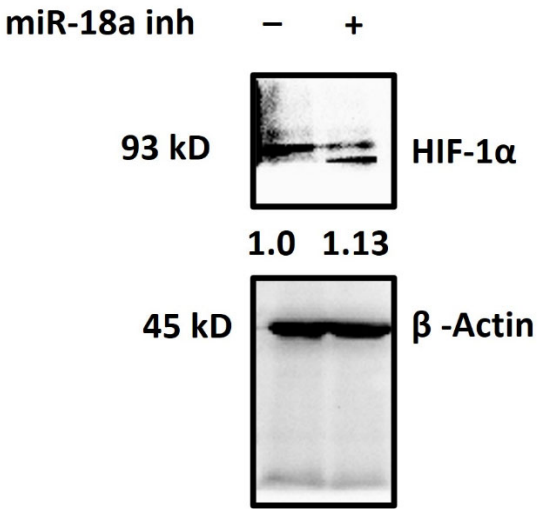




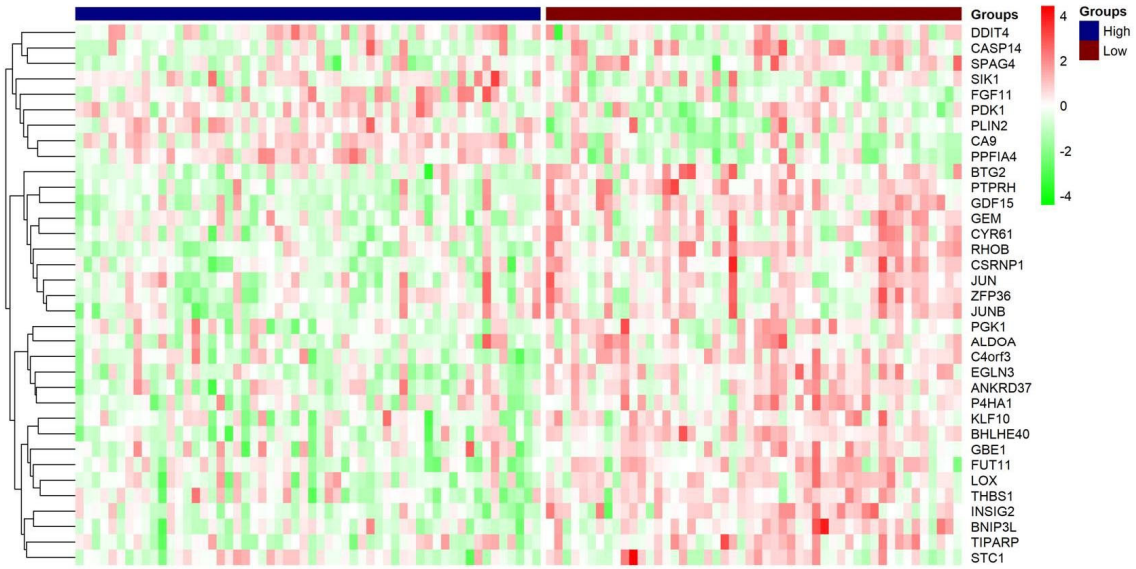
Supplementary Figure S7a: **CIBERSORT analysis depicting the proportions of M1 and M2 macrophages in miR-18a/high and miR-18a/low tumours of TCGA and METABRIC cohorts respectively, immune cell AI analysis depicting proportion of Type 2 T helper cells and Type 1 regulatory T cells in the METABRIC cohort.**



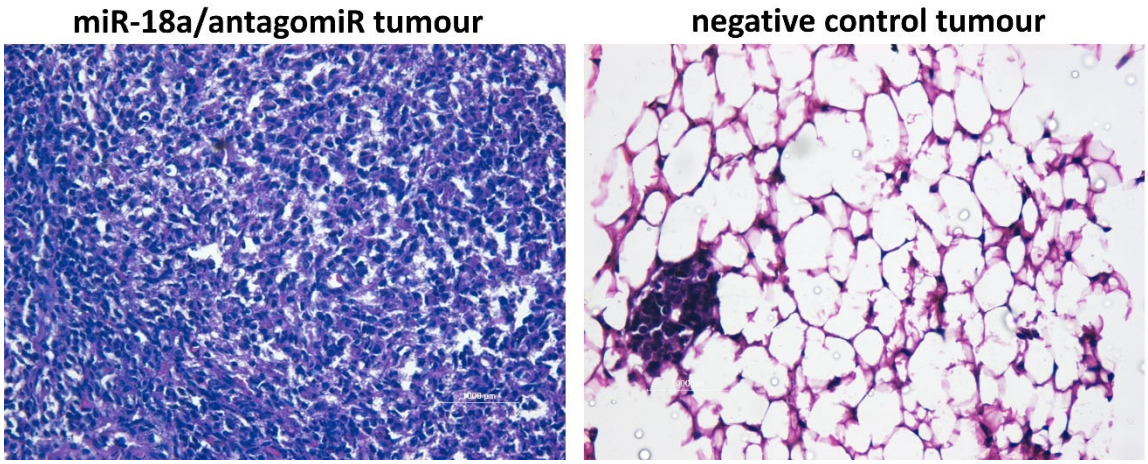
Supplementary Figure S7b: Change in protein expression levels of HIF-1α levels in MDA-MB-231/miR-18a/cont and MDA-MB-231/miR-18a/inh cells.



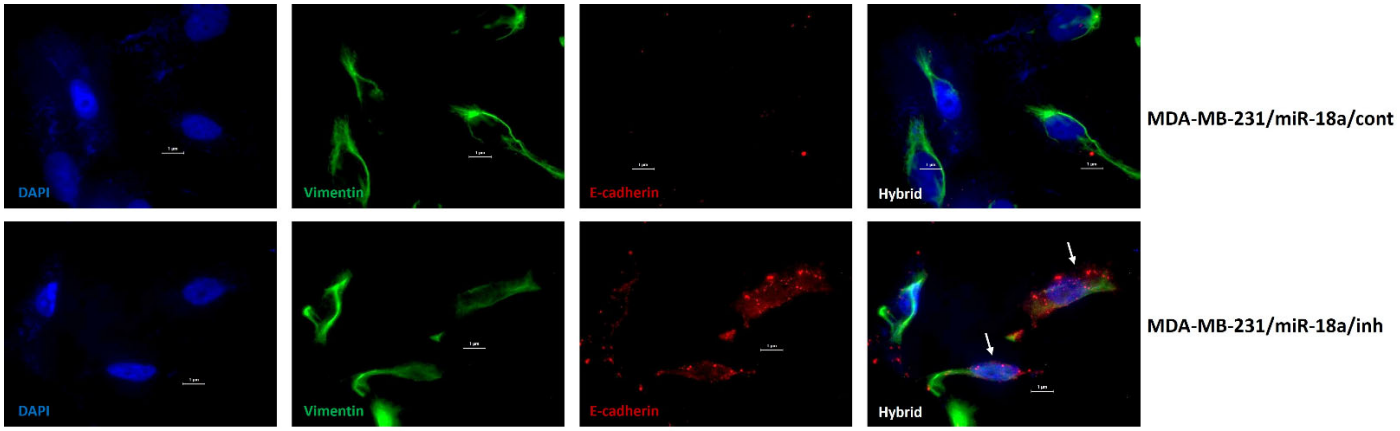
Supplementary Figure S7c: **The heatmap representing the pattern of expression of filtered hypoxia genes in the miR-18a/low tumors of TCGA**



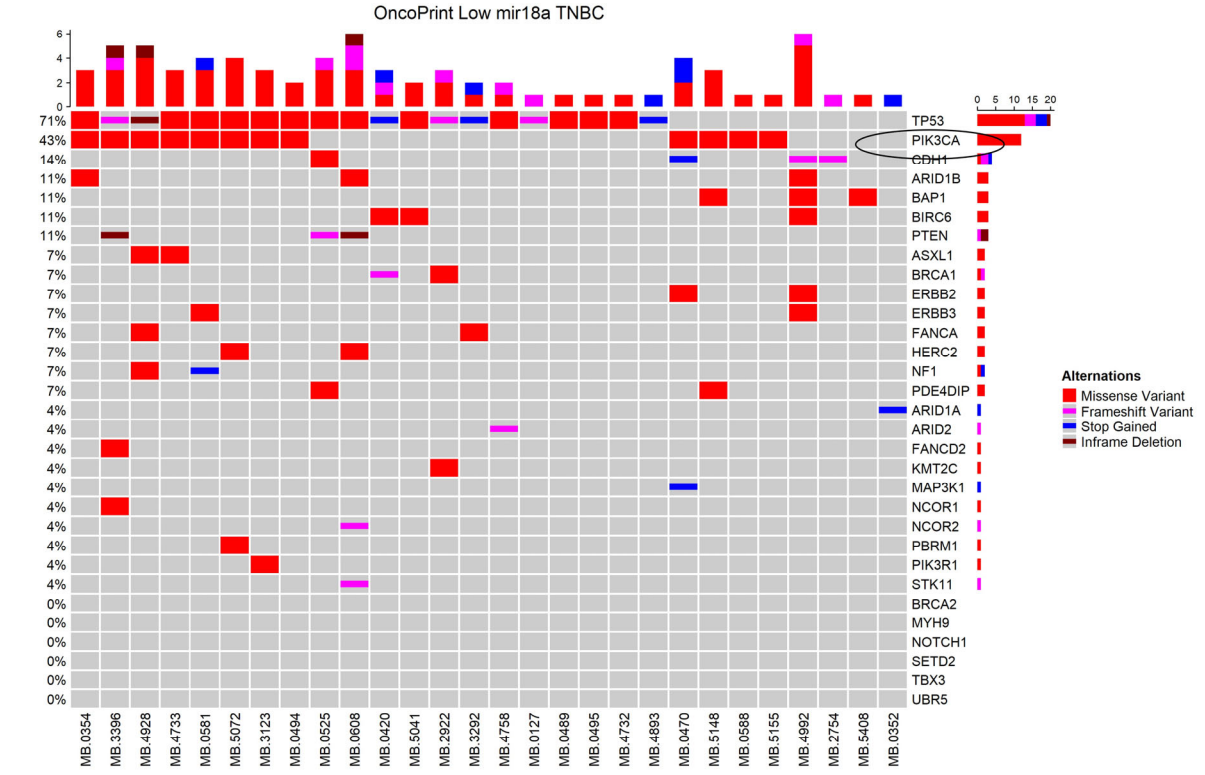
Supplementary Figure S7d: Representative H&E stained image of tumour from mice injected with miR-18a/antagomiR and and antagomiR negative control cells.

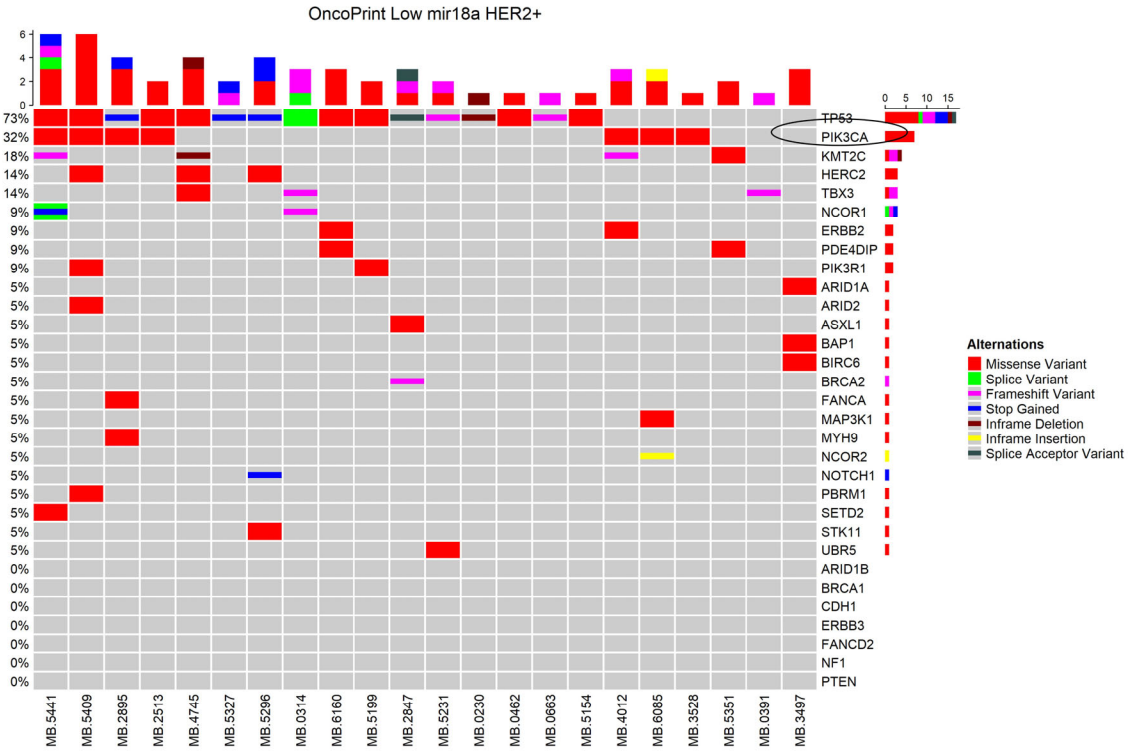


Supplementary Figure S7e: Change in protein expression levels of E-cadherin and Vimentin levels in MDA-MB-231/miR-18a/cont and MDA-MB-231/miR-18a/inh cells.

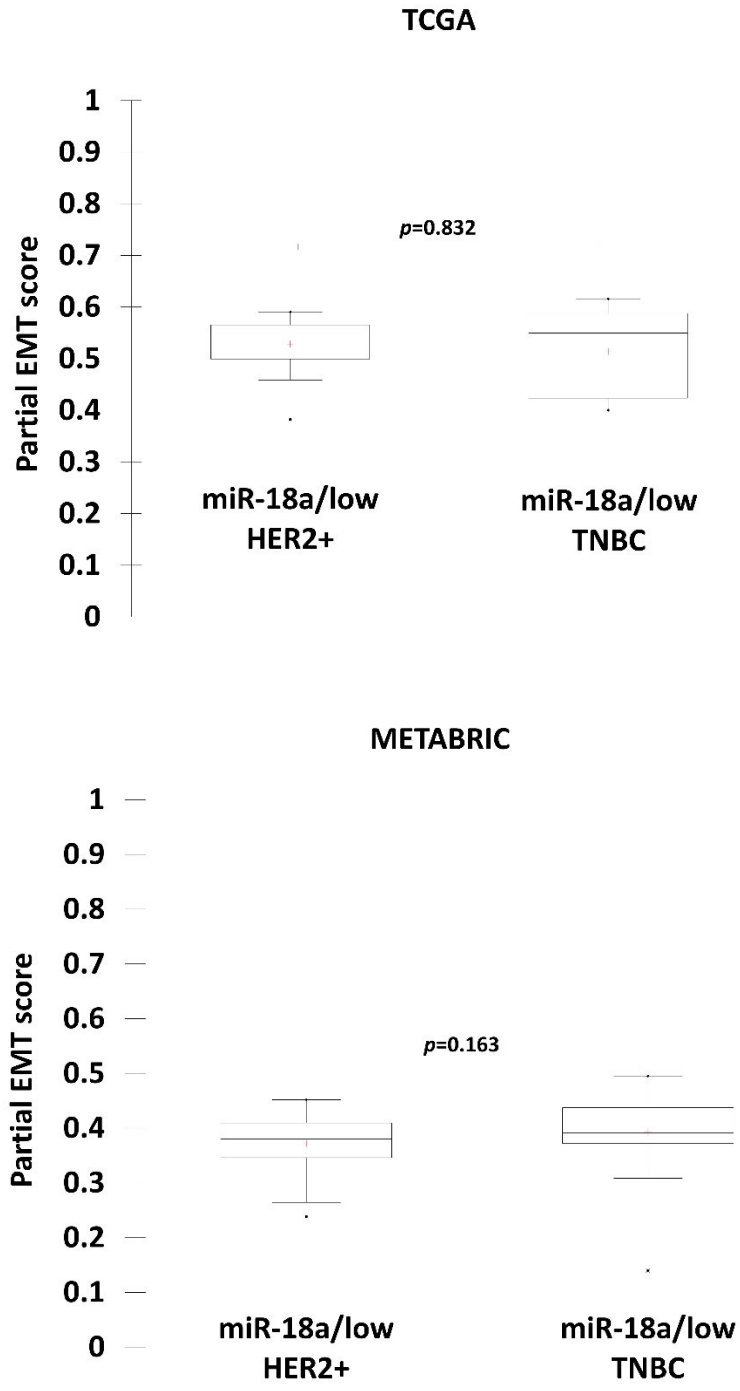


Supplementary Figure S8: Mutational spectrum analysis depicting higher PIK3CA mutation load in both TNBC and ER-HER2+ tumors of the miR-18a/low ER-negative tumours of METABRIC cohort.



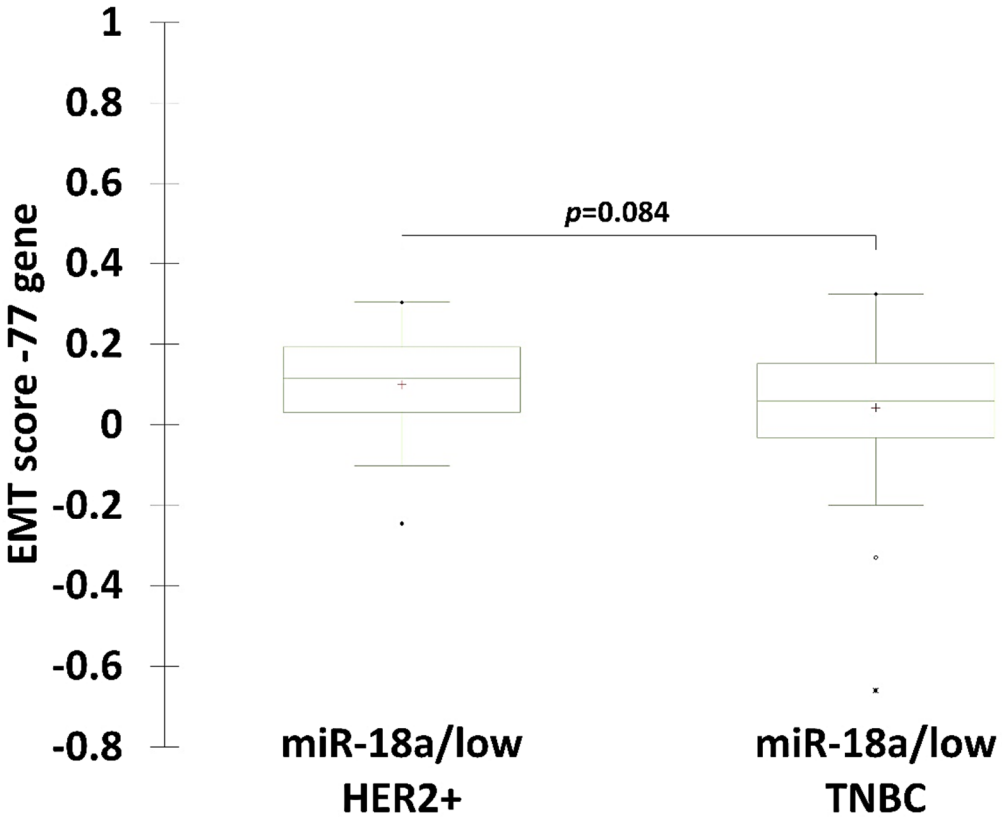


Supplementary Figure S9: Partial EMT score between the TNBC and ER-HER2+ tumors of the miR-18a/low ER-negative tumours of TCGA and METABRIC cohort.



Partial EMT Score derived from - Puram SV, Tirosh I, Parikh AS, Patel AP, Yizhak K, Gillespie S, Rodman C, Luo CL, Mroz EA, Emerick KS, Deschler DG, Varvares MA, Mylvaganam R, Rozenblatt-Rosen O, Rocco JW, Faquin WC, Lin DT, Regev A, Bernstein BE. Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. Cell. 2017 Dec 14;171(7):1611-1624.e24. doi: 10.1016/j.cell.2017.10.044. Epub 2017 Nov 30. PMID: 29198524; PMCID: PMC5878932.

Supplementary Figure S10: 77 gene EMT Score between the TNBC and ER-HER2+ tumors of the miR-18a/low ER-negative tumours of the TCGA and METABRIC cohort.



Supplementary Table S1 : List of antibodies and their dilutions

Sl. No	Antibody	Purpose	Product details	Dilution
1	TNFAIP3	Western Blot	Rabbit monoclonal EPR2663- ab92324 Abcam	1:1000
2	CD49f	Western Blot	Rabbit monoclonal EPR5578- ab124924 Abcam	1:5000
3	Cytokeratin-19	Western Blot	Rabbit monoclonal EP1580Y- ab52625 Abcam	1:10000
4	MMP9	Western Blot	Rabbit monoclonal- PA5-27191 Invitrogen	1:1000
5	RAC3	Western Blot	Rabbit monoclonal EPR6679(B)- ab124943 Abcam	1:5000
6	ALDH1	Western Blot	Mouse monoclonal- 611194 BD Biosciences	1:500
7	HIF1-α	Western Blot	Rabbit monoclonal EP1215Y- ab51608 Abcam	1:500
8	CD44	Flow cytometry	CD44 (156-3C11) Mouse mAb- 3570 Cell signalling technology	1:100
9	CD24	Flow cytometry	CD24 (M1/69) Rat mAb PE Conjugate- 90378 Cell Signalling technology	1:660
10	E-cadherin	Western Blot /Immunofluorescence	Rabbit monoclonal EP700Y- ab40772 Abcam	1:5000/1 :500
11	Vimentin	Immunofluorescence	Mouse clone V9- AM074-5M Bio-genex	1:25
12	Integrin β3	Immunohistochemistry	Rabbit monoclonal EPR2417Y- ab75872 Abcam	1:500

Supplementary Table S2. Clinico-pathological characteristics of 211 ER-negative patients used for analysis from TCGA dataset.

	All N (%) (n = 211)	miR-18a/low (n = 50)	miR-18a/high (n = 57)
Age (y)			
Mean	55	57	52
Median	55	58	51
Tumor Size (cm)			
T1	33 (22)	9 (26)	4 (10)
T2	100 (66)	19 (56)	34 (83)
T3	13 (9)	3 (9)	2 (5)
T4	5 (3)	3 (9)	1 (2)
Stage			
1	20 (14)	3 (9)	2 (5)
2	96 (65)	20 (63)	31 (76)
3	29 (20)	9 (28)	8 (19)
4	2 (1)		
Lymph Node status			
Positive	71 (39)	17 (49)	15 (30)
Negative	112 (61)	18 (51)	35 (70)
Menopausal status			
Pre	46 (24)	12 (26)	18 (36)
Post	142 (76)	35 (74)	32 (64)
Her2 positivity			
Negative	111 (62)	17 (40)	39 (76)
Positive	37 (21)	18 (43)	4 (8)
Equivocal	31 (17)	7 (17)	8 (16)

Supplementary Table S3. Clinico-pathological characteristics of 265 ER-negative patients used for analysis from METABRIC dataset.

	All N (%) (n = 265)	miR-18a/low (n = 54)	miR-18a/high (n = 62)
Age (y)			
Mean	55	59	53
Median	55	60	52
Tumor Size (cm)			
Mean	2.9	2.4	2.9
Median	2.5	2.2	2.5
Stage			
0	2 (1)	1 (2)	0 (0)
1	63 (27)	17 (34)	9 (15)
2	127 (55)	22 (44)	42 (71)
3	39 (17)	10 (20)	8 (14)
Grade			
I	2 (1)	1 (2)	0 (0)
II	33 (13)	11 (22)	3 (5)
III	223 (86)	38 (76)	58 (95)
Lymph Node status			
Positive	139 (52)	27 (50)	33 (53)
Negative	126 (48)	27 (50)	29 (47)
Menopausal status			
Pre	99 (37)	13 (24)	27 (44)
Post	166 (63)	41 (76)	35 (56)
Her2 positivity			
Negative	187 (71)	32 (59)	59 (95)
Positive	78 (29)	22 (41)	3 (5)

Supplementary Table S4: List of luminal and basal genes

Luminal genes	Basal genes
ESR1	*CK5/6*
GATA3	*Laminin*
TFF3	*CENPI*
FOXA1	*CENPK*
LIV-1	*CDC7*
KRT8	*KIF18A*
GRM4	*CCNE2*
GRM8	*STIL*
KRT18	*CDCA7*
PGR	*CKS2*
NMUR1	*MIA*
MUC1	*ANLN*
CX3CL1	*FABP7*
NCAM1	*KRT17*
XBP1	*KRT6b*
	DCS2

Supplementary Table S5. Clinico-pathological characteristics of 105 ER-negative patients used for analysis from our case series.

	All N (%) (n= 105 patients)
Age (y)	
Mean	54
Median	54
Tumor Size (cm)	
Mean	3.5
Median	3
Stage	
I	16 (15)
II	53 (50)
III	32 (30)
IV	4 (4)
Grade	
I	6 (6)
II	38 (39)
III	54 (55)
Lymph Node status	
Positive	54 (51)
Negative	49 (47)
Nx	2 (2)
Menopausal status	
Pre	28(27)
Post	77(73)
Her2 positivity	
Equivocal	4 (4)
Negative	72 (68)
Positive	29 (28)

Supplementary Table S6. Clinical characteristics of post NACT residual tumors: Clinico-pathological characteristics of (n=54) post NACT residual tumors and (n=43) tumors with adequate tissue available for estimation of integrin β3 and miR-18a

	n = 54 patients (%)	n = 43 patients (%)
Age		
Mean (Yrs)	49	49
Median (Yrs)	48	48
Tumor Size		
Mean (cm)	6	6
Median (cm)	6	6
Stage		
III	38 (70)	31 (72)
IV	14 (26)	10 (23)
Nx	2 (4)	2 (5)
Menopausal status		
Pre	24 (44)	19 (44)
Post	30 (56)	24 (56)
Estrogen Receptor		
Positive	30 (56)	22 (51)
Negative	24 (44)	21 (49)
Progesterone Receptor		
Positive	28 (52)	21 (49)
Negative	26 (48)	22 (51)
HER2		
Positive	18 (33)	13 (30)
Negative	29 (54)	25 (58)
Equivocal	7 (13)	5 (12)

Supplementary Table S7: List of primer sequences used in q-RT-PCR analysis

Sl No.	Gene	PRIMER SEQUENCE
1	*ACTB*	5’-TTCCTGGGCATGGAGTC-3’ 3’-CAGGTCTTTGCGGATGTC-5’
2	*ANLN*	5’-ACAGCCACTTTCAGAAGCAAG-3’ 3’-CGATGGTTTTGTACAAGATTTCTC-5’
3	*BCL2*	5’-TACCTGAACCGGCACCTG-3’ 3’-GCCGTACAGTTCCACAAAGG-5’
4	*BIRC3*	5’-CCATGGGTTCAACATGCCAAGTGGT-3’ 3’-GGGTAACTGGCTTGAACTTGACGG-5’
5	*BMPR1B*	5’-ATTCCCAAACCGGTGGAGCAGT-3’ 3’-TTGATGCAGGATTGTGAGCCCAGC-5’
6	*CDK19*	5’-TACCTCCATGCAAATTGGGTGCT-3’ 3’-R-TTTGACTCTCCCCCTCTCAGGA-5’
7	*DICER*	5’-TTAACCTTTTGGTGTTTGATGAGTGT-3’ 3’-GCGAGGACATGATGGACAATT-5’
8	*HIF1A*	5’-TGCTTACACACAGAAATGGCCT-3’ 3’-TAGTTAGGGTACACTTCATTCTGAG-5’
9	*ITGB3*	5’-CCCACCAGAGGCCCTCGAAA-3’ 3’-AAGCGGGTCACCTGGTCAGT-5’
10	*LGR5*	5’-GACCATTGCCTACACCAAGC-3’ 3’-GAGCAACAGGGCAATGTGTT-5’
11	*PGR*	5’-TTATAATTCGAGGCGGTTAGTGTTT-3’ 3’-TCGAACTTCTACTAACTCCGTACTACGA-5’
12	*PUM1*	5’-CCGGAGATTGCTGGACATATAA-3’ 3’-TGGCACGCTCCAGTTTC-5’
13	*RPLP0*	5’-GGCTGTGGTGCTGATGGGCAAGAA-3’ 3’-TTCCCCCGGATATGAGGCAGCAGT-5’
14	*SALL4*	5’-GGCCAATAGTCAAGCCGAAA-3’ 3’-TCCGACCTTCCATCTCAGTG-5’
15	*ZEB2*	5’-TGCACAGAGTGTGGCAAGGC-3’ 3’-TGGGCACTCGTAAGGTTTTTCACC-5’

Details on literature survey:

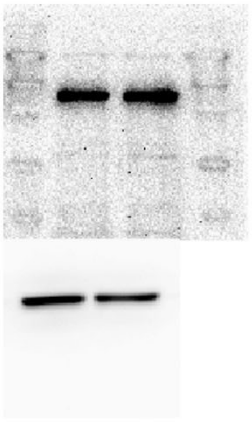
The literature survey as part of the manuscript was conducted using Pubmed search and Google scholar search. The key words employed for the search was ‘miR-18a ER-Negative breast cancer’, ‘miR-18a cancer’, ‘miRNA targeting’.

The time period for literature survey was 2009-2022.

Western blot - Densitometric analysis and raw blot images

Fig 1B
TNFAIP3

	Control	miR-18a inhibitor
TRIAL 1	100	103.1
TRIAL 2	100	157.5
TRIAL 3	100	155.8959
TRIAL 4	100	164.5387
TRIAL 5	100	147.2625
AV	100	145.6594
STD ERROR	0	10.98928
P VALUE		0.000236



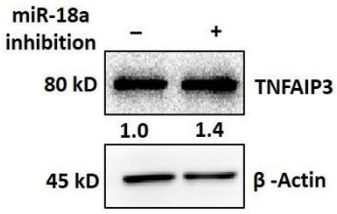
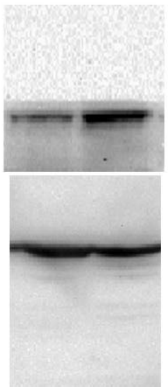


Fig 1B
CD49f

	Negative control	miR-18a inhibitor
TRIAL 1	100	270
TRIAL 2	100	258
TRIAL 3	100	158
AV	100	208
STD ERROR	0	40.82483
P VALUE	0.000668	



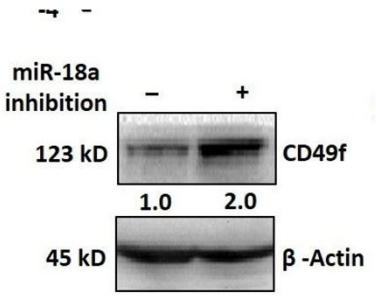
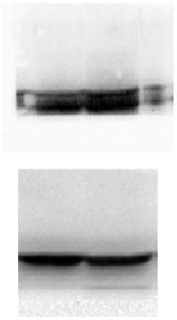


Fig 1B
Cytokeratin 19

	Negative control	miR-18a inhibitor
TRIAL 1	100	181
TRIAL 2	100	149
TRIAL 3	100	125
TRIAL 4	100	151.6667
AV	100	151.6667
STD ERROR	0	16.2207
P VALUE	0.016684	



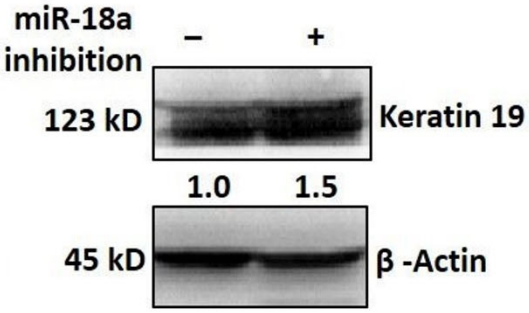
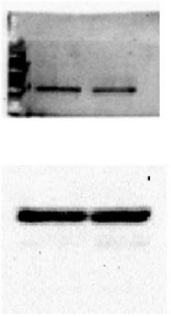


Fig 2C
E-cadherin

	Negative control	miR-18a inhibitor
TRIAL 1	100	86
TRIAL 2	100	73.2
TRIAL 3	100	94.5
AV	100	84.56666667
STD ERROR	0	6.190404761
P VALUE	0.03363	



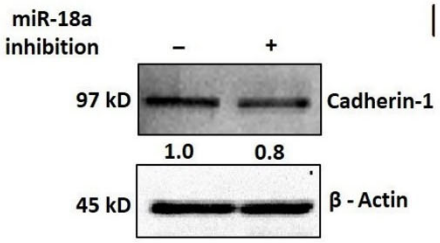
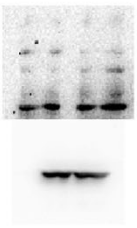


Fig 2C MMP9

	Negative control	miR-18a inhibitor
TRIAL 1	100	137.2
TRIAL 2	100	171.8
TRIAL 3	100	157.8
AV	100	155.6
STD ERROR	0	10.04855
P VALUE	0.002607	



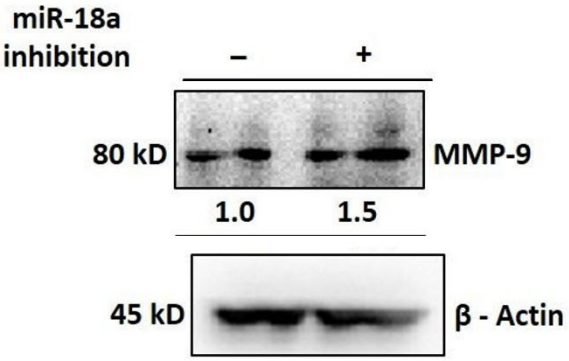


Fig 2C RAC3

	Negative control	miR-18a inhibitor
TRIAL 1	100	147
TRIAL 2	100	150
TRIAL 3	100	131.5
AV	100	142.8333
STD ERROR	0	5.732461
P VALUE	0.000857	

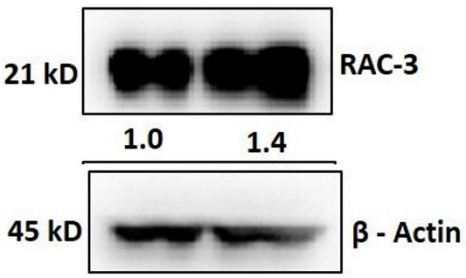
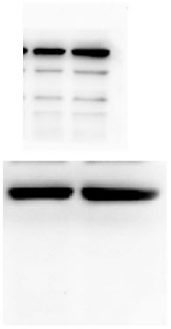


Fig 3H ALDH1A1

	Negative control	miR-18a inhibitor
TRIAL 1	100	115
TRIAL 2	100	191
TRIAL 3	100	144
AV	100	150
STD ERROR	0	22.14347
P VALUE	0.043436135	



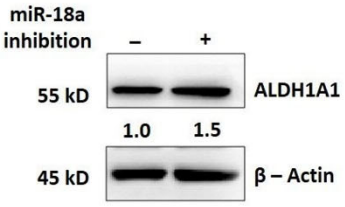
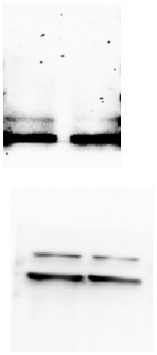


Fig 5C HIF1A

	Negative control	miR-18a inhibitor
TRIAL 1	100	331.2888
TRIAL 2	100	179.2266
TRIAL 3	100	147.3008
AV	100	219.2721
STD ERROR	0	56.76155
P VALUE	0.051752	



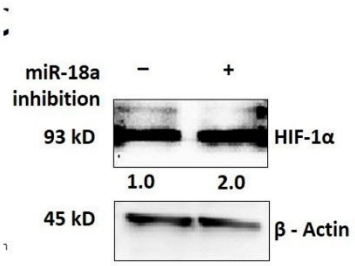
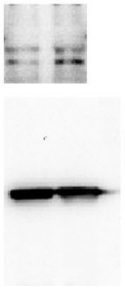
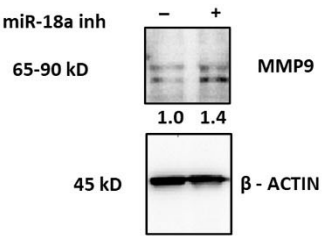


Fig S3 MMP9

	Negative control	miR-18a inhibitor
TRIAL 1	100	144.5632
TRIAL 2	100	191.7947
TRIAL 3	100	110.622
AV	100	148.9933
STD ERROR	0	23.53699
P VALUE	0.052919