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Supplemental Material to:

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Oncogenic miR-181a/b affect the DNA damage response in aggressive breast cancer

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SUPPLEMENTARY METHODS

Constructs and antibodies.

The 3'UTR sequence of ATM was obtained from UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) database. The luc-ATM 3'UTR wt was generated by cloning the 3'UTR sequence of ATM, amplified from human genomic DNA extracted from U2OS cells downstream of the luciferase coding sequence into the pGL3 vector (Promega). In the luc-ATM 3'UTR mut181 reporter, the two miR-181 putative binding sites were mutated into an AgeI and XbaI restriction sites by using Quick change II XL Site-Directed Mutagenesis kit (Stratagene, CAT#200521-5), following manufacturer's instructions. shATM construct was kindly provided by Fabrizio d'Adda di Fagagna.

Oligo used:

ATM 3'UTR:

F:GCGGACGTCTGATCTTCAGTATATGAATTACCCTTTCATTCAGCC

R:CGCACCGGTGCTTTTAGAATTATTTATTCAAAAACACTTTATAA

miR-181BS1mut:

F:GCCAACATACTTTAAGTAGGGATTAATATTTAAGTGAACTATTGTGGGTTTTTTACCGGTTTGGTT
TTAATACTTGATTTAATCACCAC

R:GTGGTGATTAAATCAAGTATTAAAACCAAACCGGTAAAAAACCCACAATAGTTCACTTAAATAT
TAATCCCTACTTAAAGTATGTTGGC

miR-181BS2mut

F:CAAGTATTTTAATTGCACCTTAATGAAATTATCTATTTTCTATAGATTTTAGTACTATTTCTAGAA
TTACTTTACTGTTACCTGAATTTATTATAAAGTGT

R:ACACTTTATAATAAATTCAGGTAACAGTAAAGTAATTCTAGAAATAGTACTAAAATCTATAGAA
AATAGATAATTTCATTAAGGTGCAATTAAAATACTTG

Western Blot.

Total cell extracts were prepared in Lysis buffer (50 mM Tris-HCl pH 7,5, 300 mM NaCl, 1% NP40. 1 mM EDTA) supplemented with 1 mM PMSF, 5 mM NaF, 1 mM Na_3VO_4, 10µg/ml CLAP. Protein concentration was determined with Bio-Rad Protein Assay Reagent. Western blot analysis was performed according to standard procedures using the following primary antibodies: anti-HSP90 (SantaCruz SC13119), anti-ATM (Sigma A1106), anti-Actin (Sigma A2066), anti-Vinculin (Sigma V4505), anti-BRCA (Cell signalling #9010S), anti-phospho-Ser1524-BRCA1 (Cell signalling #9009S), Cleaved Caspase (Cell signalling #9664S), anti-phospho-Ser139-H2AX (γ-H2AX, Millipore #05636), anti-RAD51(H92) (SantaCruz SC8349). The secondary antibodies used for western blot were anti-mouse IgG (Sigma A4416) and anti-rabbit IgG (Sigma A6154).

Luciferase Assays.

For all the other luciferase assays, H1299 cells were seeded in 24-well plates ($6*10^4$ cells/well). The following day cells were transfected with 3nM of a mix of synthetic miR-181a and miR-181b or control siRNA with 200ng of luc-ATM 3'UTR wt or luc-ATM 3'UTR mutated reporters. In all experiments 25 ng of CMV-renilla construct were cotransfected in each point for normalization of transfection efficiency. Luciferase activity was measured 24h after transfection with Dual-Luciferase Reporter Assay System (Promega, # E1910). Relative Luciferase Units (RLU) were calculated by normalizing the luciferase units measured for the firefly (Photinus pyralis) luciferase with the amount of Renilla luciferase in each sample.

104 BC dataset.

Total RNA (including miRNAs) was extracted from 104 snap-frozen breast tumor samples and 12 normal breast tissues by using Ambion MirVANA kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA's purity and concentration were determined

from OD260/280 readings using a spectrophotometer (Bio-Rad, Laboratories, Hercules, CA, USA). Integrity of RNA was determined by using an experion automated electrophoresis system (Bio-Rad). RNA with an integrity number >6 underwent in further analysis. Quantitative RT-PCR of microRNAs was performed using Taqman MicroRNA assays (Applied Biosystems, Foster City, California) according to the manufacturer's instructions using expression levels of the small nuclear RNA, U6, as housekeeping gene. All assays Quantitative RT-PCR for all miRNAs was performed starting from 10 ng total RNA and using the looped primers in a final 20 μL PCR volume. Real-time PCR was performed in triplicate for each case. The two human mature forms of miR-181a and miR-181b (Applied Biosystems) were analyzed by using the standard TaqMan miRNA assays protocol on a CFX96 real-time RT-PCR detection system as described by the manufacturer (Bio-Rad Laboratories). Forty cycles of amplification were performed and fluorescent signals of probes were used to generate threshold cycle (Ct) to calculate miRNA expression levels. The ΔΔCt method for relative quantitation of gene expression was used to determine miRNA expression levels. The ΔCt was calculated by subtracting the Ct of U6 RNA from the Ct of the miRNA of interest. The ΔΔCt was calculated by subtracting the ΔCt of the reference sample (normal breast) from the ΔCt of each sample. A pool of 12 normal breasts was used for the standard curve calculation and as reference sample for the ΔΔCt.

123 BC dataset.

The dataset consisted in 123 node negative Breast Cancer patients developing distant metastases within 5 years from surgery (59 patients) and being free of distant metastases (64 patients) for at least 5 years. In order to control the effect of classical prognostic factors on the clinical outcome, patients were selected to obtain similar distributions across metastatic and metastasis-free group for age and tumor size. Total RNA was extracted from 100 mg of tissue from the primary tumor using Trizol (Life Technologies, Frederick, MD, USA) following the

manufacturer's instructions. Integrity of the RNA was checked on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). miRNA profiling was done at the Functional Genomics core facility at the Fondazione IRCCS Istituto Nazionale Tumori of Milano using the humanMI_V2 (Illumina, Inc, San Diego, CA). Only expression data of miR-181 family extrapolated from array data were used for the present study. Raw data were normalized using the Robust Spline Normalization algorithm implemented in the lumi R package. Kaplan-Meier curves were built comparing patients subdivided according expression of selected microRNAs stratified by tertiles.

Immunohistochemistry.

Tumors and patients' characteristics are reported in Supplementary Table 1. The ATM protein was evaluated on Glyofix fixed paraffin-embedded breast tumor tissues previously characterized for ER, PR, HER2 and Cytocheratin 5-6. Ki67 expression was analysed as previously reported (Cheang MC, et al. *Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009;101(10):736-750*). All tissues sections were cut at 4 μm, mounted on glass slides, and air dried overnight. Adjacent sections were used, one section for immunostaining and the other as a control without the primary antibody. After deparaffinization, slides were rehydrated and antigen retrieved in a calibrated steam pressure cooker with citrate buffer (pH 6.0) and processed by an automatic Dako Stainer System (DAKO Denmark A/S - Glostrup, DK) according to the manufacturer's instructions: endogenous peroxidases were inactivated, slides rehydrated and incubated with the primary Ab at 1:100 concentration and pH 6 overnight at 4° C. The immunostaining was scored semiquantitatively. Only nuclear staining was taken into account. The scores for IHC were: 0 (-), no staining; 1 (+), few nuclear staining of tumor cells (<10% of positive tumor cells); 2 (++), 10-30% of positive tumor cells; 3 (+++), >30% positive tumor cells. For pairwise

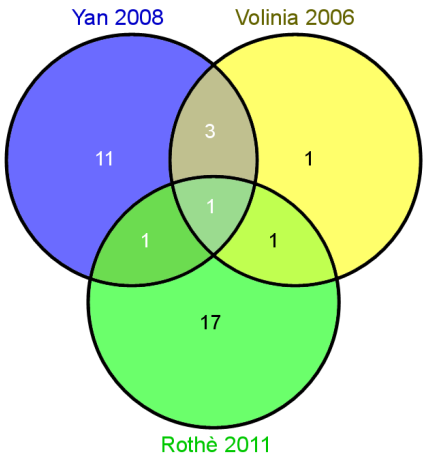
comparisons, the scores were collapsed to low (score, 0-1) versus high (score, 2-3) expression, excluding not interpretable samples.

Bisso et al - Supplementary fig.1

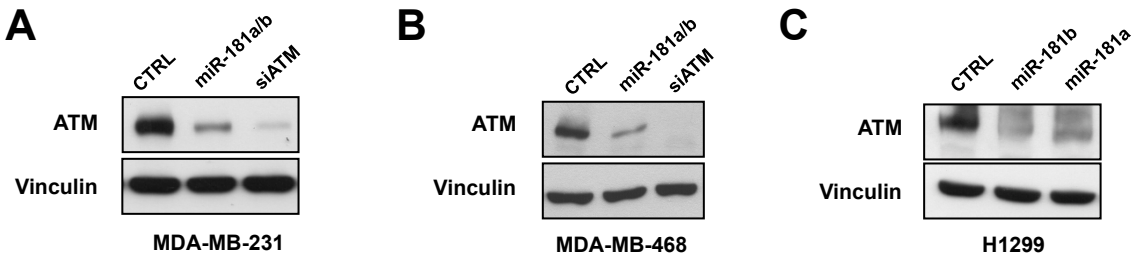
A

Yan et al, 2008	Volinia et al, 2006	Rothè et al, 2011
miR-21	miR-21	miR-181b
miR-365	miR-17	miR-130b
miR-181b	miR-181b	miR-146a
let-7f	miR-155	miR-146b
miR-155	miR-146a	miR-93
miR-29b	miR-29b	miR-181a
miR-181d		miR-20b
miR-98		miR-423
miR-29c		miR-25
miR-497		miR-106b
miR-31		miR-186
miR-335		miR-148a
miR-320		miR-181d
miR-140a		miR-128a
miR-127		miR-34a
miR-30a-3p		miR-363
		miR-130a
		miR-345
		miR-362
		miR-22

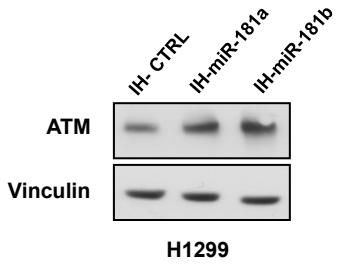
B



S1 – miR-181b is overexpressed in breast cancer.
A) List of miRNAs overexpressed in breast cancer detected in three different dataset.
B) Venn diagram showing the numbers of shared and not shared miRNAs between the three datasets.

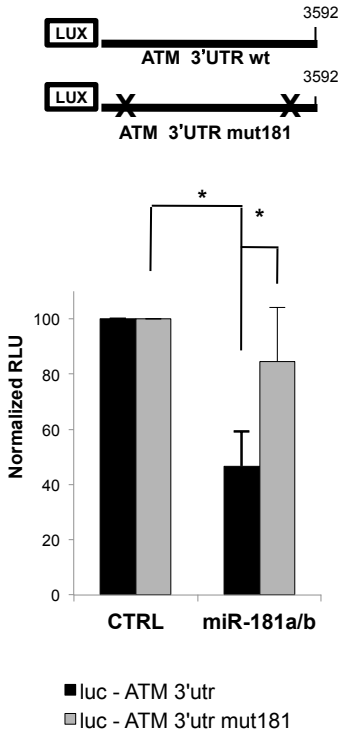


S2 - ATM protein levels are regulated by miR-181a/b.
A) MDA-MB-231, B) MDA-MB-468 and C) H1299 cells were transfected with miR-181a, miR-181b or a combination of miR-181a/b, ATM (siATM) or control siRNA (CTRL). Western blot analysis was performed to detect ATM and vinculin (loading control) protein levels.



S3 – Inhibition of miR-181a and miR-181b increases ATM protein levels.
H1299 cells were transfected with miR-181a (IH-miR-181a), miR-181b (IH-miR-181b) or control inhibitors (IH-CTRL). Western blot analysis was performed 96h hours later to detect ATM and vinculin (loading control) protein levels.

A



B

```
                3'- UGAGUGGCUGUCGCAACUUACAA -5'   miR-181a
                3'- UGGGUGGCUGUCGUUACUUACAA -5'   miR-181b
                                  |||||||
          …AAGUGAACUAUUGUGGG-UUUUUUUGAAUGUUGGUUUUAAUAC…   H. sapiens
miR-181 BS1 …AAGUGAACUAUUGUGGG-UUUUUUUGAAUGUUGGUUUUAAUAC…   P. troglodytes
          …AAGAGAACUAUUGUGGGUUUUUUUUGAAUGUUGGUUUUAAUAC…   M. mulatta
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          3'- UGAGUGGCUGUCGCAACUUACAA -5'   miR-181a
           3'-UGGGUGGCUGUCGUUACUUACAA -5'   miR-181b
                             |||||||
              UUU----UAGUACUAUUGAAUGUAUUAC-UUUACUGUU…   H. sapiens
miR-181 BS2   UUU----UGGUACUAUUGAAUGUAUUAC-UUUACUGUU…   P. troglodytes
              UUUAUUUUAGUACUAUUGAUUGUAUUAC-UUUACUGUU…   M. mulatta
              UUU-GUUUAGUGCUAGUAAAUGUUUUUCAGUUA-UGCU…   M. musculus
              UUU----UAGUACCAUUGAAUGUAUUUC-UU-AGCAUG…   F. catus
              UUU-GAGUA---CUAUUGAAUGUAUUUC-UUUAUUACA…   E. caballus
              UUU----CAGUACUAUUGAAUGUAUAUCUU--UAUUAU…   B. primigenius
```
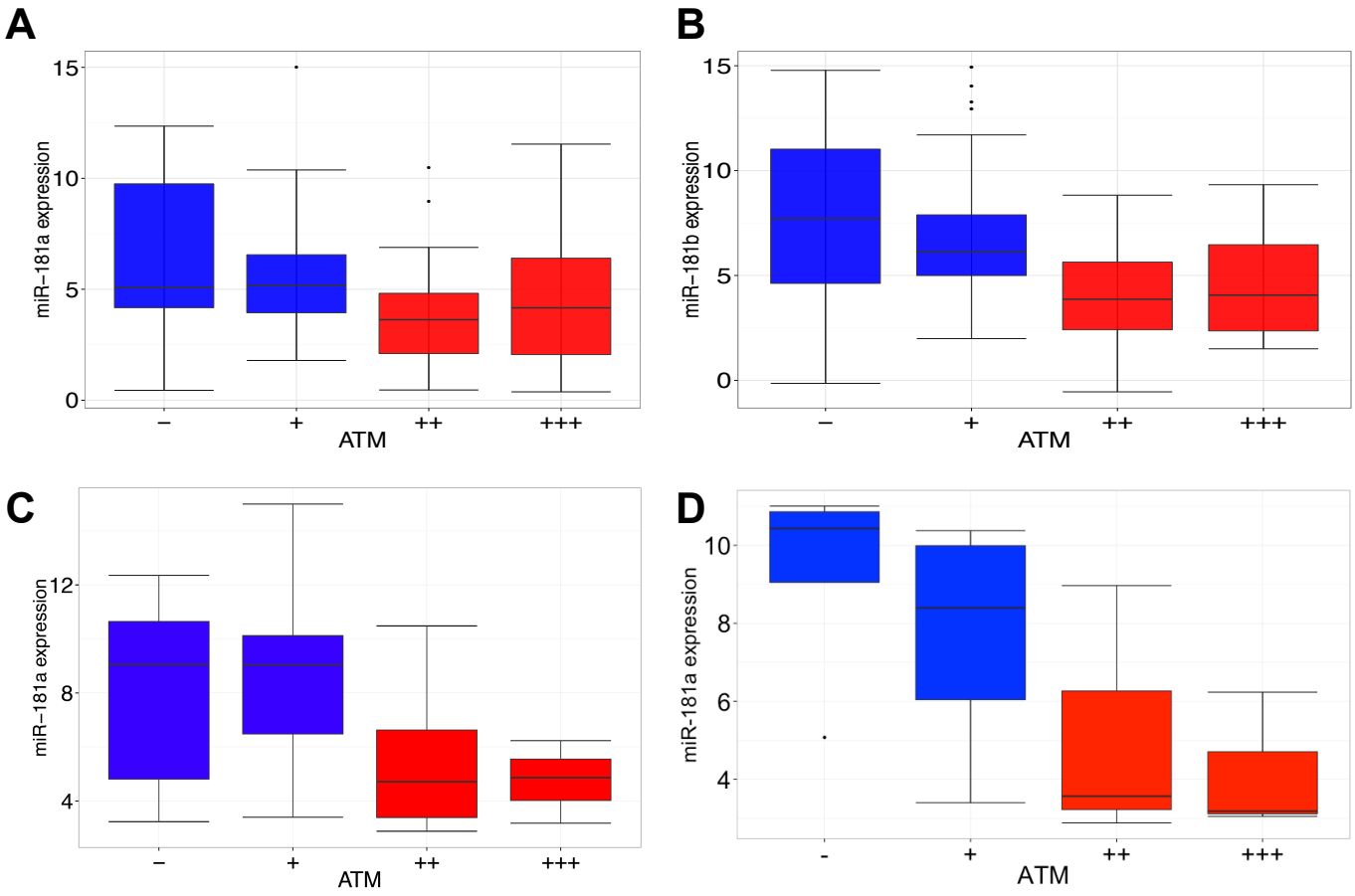
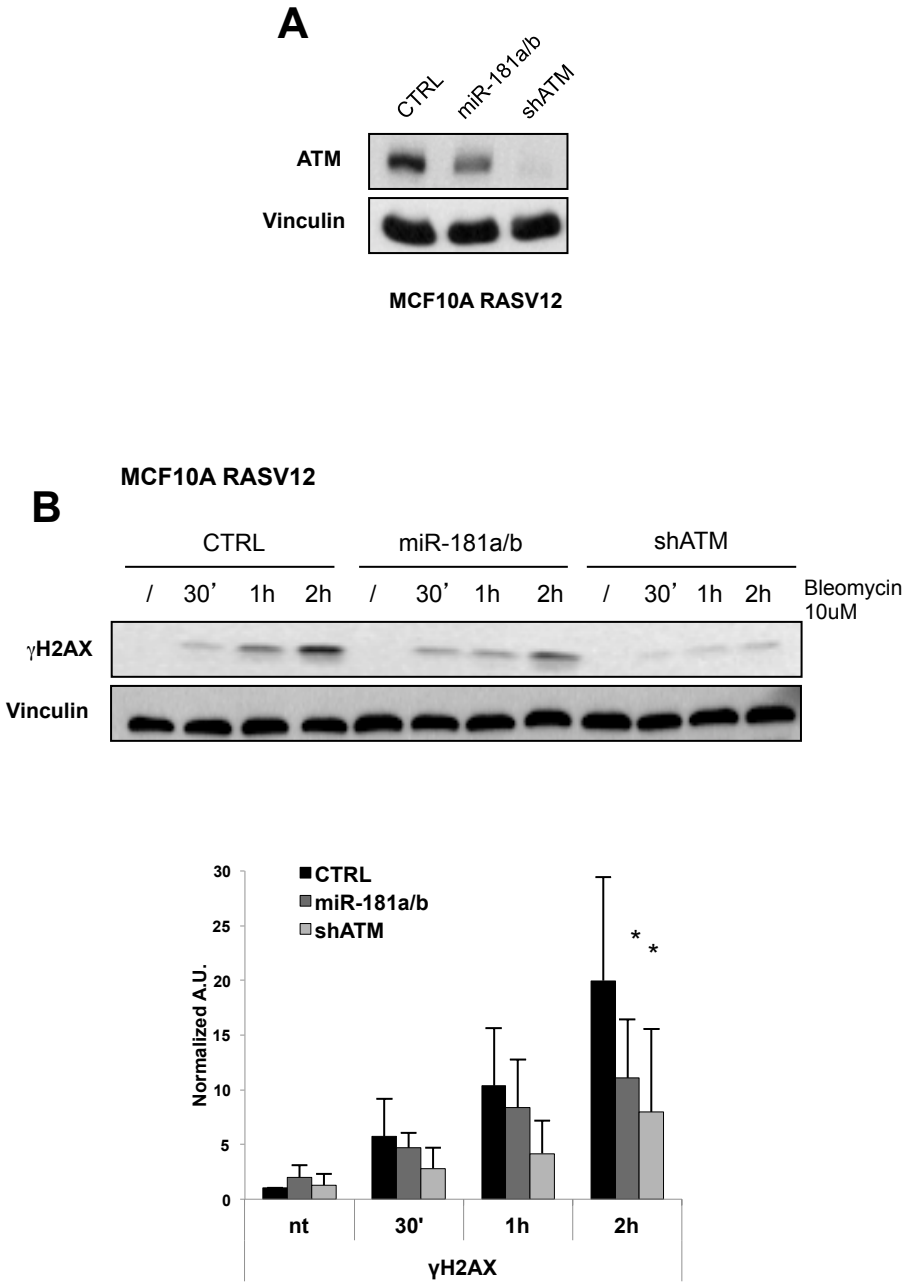

S4 – ATM is a direct target of miR-181a/b.

A) Luciferase reporter assay in H1299 cells transfected for 24h with luc-ATM 3'UTR wt or luc-ATM 3'UTR mut181 constructs along with a plasmid encoding both miR-181a and miR-181b (miR-181a/b). RLU, relative luciferase units. Graph shows mean and s.d. for at least three independent experiments. p-values were calculated with two tailed t-test, *= $p<0.05$.

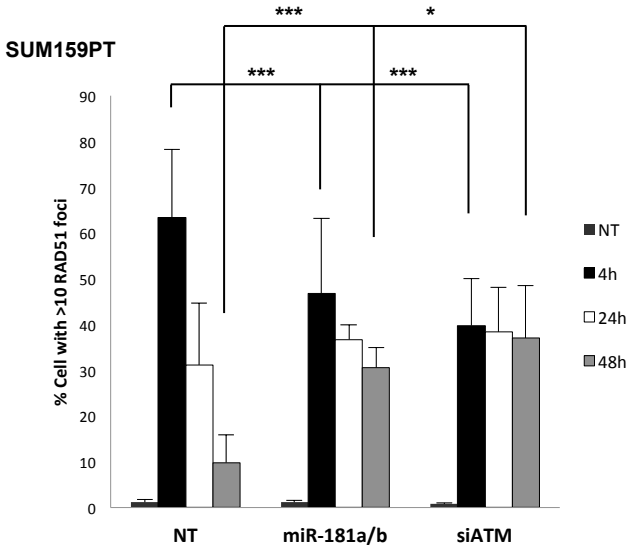
B) miR-181a/b predicted target sites in ATM 3'UTR.



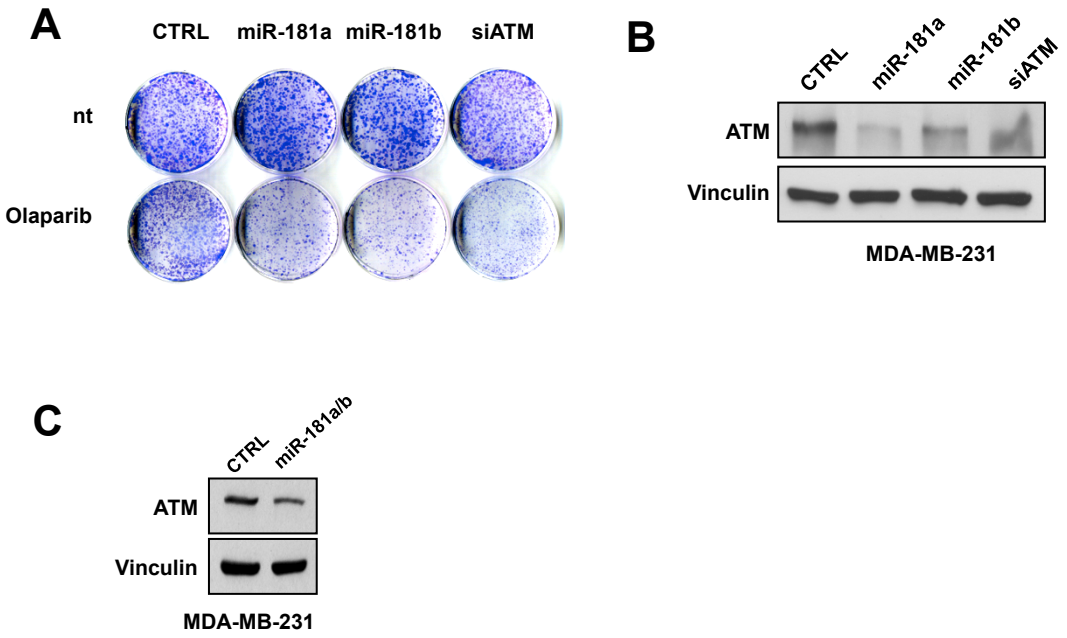
S5 –miR-181a/b correlate with ATM levels in Grade 3 and Triple negative breast cancers.
A) Plots of miR-181a and B) miR-181b expression (ΔΔCt) according to ATM staining intensity in breast cancer samples. p=0.002801 for miR-181a and $p<10^{-5}$ for miR-181b, anova test on linear regression models.
C) Plots of miR-181a expression (ΔΔCt) according to ATM staining intensity in grade 3 breast cancer samples. p= 0.1741, anova test on linear regression models.
D) Plot of miR-181a expression (ΔΔCt) according to ATM staining intensity in triple-negative breast cancer samples. p=0.09543, anova test on linear regression models.



S6 – miR-181a/b regulate ATM activation.
A) MCF10A cells transformed with RASV12 were transduced with retroviral constructs expressing miR-181a/b precursor, shATM or empty vector. Western blot analysis was performed to detect ATM and vinculin (loading control) protein levels. B) Cells were treated with bleomycin (10μM) for indicated times and Western blot analysis was performed to detect phosphorylated H2AX and vinculin levels (loading control). Graphs represent the mean and s.d. of three independent experiments. Phosphorylated H2AX and vinculin levels were measured with ImageJ software. p-value was calculated with two tailed t-test, *= $p<0.05$.



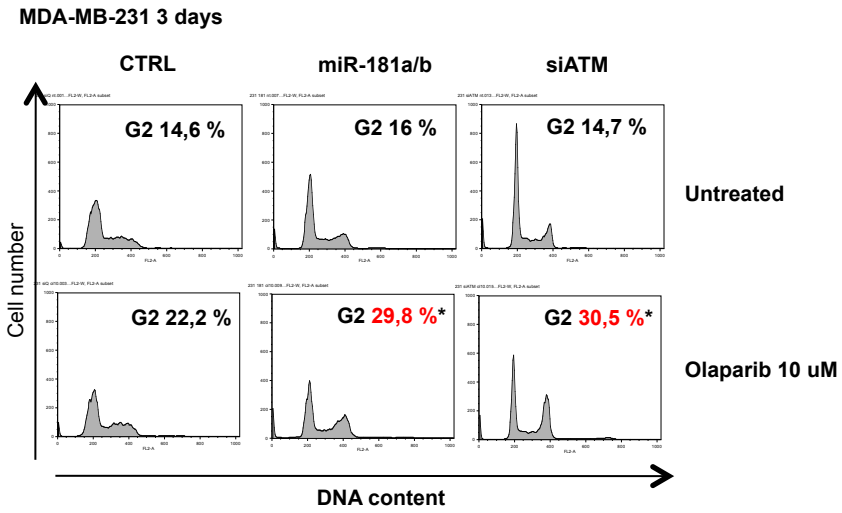
S7 – Relative to Figure 4 - miR-181a/b alters the assembly of RAD51 foci.
SUM159PT cells were transfected with a combination of miR-181a/b, ATM (siATM) or control siRNA and treated with 10Gy of IR. Cells were fixed after 4, 24 and 48 hours and immunofluorescence assay was performed to detect cells positive for RAD51 foci. Graph represents percentage of cells positive for RAD51 foci and shows means and s.d. for three independent experiments. p-values were calculated with two tailed t-test, *= $p<0.05$; **= $p<0.001$.



S8 - Relative to Figures 5A and 5B. - miR-181a/b sensitize cancer cells to Olaparib treatment.

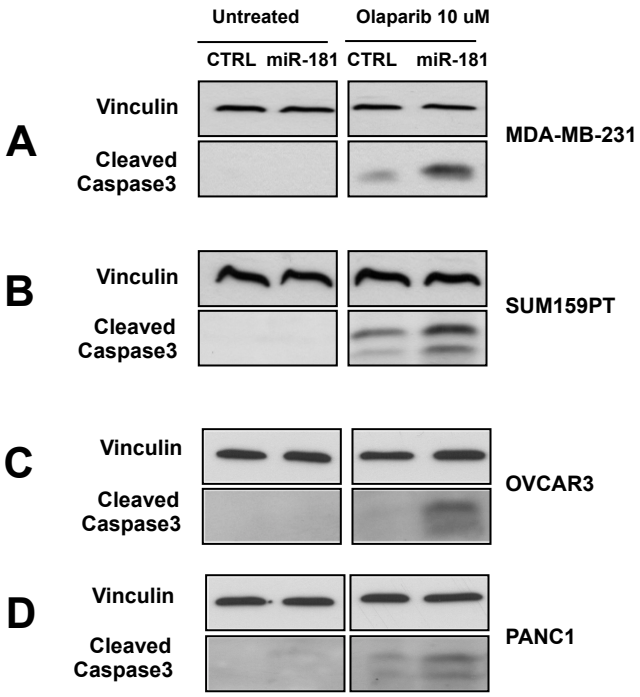
Representative images (A) and western blot analysis of ATM and vinculin (loading control) protein levels (B) in MDA-MB-231 cells analysed in experiments of colony formation reported in Figures 5A.

(C) Representative images and western blot analysis of ATM and vinculin (loading control) protein levels in MDA-MB-231 cells analysed in experiments reported in Figures 5B.

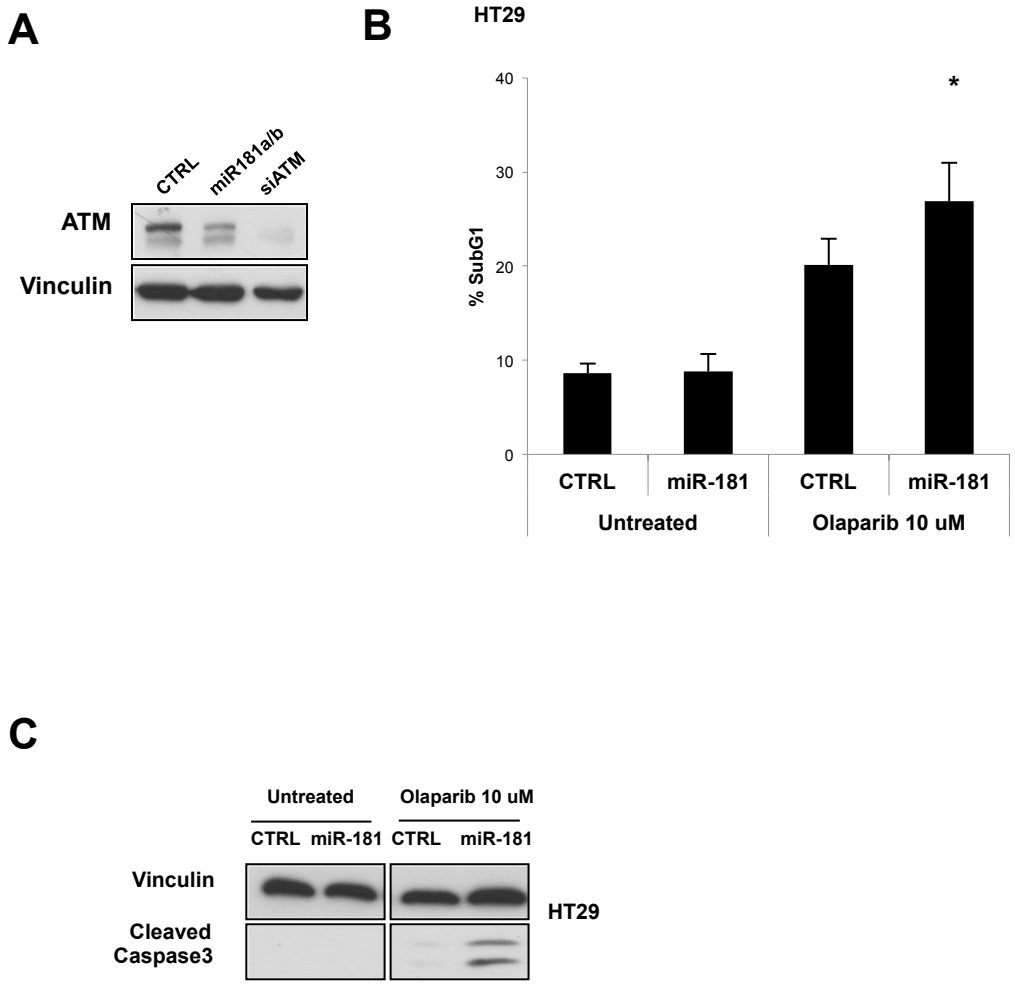


S9 – Treatment with Olaparib caused G2/M cell cycle arrest in miR-181a/b overexpressing cells.

MDA-MB-231 cells transfected with a combination of miR-181a and miR-181b, ATM (siATM) or control siRNA (CTRL) were treated with 10 μM Olaparib. After 3 days of treatment cells were harvested, stained with propidium iodide and subjected to FACS analysis. The mean of the percentage of G2/M population obtained in three independent experiments is reported for each sample. p-value was calculated with two tailed t-test, *= $p<0.05$.



S10 - miR-181a/b enhance Caspase 3 cleavage upon Olaparib treatment.
A) MDA-MB-231, B) SUM159PT, C) OVCAR-3 and D) PANC1 cells transfected with a combination of miR-181a/b or control siRNA were treated with 10 μM Olaparib for 4 (SUM159PT and PANC1) or 7 (MDA-MB-231 and OVCAR-3) days. Western blot analysis was performed to detect cleaved caspase-3 (apoptotic marker) and vinculin (loading control) protein levels.



S11 - miR-181a/b enhance Olaparib sensitivity in HT29 colon cancer cells

A) HT29 cells were transfected with a combination of miR-181a/b, ATM (siATM) or control siRNA(CTRL). Western blot analysis was performed after 72h to detect ATM and vinculin (loading control) protein levels.

B) HT29 cells transfected with a combination of miR-181a/b or control siRNA(CTRL) were treated with 10 μM Olaparib. After 7 days cells were harvested, stained with propidium iodide and subjected to FACS analysis. The percentage of SubG1 population is reported.

C) HT29 cells transfected with a combination of miR-181a/b or control siRNA and treated with 10 μM Olaparib for 7 days were subjected to Western blot analysis to detect cleaved caspase-3 and vinculin (loading control) protein levels.

Supplementary Table 1

104 BC dataset - Clinico-pathological characteristics

Sample size, No.		104
Age	**Mean (mini-max)**	63 (37-82)
Tumor size		
	≤ 2cm	78
	≥2cm	26
Lymph node status		
	node-positve	42
	node-negative	62
	unknown	0
Histologic grade		
	1	17
	2	60
	3	27
Estrogen Receptor (ER) status*		
	ER-positive	69
	ER-negative	24
HER-2 status*		
	HER-2-positive	8
	HER-2-normal	95
	Unknown	1

*Receptor status was assessed by the pathology lab as routine care.

Supplementary Table 2

123 BC dataset - Clinico-pathological characteristics

	Primaries from patients developing distant metastases	Primaries from patients with no evidence of distant metastases for more than 60 months	P value
Size			
≤ 2 cm	26	38	0.106
> 2 cm	33	26	
Age			
≤ 50 years	18	27	0.195
> 50 years	41	37	
ER status*			
positive	47	53	0.817
negative	12	11	
Total	59	64	

*ER status defined based on *ESR1*gene expression levels