Amplification and overexpression of *MAP3K3* gene in human breast cancer promotes formation and survival of breast cancer cells

Running Title: MAP3K3 amplification in human breast cancer

Keywords: MAP3K3, kinase, oncogene, breast cancer

Yihui Fan[1,2,11], Ningling Ge[1,2,8,11], Xiaosong Wang[3,4,11], Wenjing Sun[1,2,11], Renfang Mao[5], Wen Bu[3], Chad J. Creighton[4], Pingju Zheng[3], Sanjeev Vasudevan[6], Lei An[5], Jinshu Yang[7], Yi-Jue Zhao[1,2], Huiyuan Zhang[9], Xiao-Nan Li[1,2], Pulivarthi H. Rao[1,2], Eastwood Leung[1,2], Yong-Jie Lu[7], Joe W. Gray[10], Rachel Schiff[3], Susan G. Hilsenbeck[3,4], C. Kent Osborne[3,4], Jianhua Yang[1,2,4], Hong Zhang[5,9,*]

Supplementary Materials and Methods

Analysis of paired-end transcriptome sequencing data

Mate pair transcriptome reads were mapped to the human genome (hg18) and Refseq transcripts using Burrows-Wheeler Aligner （Li H, Bioinformatics 2009; 25: 1754-1760）. Chimerical sequences were extracted based on the approach applied in our previous study（Wang XS，Nat Biotechnol 2009; 27: 1005-1011）.

Quantitative RT-PCR, genomic PCR and sequencing

RNAs from breast cancer cell lines were reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) and random plus oligo-dT primers. Polymerase chain reaction was performed using MYO15B-MAP3K3 specific fusion primers for 35 cycles. The primers are listed in Supplementary Table 1. The PCR product was purified and sequenced (by capillary sequencing) bidirectionally using both forward and reverse primers.

100 ng of total RNA isolated using Trizol (Invitrogen, Carlsbad, CA) was used for quantitative RT-PCR to detect the mRNA levels of MAP3K3 and GAPDH (internal control) with one-step SYBR Green real-time qRT-PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The specific primers for MAP3K3 were 5'-GTTGAAGGCTTACGGTGCTC-3' and 5'-ACATTCCCAGCAGAGTCTCG-3'. The primers for GAPDH were 5'-AAGGTGAAGGTCGGAGTCAA-3' and 5'-TGGACTCCACGACGTACTCA-3'. The mRNA level of MAP3K3 in each sample was normalized to GAPDH and the relative expression level of MAP3K3 is

represented as fold change relative to MCF-10A. 100 ng of genomic DNA extracted cells using standard protocol was used for real-time qPCR to detect the genomic DNA level of MAP3K3 with SYBR Green real-time qPCR kit from Qiagen. The specific primers for MAP3K3 genomic DNA were: 5'-GAGAACATGGGTCTGGCTGT-3' and 5'-CTTGGTTGGCACATTCCTCT-3'. Copy number was calculated relative to normal breast tissue control in patient specimens. Each sample was determined from triplicate reactions.

Immunoblotting

Cells or tumors were scraped into cold PBS buffer, pelleted and lysed in protein lysis buffer (25 mM HEPES, pH 7.7, 135 mM NaCl, 3 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Benzomidine, 20 mM disodium *p*-nitrophenylphosphate, 1 mM phenylmethylsulfonyl fluoride) on ice for 30 minutes, clarified by centrifugation at 15,000 g for 10 minutes at 4°C. Equal amounts of protein in the supernatants (100 μg) were subjected to 10% SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were probed with the antibodies as indicated. Proteins were visualized using the ECL detecting system (Amersham Biosciences).

Colony formation assay

Aliquots of 1×10^4 cells were mixed with a final concentration of 0.3% Sea Plaque agarose (Hoefer Scientific Instruments, San Francisco, CA) and DMEM containing 12% FBS and were overlaid on a 0.5% agarose layer containing DMEM and 12% FBS in 6-well plates. The plates were put in a humidified incubator at 37°C with 5%

CO_2 for 2 weeks. Each well was stained with 0.5 ml 5 mg/ml sterile-filtered MTT solution for 4 hours and stained colonies (≥ 15 mm) were counted. All samples were assayed in triplicate.

Statistical analyses

Comparisons of RT-PCR data, proliferation, colony formation, and apoptosis were analyzed using two-tailed student t tests. The p values of 0.05 or less were defined as statistically significant. Error bar represents standard deviations or standard error of the mean.

Supplementary Table 1. Reverse transcription PCR primers specific to MYO15B-MAP3K3 fusion

Primers	Type	Sequence (5'->3')
MYO15B-MAP3K3_F1	Forward	AGCATTGGCACCGTCACTGTCG
MYO15B-MAP3K3_R1	Reverse	CCTGTCCAAGGATTGGCAGCTTCCA
MYO15B-MAP3K3_F2	Forward	ACTGTCGTGGATGCCTACGGCT
MYO15B-MAP3K3_R2	Reverse	TGTCTGCTGACCTGTCCAAGGATT
MYO15B-MAP3K3_F3	Forward	AGAACCAATGCACGGCTGGCAC
MYO15B-MAP3K3_R3	Reverse	AATTTTCTGCACTGCTCAGGGGATC

Supplemental Figure Legends

Figure S1. *MAP3K3* is amplified and overexpressed in breast cancer cell lines.

(A) Representative FISH images of *MAP3K3* in MAP3K3 non-amplified MDA-MB-435 cells. (B) Summary of important breast cancer biomarkers in the above analyzed breast cancer cell lines. (C) The gene copy number of *MAP3K3* in Luminal and Basal B subtypes is higher than it in Basal A subtype. (D) The mRNA level of *MAP3K3* in Luminal and Basal B subtypes is higher than that in Basal A subtype.

Figure S2. MAP3K3 is not associated with ER, PR, or HER2 in human breast cancers. Data were collected and analyzed using Oncomine (https://www.oncomine.com). After Oncomine datasets were filtered for breast cancer, clinical specimen, > 151 samples, mRNA, and with ER, PR, and HER2 status, the Bittner Breast dataset (n=336) met all these criteria. Relative *MAP3K3* expression levels are shown among samples with No value (0), Positive (1), or Negative (2) expression of ER, PR, and HER2. P values were derived by ANOVA.

Figure S3. Identification of *MYO15B-MAP3K3* gene fusion in the MDA-MB-453 breast carcinoma cell line.

(A) Representative copy number data show high-level amplifications and unbalanced rearrangements at the *MAP3K3* locus in primary breast cancer tissues (data from The

Cancer Genome Atlas) and cell lines (data from Dr. Joe Gray). Relative quantifications of copy number data for paired tumor and normal tissues are shown. Copy number data are visualized by Integrative Genomics Viewer (IGV). (B) RT-PCR validation of *MYO15B-MAP3K3* gene fusion in MDA-MB-453. Two negative controls (MDA-MB-361 and MCF-7) were included in the analysis. At the right is the capillary sequencing result of the PCR products (primers F1 and R1 as shown in Supplementary Table 1). The sequence of *MYO15B* is shown in brown color and that of *MAP3K3* in green color. (C) A diagram showing fusion of exon 9 of *MYO15B* with exon 7 of *MAP3K3*.

Figure S4. The effect of knockdown of *MAP3K3* expression on cell proliferation and colony formation in MCF-7, MDA-MB-361 and MDA-MB-231 breast cancer cells.

(A) Colony assay showing reduced colony formation in *MAP3K3*-knockdown MCF-7 cells (Day 14). (B) Quantification of mean colony numbers in (A). Error bars represent standard deviation from triplicate samples. (C) Colony assay in *MAP3K3*-knockdown MDA-MB-231 cells (Day 14). (D) Quantification of mean colony numbers in (C). Error bars represent standard deviation from triplicate samples. (E) Immunoblotting analysis confirming *MAP3K3* knockdown in MDA-MB-361 cells. (F) Cell growth assay showing reduced growth of *MAP3K3* knockdown cells. Cell Counting Kit-8 (CCK-8) was used to determine relative cell numbers. Error bars represent standard deviation from triplicate samples. (G) Colony formation assay showing reduced colony forming potential of *MAP3K3*-knockdown cells. After 14 days of growth, the colonies were stained with MTT. (H) Quantification of mean colony numbers in (G). Error bars represent standard deviation from triplicate

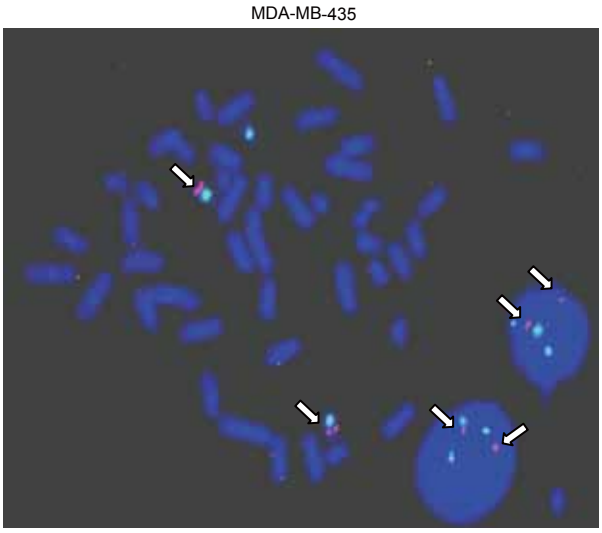
samples. *<0.05, ** P<0.01, t-test. Results from (E) and (G) are representative of three independent experiments.

Figure S5. The effect of knockdown of MAP3K3 expression on Dox- and VP16-induced cell death in MDA-MB-468 and MDA-MB-453 cells.

(A, B) Quantification of cell numbers (by CCK-8) of MDA-MB-468 (A) and MDA-MB-453 (B) cells in cell cultures treated with the indicated concentrations of doxorubicin. (C, D) Quantification of cell numbers (by CCK-8) of MDA-MB-468 (C) and MDA-MB-453 (D) cells in cell cultures treated with the indicated concentrations of VP-16.

Figure S1

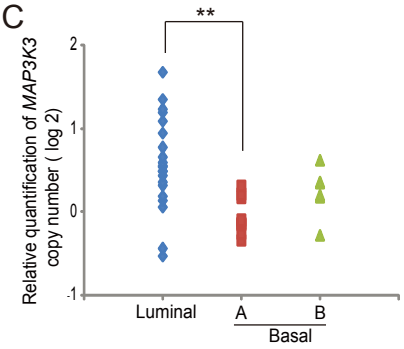
A



B

Cell line	ER	PR	HER2	TP53	MAP3K3		
					Amplification	RNA level	Protein level
MCF-10A	+	+	-	WT	No	Low	Low
MCF-7	+	+	-	WT	Yes	High	High
MDA-MB-361	+	+	+	Mutant	Yes	High	High
MDA-MB-231	-	-	-	Mutant	No	Low	High
MDA-MB-468	-	-	-	Mutant	no	Low	Low
SK-BR-3	-	-	+	Mutant	No	Low	High

C



E

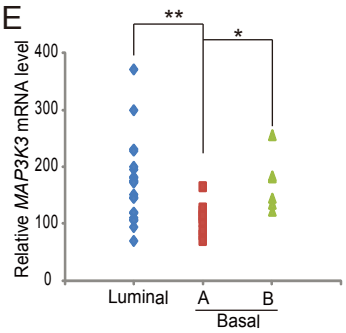
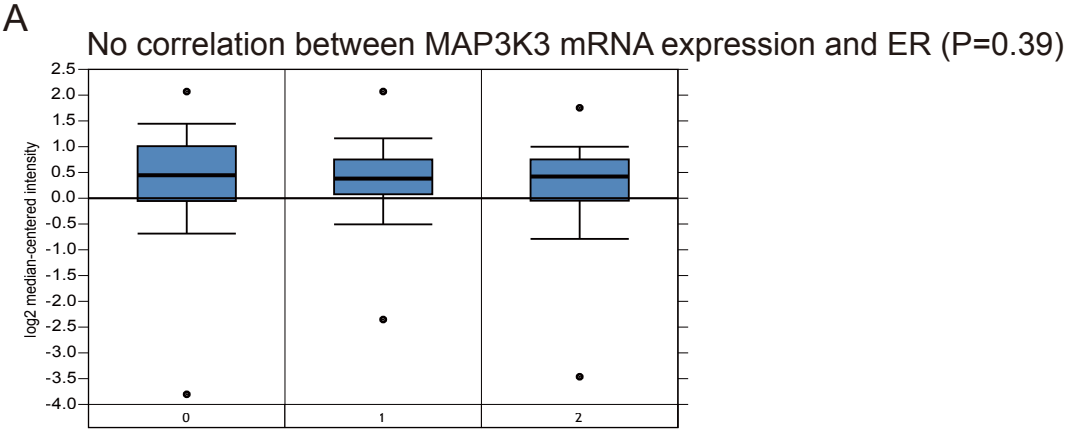
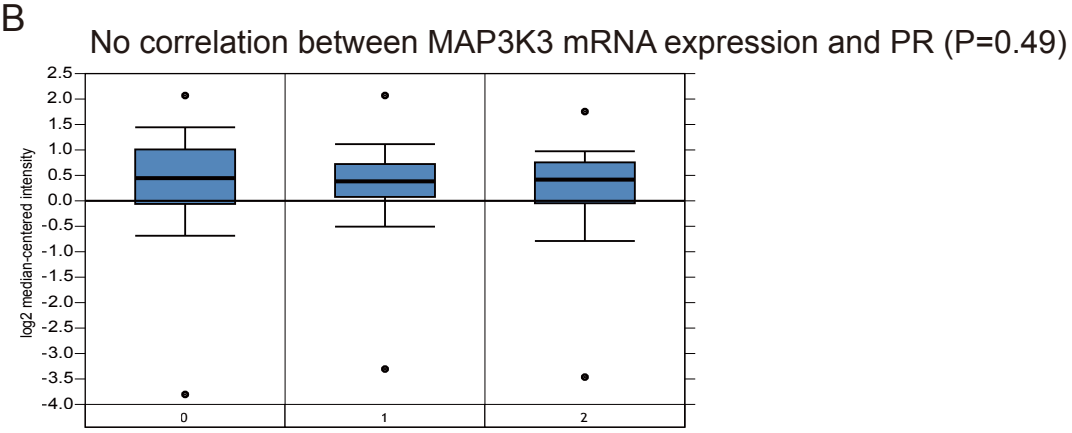


Figure S2

A



B



C

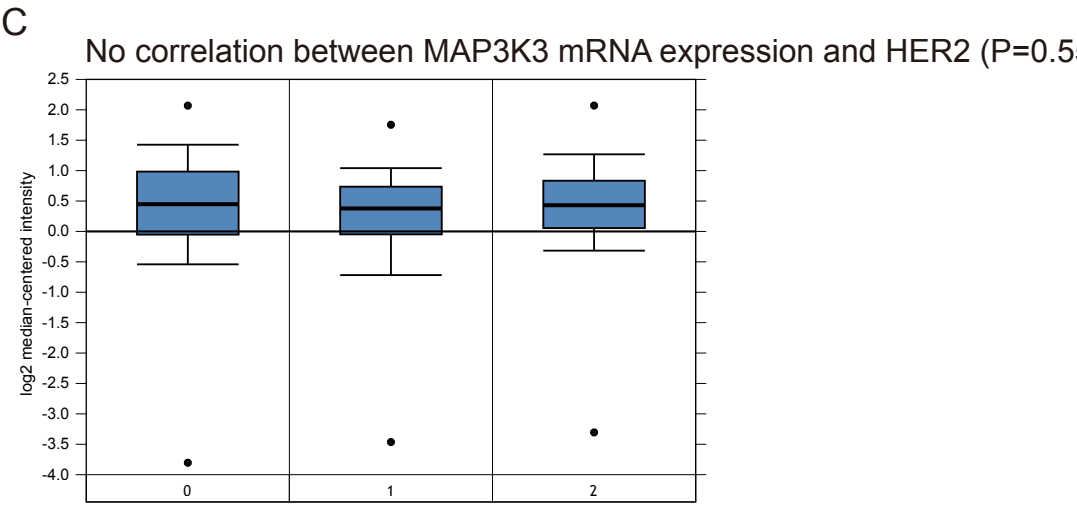


Figure S3

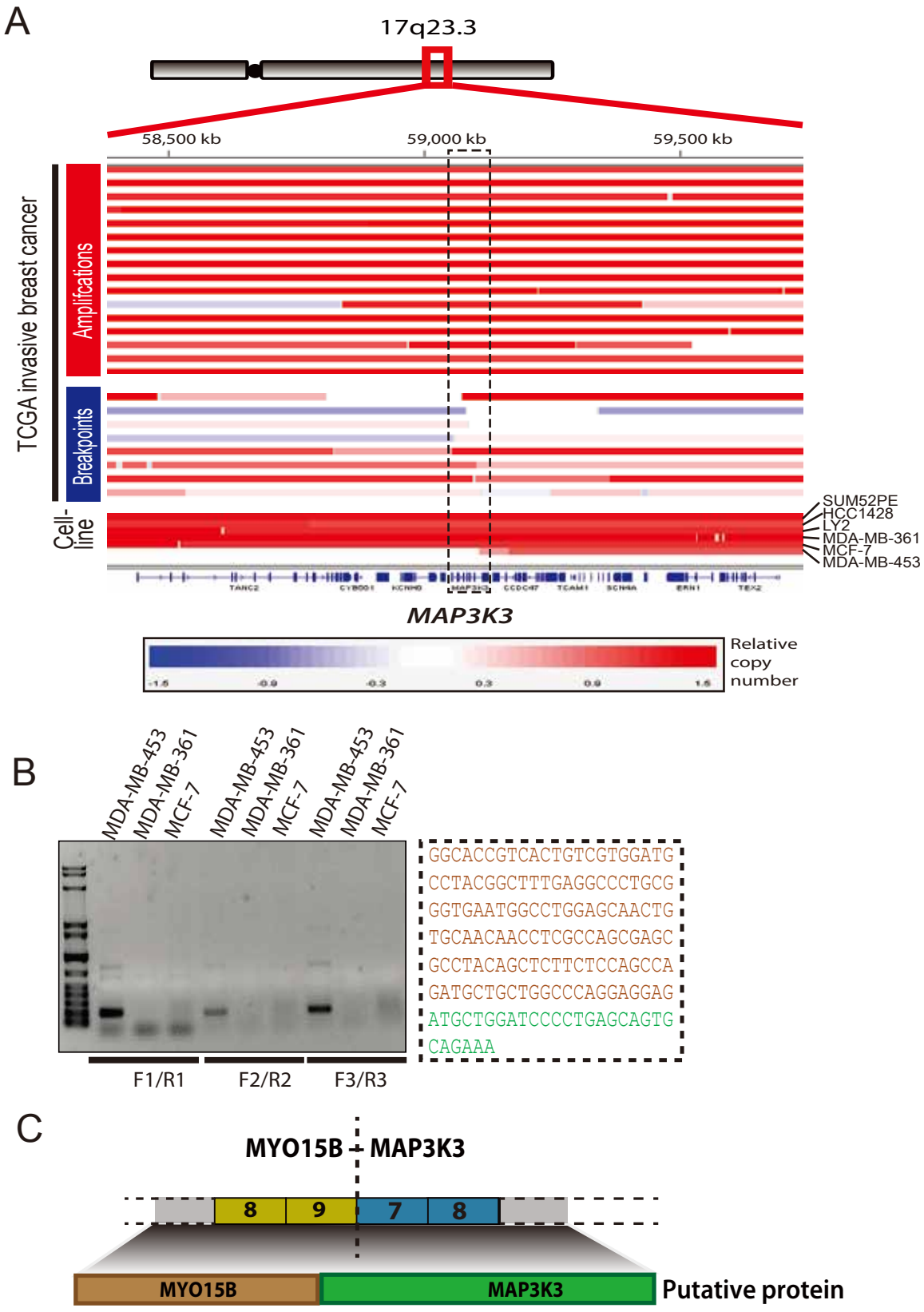

A
17q23.3
58,500 kb
59,000 kb
59,500 kb
TCGA invasive breast cancer
Amplifications
Breakpoints
Cell-line
SUM52PE
HCC1428
LY2
MDA-MB-361
MCF-7
MDA-MB-453
MAP3K3
Relative copy number
B
MDA-MB-453
MDA-MB-361
MCF-7
F1/R1
F2/R2
F3/R3
GGCACCGTCACTGTCGTGGATG
CCTACGGCTTTGAGGCCCTGCG
GGTGAATGGCCTGGAGCAACTG
TGCAACAACCTCGCCAGCGAGC
GCCTACAGCTCTTCTCCAGCCA
GATGCTGCTGGCCCAGGAGGAG
ATGCTGGATCCCCTGAGCAGTG
CAGAAA
C
MYO15B
MAP3K3
8
9
7
8
MYO15B
MAP3K3
Putative protein

Figure S4

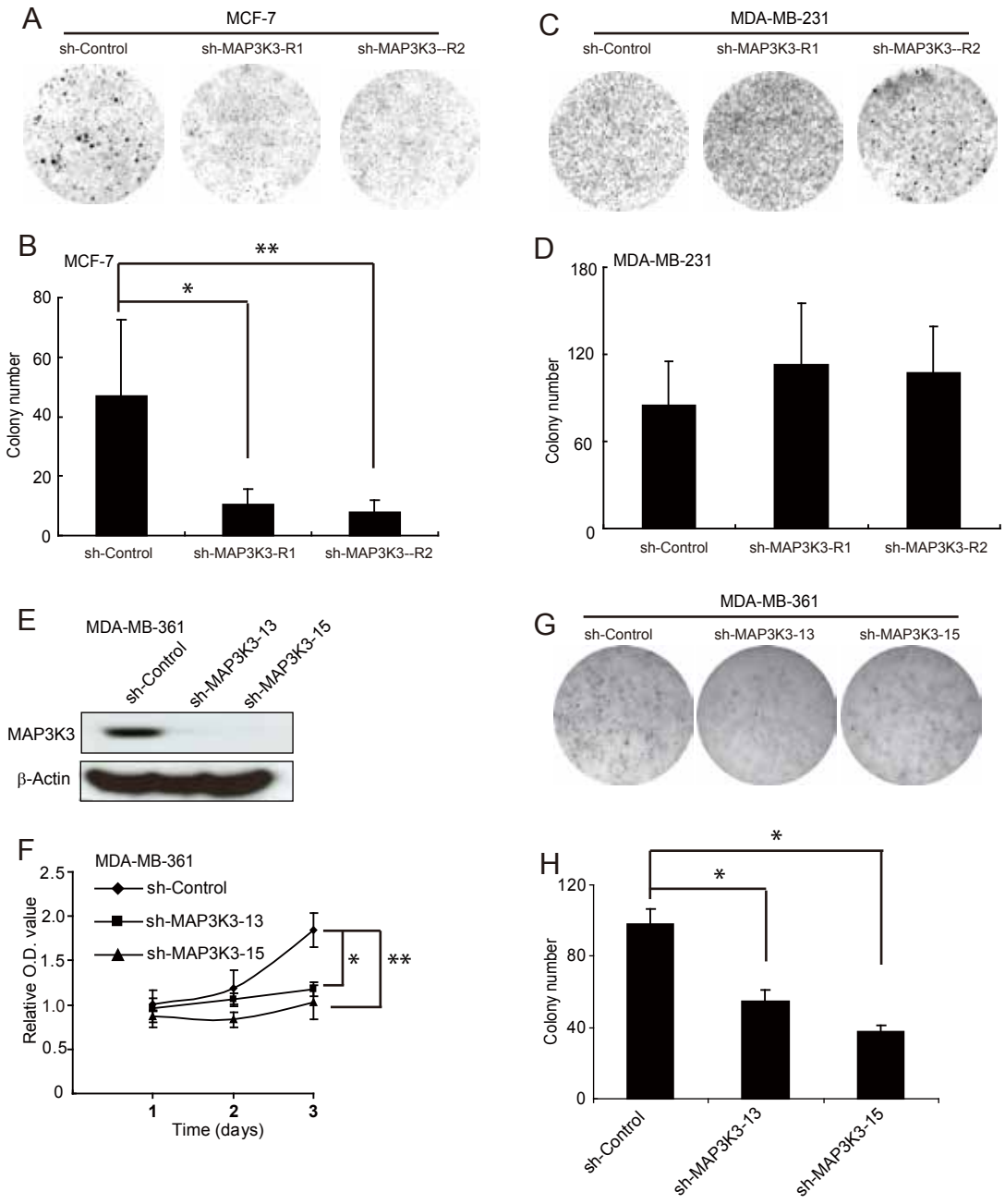
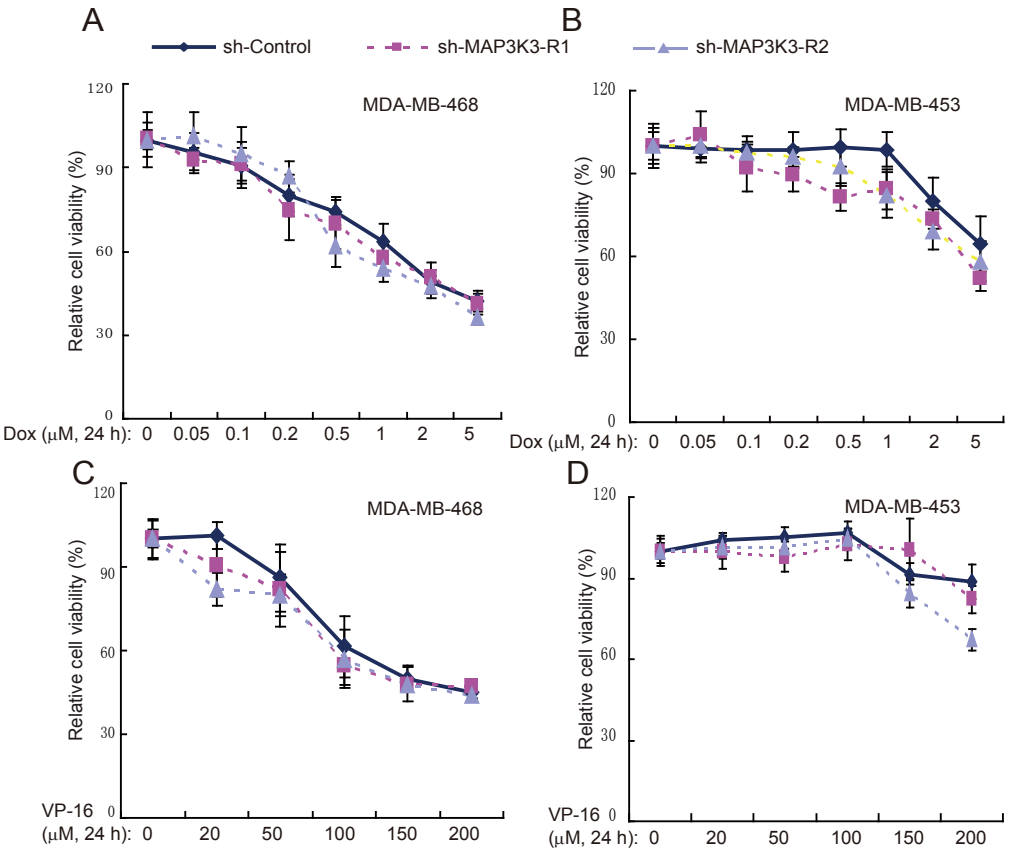

A
MCF-7
sh-Control
sh-MAP3K3-R1
sh-MAP3K3--R2
C
MDA-MB-231
sh-Control
sh-MAP3K3-R1
sh-MAP3K3--R2
B
MCF-7
**
*
Colony number
80
60
40
20
0
sh-Control
sh-MAP3K3-R1
sh-MAP3K3--R2
D
MDA-MB-231
Colony number
180
120
60
0
sh-Control
sh-MAP3K3-R1
sh-MAP3K3-R2
E
MDA-MB-361
sh-Control
sh-MAP3K3-13
sh-MAP3K3-15
MAP3K3
β-Actin
G
MDA-MB-361
sh-Control
sh-MAP3K3-13
sh-MAP3K3-15
F
MDA-MB-361
sh-Control
sh-MAP3K3-13
sh-MAP3K3-15
Relative O.D. value
2.5
2.0
1.5
1.0
0.5
0
*
**
1
2
3
Time (days)
H
*
*
Colony number
120
80
40
0
sh-Control
sh-MAP3K3-13
sh-MAP3K3-15

Figure S5


A
B
sh-Control
sh-MAP3K3-R1
sh-MAP3K3-R2
MDA-MB-468
MDA-MB-453
Relative cell viability (%)
120
90
60
30
0
Dox (μM, 24 h): 0 0.05 0.1 0.2 0.5 1 2 5
C
D
VP-16 (μM, 24 h): 0 20 50 100 150 200