SUPPLEMENTARY MATERIAL AND METHODS.

Proliferation, Adhesion and Migration assays.

Cell cycle distribution was evaluated by Propidium Iodide staining of ethanol fixed cells, using a FACSscan Becton Dickinson cytometer interfaced with the Cell Quest software. MDA-MB-231 and SKBR3 cells were serum starved for 24 hours and then cultured in the presence of the indicated concentration of PS or WF for the following 12 hours. Cells were then fixed and evaluated for their DNA content by FACS analysis.

MTT (Sigma) assay was used to evaluate cell growth over a period of 4 days, following the manufacturer's instruction. Briefly, 1000 or 5000 cells/well of each cell line were plated in 96-well plates. Cells were allowed to grow in serum-reduced medium supplemented with 1% PS or WF, as indicated. At indicated times, cells were incubated with MTT for 4 hours at 37°C. The amount of MTT incorporated into the cells represents a direct expression of the viable cell number in each well, and was evaluated by dissolving MTT crystals in 200 ml DMSO and then reading the plates at 560 nm.

MTT assay on HMEC was performed as above, except for the different use of serum free medium. To prevent cell death by serum deprivation cells were cultured in 30% complete medium (MEBM)-70% serum-reduced medium. This was used as basal medium to which 1% PS or WF was added to evaluate ability to stimulate cell growth.

To evaluate the growth of mammary normal and cancer cells within a 3D-matrix we used a published protocol (Debnath et al. 2003). Briefly, 8 well Labtek chamber slides (Becton Dickinson) were filled with 100 μl of Collagen I (2mg/ml) or Matrigel™ (10mg/ml)/well and the matrices allowed to polymerize. 1000 cells for each well were then resuspended in 100 μl Collagen I (0.85mg/ml) or Matrigel™ (0.25mg/ml) gel and layered on top of the polymerized matrices. Complete medium, or serum free medium supplemented with 2% PS or WF was then added on top of the matrix-included cells and incubation was carried out at 37°C for 15 days. The appropriate medium was then replaced every 3 days, till the end of the experiment. After 15 days, wells were

fixed in 4% PFA, permeabilized with PBS-0.2%Triton and stained with cytox green (nuclear staining) and texas-red conjugated phalloidin (Molecular Probes) for 30 minutes at RT. In some experiments only cytox green was used to stain the colonies. After several washes in PBS, labelled cells were observed using a confocal laser-scanning microscope (TSP2 Leica) interfaced with a Leica DMIRE2 fluorescent microscope. Colony area was calculated using the Leica LAS Software. Migration and invasion assays were performed essentially as previously described (Baldassarre et al., 2005). Briefly, for migration, HTS Fluoroblok transwells (Becton Dickinson) were saturated 2 hrs at room temperature with PBS 1% BSA. Serum starved cells were labelled with DiI (Molecular Probes) for 20 minutes at 37°C before being seeded in the Fluoroblok^{TM} upper chamber (1×10^5 cells/chamber) and then incubated at 37°C for the indicated time. The lower chamber was filled with serum free medium supplemented with 2.5% PS or WF, as indicated. Migrating cells were evaluated by reading at different time points with a Spectrafluor (Tecan) the lower and the upper sides of the membrane. For single serum analysis, the experiment was performed in duplicate, while the experiments in which sera pools were used as attractants was performed at least 3 times in duplicate .

For invasion experiments, HTS Fluoroblok transwells (Becton Dickinson) were coated overnight at 4°C with 80μg/ml Matrigel^{TM} and then saturated 2 hrs at room temperature with PBS 1% BSA. MDA-MB-231 cells were then processed as described above and allowed to invade the matrix up to 24 hours.

Time lapse microscopy.

Time lapse microscopy for 3D motility was performed essentially as previously described (Friedl et al., 1997).

Briefly, MDA-MB-453 cells from subconfluent cultures were detached using EDTA (2mM), washed in serum-free medium, and suspended in a buffered Collagen I solution, pH 7.4, at 1.67mg/ml. The suspension was dispensed in a self-constructed 3D chamber, allowed to polymerize

for 30 minutes at 37°C in a 5% CO_2 atmosphere and then overlaid with serum-free medium supplemented with 5% PS or WF, as indicated.

The pictures/images were collected every 4 minutes for 18 hours using a CCD camera mounted onto the microscope. A 10X objective was used. During the recording time the cell migration chambers were maintained at 37°C using a remote temperature control system. Collected images were used to create a movie (10 images per second, Quicktime) and analyzed with a cell tracking software to collect different locomotion parameters (Friedl el al., 1993; Maaser et al., 1999). In brief, 40 cells were randomly selected from the computer screen and their X/Y coordinates were obtained for each step (12 minutes step interval). After calibration and calculation of the pixel resolution factor, the pixel data were converted in micrometers and several migration parameters were obtained for each cell population. In particular, the total distance covered and the cell speed, as averages of the single cells values, were analyzed.

Supplementary References.

Baldassarre, G. *et al*. p27(Kip1)-stathmin interaction influences sarcoma cell migration and invasion. *Cancer Cell* 7, 51-63, (2005).

Debnath, J., Muthuswamy, S.K. & Brugge, J.S. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* **30**, 256-268 (2003).

Friedl, P., Maaser, K., Klein, C.E., Niggemann, B. & Zänker, K.S. Migration of highly aggressive MV3 melanoma cells in 3-D collagen lattices results in local matrix reorganisation and shedding of beta1 integrins and CD44. *Cancer Res*. **57**, 2061–2070 (1997).

Friedl, P., Noble, P.B. & Zänker, K.S. Lymphocyte locomotion in three-dimensional collagen gels. Comparison of three quantitative methods for analyzing cell trajectories. *J. Immunol. Methods* **165**, 157–165 (1993).

Maaser, K., Wolf, K., Klein, C.E., Niggemann, B., Zänker, K.S., Brocker, E.B. & P. Friedl. Functional hierarchy of simultaneously expressed adhesion receptors: integrin alpha2beta1 but not CD44 mediates MV3 melanoma cell migration and matrix reorganization within three-dimensional hyaluronan-containing collagen matrices. *Mol. Biol. Cell*. **10**, 3067–3079 (1999).

Supplementary Figure Legends

Supplementary Figure 1. *Wound fluids stimulate the growth of tumor-derived but not of normal mammary epithelial cells*. **A/B**. MTT proliferation assay of MDA-MB 231 human mammary carcinoma cells (**A**) or NMuMG normal mouse mammary epithelial cells (**B**), incubated for 4 days in the presence of Pre-operative Serum (PS) or Wound Fluid (WF) from the indicated patients, diluted in SFM at 1%. Viable cells were evaluated by reading the absorbance at 560 nm. Each sample was plated in sextuplicate. **C**. MTT proliferation assay of SKBR-3 cultured in the presence of PS or WF as indicated. 10% FBS (FBS) was used as positive control. Data represent the mean (± S.D.) of treatment of the cells with 20 different PS and WF samples/group of patients and each serum has been evaluated in sextuplicate. **D**. MTT proliferation assay of NMuMG cells cultured in the presence of PS or WF, as indicated. Data represent the mean (± S.D.) of treatment of the cells with 20 different PS and WF samples/group of patients and each serum has been evaluated in sextuplicate.

Supplementary Figure 2. *TARGIT treatment impairs cancer cell growth in 3D-matrices*.

T47D (**A**), MCF-7 (**B**) and MDA-MB-231 (**C**) cells were included in a 3D-Collagen I or Matrigel™ matrices, as indicated, and allowed to grow for 15 days. Cells were then stained with cytox green (green) to visualize cell nuclei and analyzed by confocal microscopy by performing sections every 10 μm. The maximum colony area was evaluated for at least 100 colonies and reported in the right graphs. Area of the colonies was measured using Leika LAS software and data plotted as box plots for T47D and MCF-7 and for MDA-MB 231. The median value (line within the box), inter-quartile range representing 50% of the data (boundaries of the box), the spread (vertical lines) representing the highest and lowest value (horizontal lines) are shown.

Supplementary Figure 3. *TARGIT treatment impairs wound fluid-induced stimulation of mammary carcinoma cells migration*. **A/B**. MCF-7 (**A**), and MDA-MB 453 cells (**B**) were assayed in a transwell-based motility assay for their ability to migrate toward the indicated

chemoattractants. PS and WF pools were used at a 2.5% concentration. NIH-3T3 fibroblasts conditioned medium was used as positive control (NIH CM). The percentage of migrated cells at two different time points is shown and represents the mean (± S.D.) of three independent experiments performed in duplicate. **C/D**. Percentage of MDA-MB-231 cells migrated toward sera from untreated (**C**) or TARGIT-treated patients (**D**). Data represent the mean of two independent experiments.
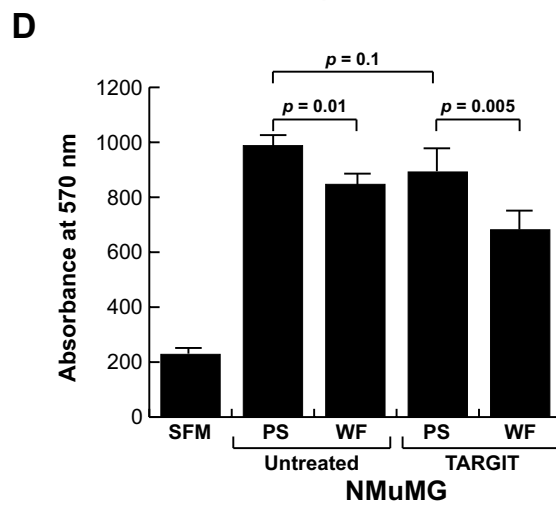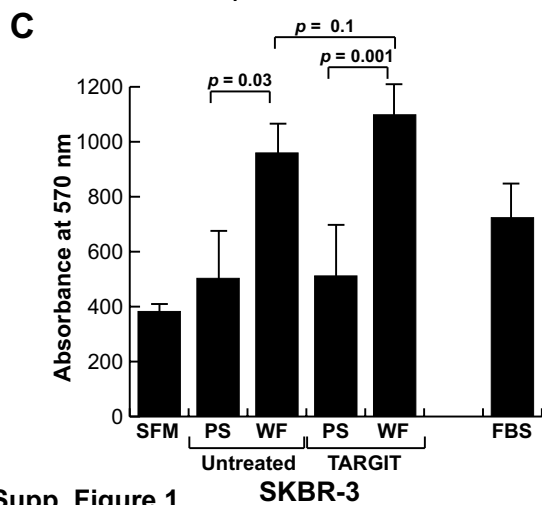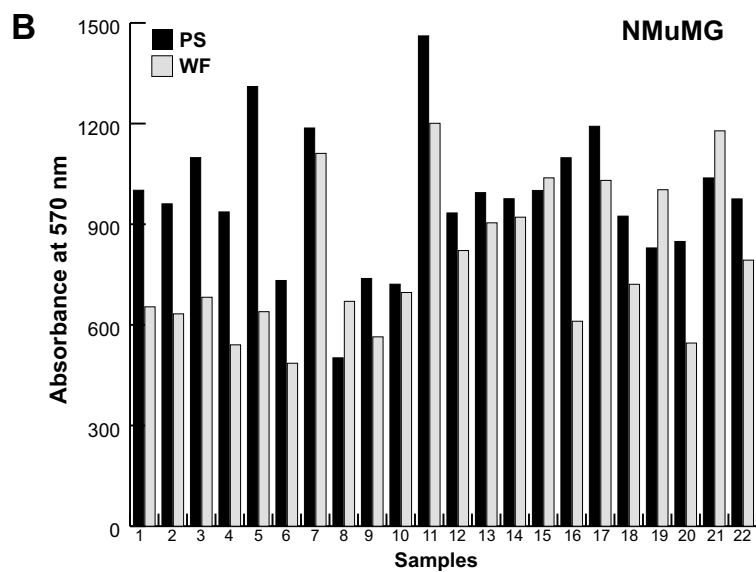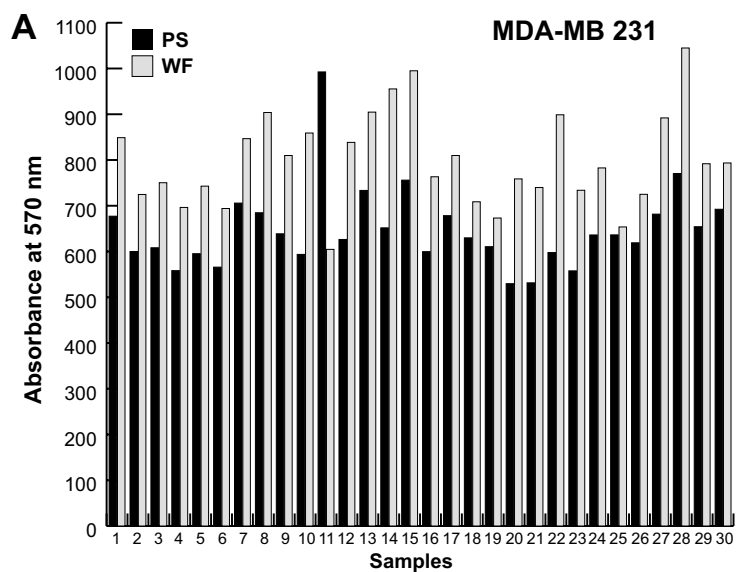
Supplementary Figure 4. *TARGIT treatment impairs wound fluid-induced stimulation of mammary carcinoma cells invasion*. **A**. Percentage of MDA-MB 231 cells invading a thick layer of Matrigel™, in response to the indicated WF used at a concentration of 2.5%. Data represents the mean (± S.D.) of two independent experiments. **B**. Percentage of MDA-MB 231 cells invading a Matrigel™ gel in response to the indicated PS- or WF-pool of sera, used at a concentration of 2.5%. Data represents the mean (± S.D.) of three independent experiments performed in duplicate. **C**. Number of moving MDA-MB 453 cells immersed in a 3D-Collagen I matrix and treated as indicated. Data represent the mean of 3 experiments, in which 40 cells/experiment were followed by cell tracking. Statistical analyses were performed using the t-student test in A and B and the Mann-Whitney U test in C.

Supplementary Figure 5. *Activation of intracellular signaling pathways in MCF-7 cells upon stimulation with wound fluids*. Western blot analysis of the indicated phosphoproteins in MCF-7 cells serum starved 24 hrs (lane 0) and then stimulated with the indicated sera at 5%, for 5, 15, 30 and 60 minutes. Vinculin (MW 105kDa) and stathmin (MW 18kDa) expression were used as loading control.

Supplementary Figure 6. *Activation of intracellular signaling pathways in T47D cells upon stimulation with wound fluids*. . Western blot analysis of the indicated phosphoproteins in T47D cells serum starved 24 hrs (lane 0) and then stimulated with the indicated sera at 5%, for 5, 15, 30 and 60 minutes. Vinculin (MW 105kDa) and stathmin (MW 18kDa) expression were used as loading control.

Belletti et al. Supp. Figure 1

A

PS Untreated   WF Untreated

PS TARGIT   WF TARGIT

T47D Collagen

B

PS Untreated   WF Untreated

PS TARGIT   WF TARGIT

MCF-7 Collagen

C   MDA-MD 231 Matrigel

Belletti et al. Supp. Figure 2

a MCF-7

p 0.02   p 0.03

p 0.45

p 0.24

■ 5 Hrs
□ 20 Hrs

Migrated Cells (% of total)

SFM   NIH CM   PS NT   WF NT   PS TARGIT   WF TARGIT

b MDA-MB 453

p 0.05   p 0.01

p 0.14

p 0.40

■ 10 Hrs
□ 20 Hrs

Migrated Cells (% of total)

SFM   NIH CM   PS NT   WF NT   PS TARGIT   WF TARGIT

c MDA-MB 231

■ PS
□ WF

Migrated Cells (% of total)

Samples
Untreated Patients

1 2 3 4 5 6 7 8 9 10 11 12

d MDA-MB 231

■ PS
□ WF

Migrated Cells (% of total)

Samples
TARGIT treated Patients

1 2 3 4 5 6 7 8 9 10 11 12

Belletti et al. Supp. Figure 3

A MDA-MB 231

WF NT
WF TARGIT

Invaded Cells (% of total)

Samples

B MDA-MB 231

p = 0.02

Invaded Cells (% of total)

PS   WF   PS   WF
Untreated   TARGIT

C

MDA-MB 453

p<0.0001   p<0.0001

Moving Cells

p = 0.6

Control   WF NT   WF TARGIT

Belletti et al. Supp. Figure 4

	Pre Serum					WF Unreated				WF TARGIT			
	0	5	15	30	60	5	15	30	60	5	15	30	60

pYpYpY-IGF1R

pY896-IRS1

pS612-IRS1

pS473-AKT

AKT

pT202/Y204-MAPK

MAPK

pT389-p70

pS235/6-S6

pS240/4-S6

pS21-GSK3α/β

pS10-p27^{kip1}

Vinculin

pY705-Stat 3

pS727-Stat 3

pY641-Stat 6

pY701-Stat 1

pS19-MLC

pS3-Cofilin

Stathmin

Belletti et al. Supp. Figure 5

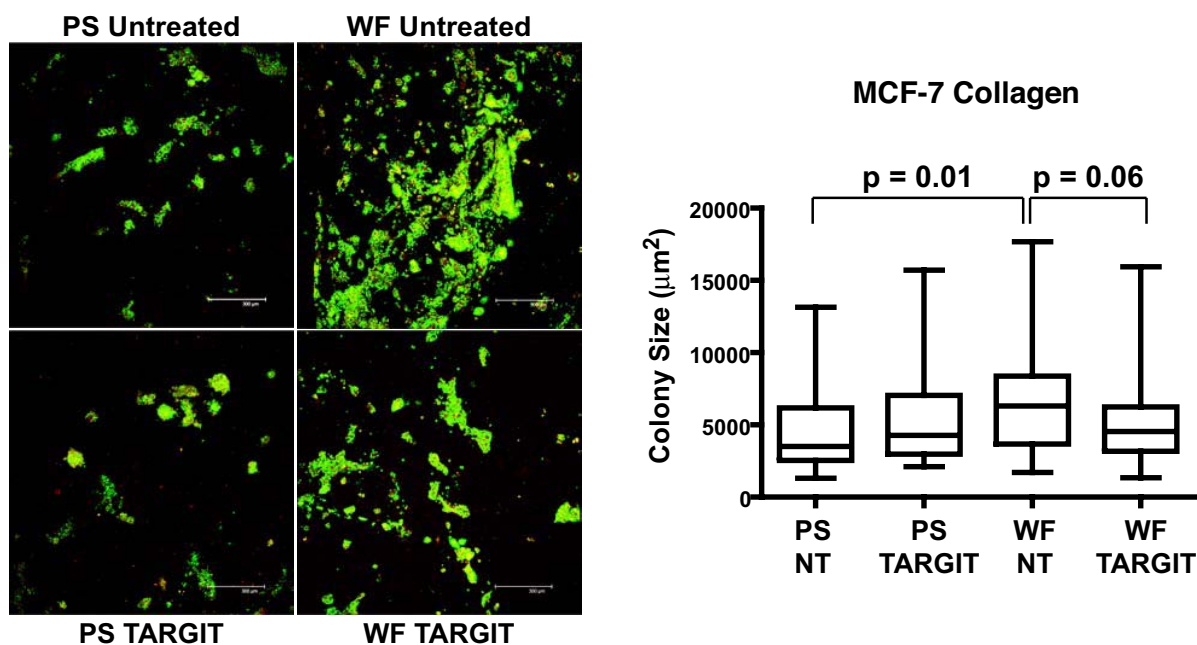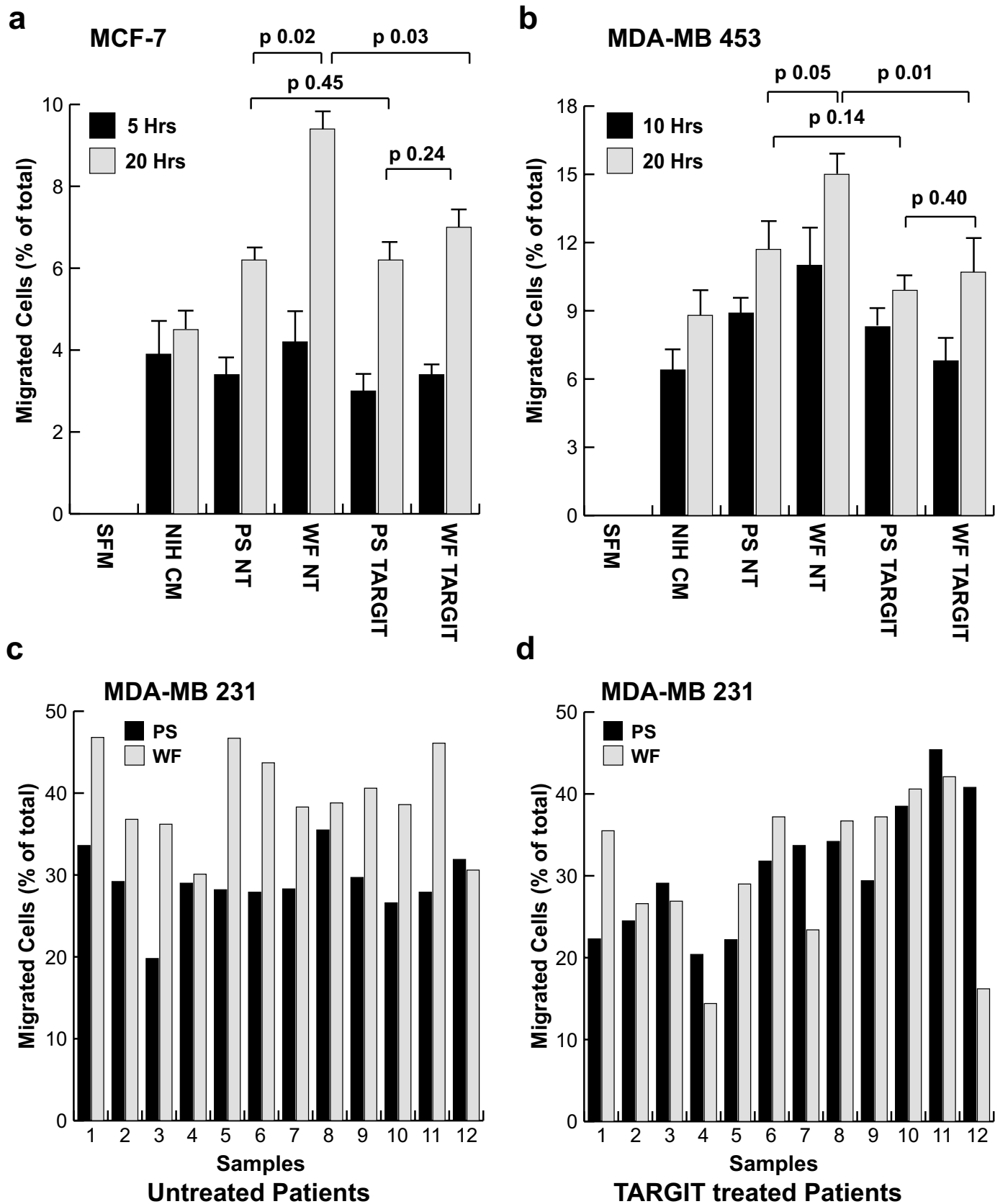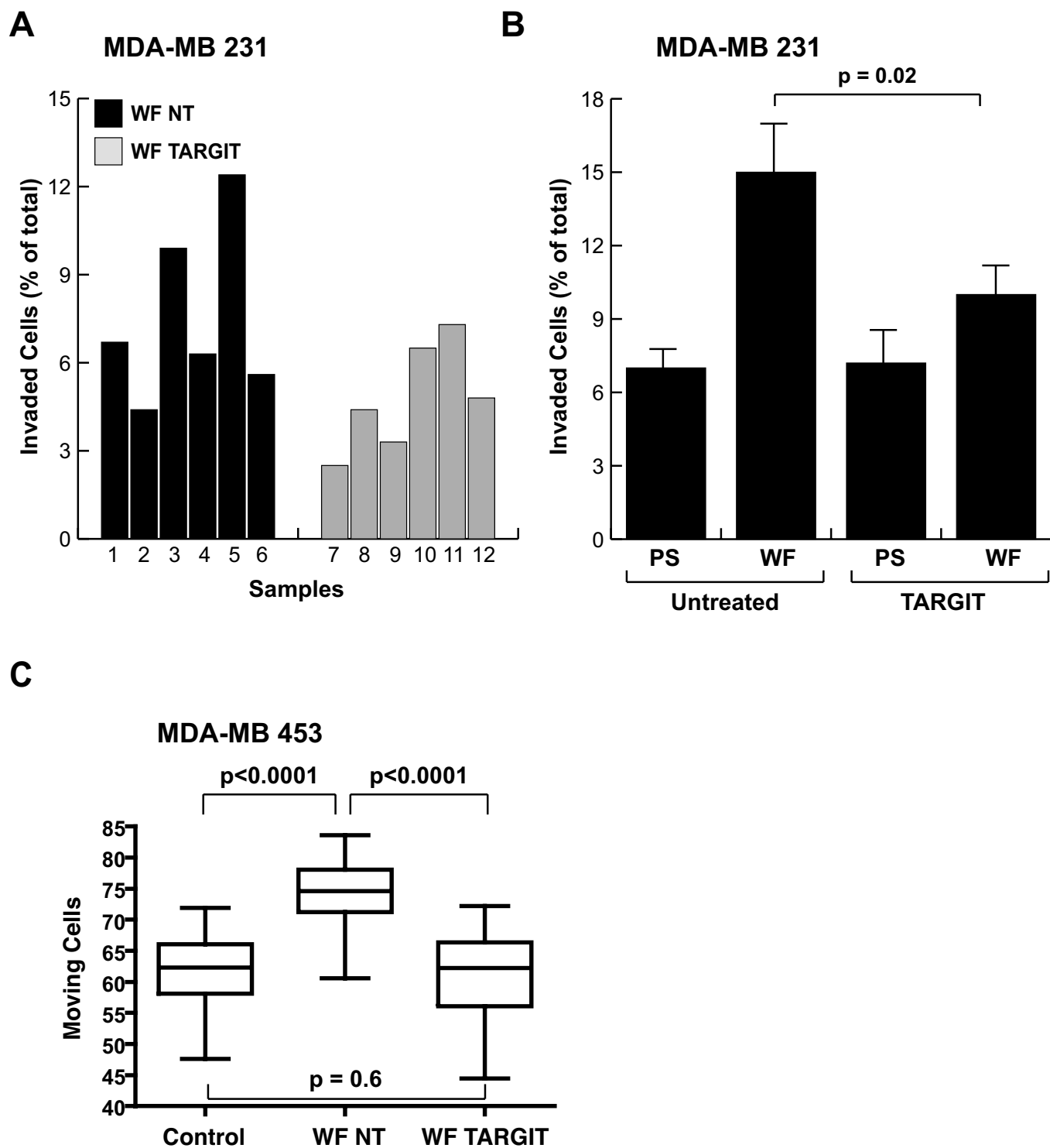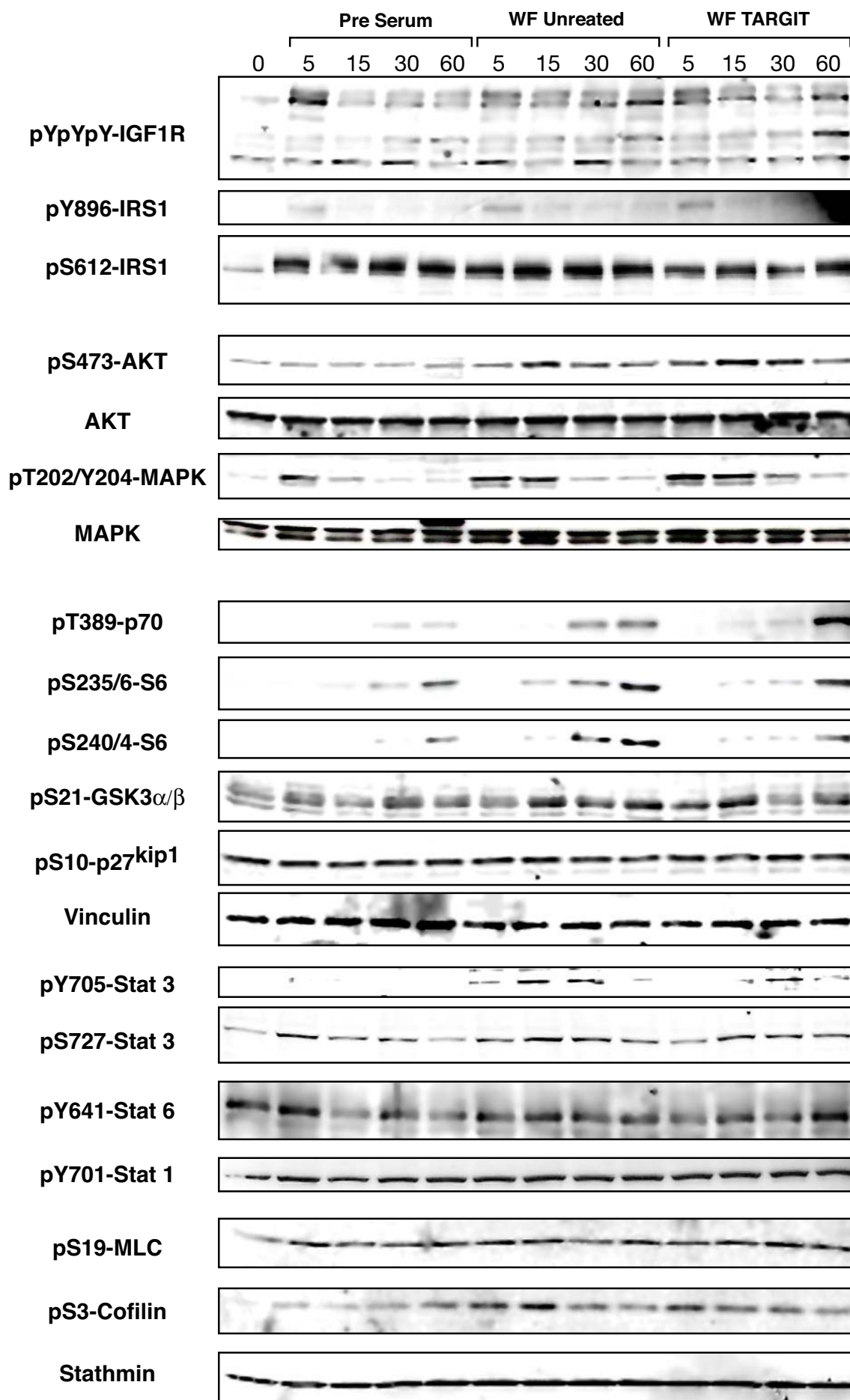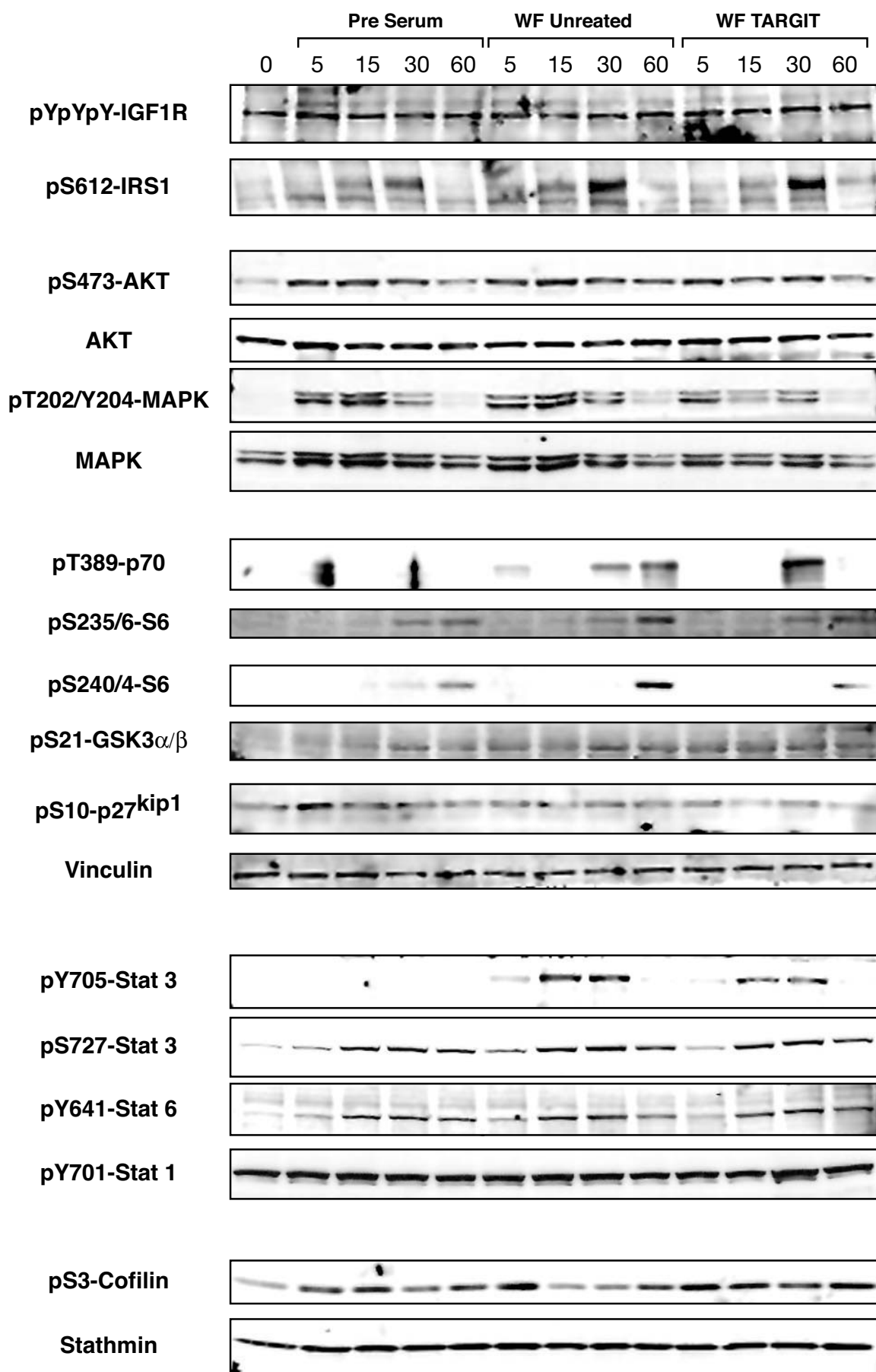|		Pre Serum				WF Unreated				WF TARGIT			
	0	5	15	30	60	5	15	30	60	5	15	30	60
pYpYpY-IGF1R													
pS612-IRS1													
pS473-AKT													
AKT													
pT202/Y204-MAPK													
MAPK													
pT389-p70													
pS235/6-S6													
pS240/4-S6													
pS21-GSK3α/β													
pS10-p27^{kip1}													
Vinculin													
pY705-Stat 3													
pS727-Stat 3													
pY641-Stat 6													
pY701-Stat 1													
pS3-Cofilin													
Stathmin													

Belletti et al. Supp. Figure 6

Supplementary Table 1

Pts n°	TARGIT	Age Year	Weight (Kg)	Height (cm).	Weight/ height	dissection
1	NO	56	66	160	0.41	no
3	NO	57	49	165	0.30	no
5	NO	51	56	162	0.35	no
8	NO	65	71	160	0.44	no
9	NO	49	70	168	0.42	no
10	NO	48	52	160	0.33	no
12	NO	69	68	155	0.44	no
13	NO	55	45	155	0.29	no
15	NO	32	57	160	0.36	no
16	NO	60	75	150	0.5	no
17	NO	55	63	175	0.36	no
18	NO	55	65	154	0.42	yes
20	NO	66	54	155	0.35	no
25	NO	45	65	154	0.42	yes
26	NO	44	60	157	0.38	no
28	NO	74	54	153	0.35	no
29	NO	65	70	173	0.40	no
32	NO	53	78	165	0.47	no
33	NO	66	53	148	0.36	no
35	NO	62	69	158	0.44	no
38	NO	46	56	162	0.35	no
42	NO	70	70	155	0.45	no
43	NO	57	44	156	0.28	no
44	NO	46	67	164	0.41	no
45	NO	62	66	164	0.49	no

2	YES	65	52	156	0.33	no
4	YES	44	56	165	0.34	no
6	YES	51	64	162	0.40	yes
7	YES	56	57	165	0.35	no
11	YES	52	59	159	0.37	no
14	YES	68	52	155	0.34	no
19	YES	45	62	169	0.37	no

36	YES	72	69	158	0.44	no
37	YES	71	60	170	0.35	no
46	YES	61	72	163	0.44	no
47	YES	63	70	160	0.43	no
48	YES	41	58	165	0.35	no

1 = "sn" indicate the evaluation of sentinel node status

2 = For ER positivity Immunohistochemistry has been used. The percentage of positive cells and the strenght of staining (

3 = For PG positivity Immunohistochemistry has been used. The percentage of positive cells and the strenght of staining (

4 = For HER2/Neu positivity the DAKO kit has been used following the manufacturer instructions

Diameter (cm)	Histotype	Grade	Node Status[1]	ER[2]	PR[3]	HER2[4]
1.1	lobular	G2	N0 (sn)	50-75% Low	Negative	Negative
0.7	tubular	G1	N0 (sn)	>75% Moderate	>75% High	Negative
1.9	ductal	G3	N1 m i (sn)	>75% Moderate	25-50% Moderate	2+
1.2	ductal	G3	N0 (sn)	>75% Moderate	>10% Moderate	Negative
0.7	ductal	G2	N1 mi (sn)	>75% Moderate	>75% High	Negative
0.5	tubular	G1	N0 (sn)	>75% High	Negative	Negative
1.4	ductal	G3	N1 mi (sn)	>75% Moderate	>75% High	Negative
1.5	ductal	G3	N0 (sn)	>75% High	>75% Moderate	1+
2	intraductal	G3	N0 (sn)	Negative	Negative	3+
2	ductal	G1	N0 (sn)	>75% High	>75% Moderate	1+
2.2	ductal	G3	N1 mi (sn)	>75% Moderate	Negative	1+
1.5	ductal	G3	N1a (4/19)	10-25% Moderate	Negative	Negative
0.8	tubular	G2	N0 (sn)	>75% Moderate	>75% Moderate	Negative
1.5	ductal	G3	N1a (2/15)	>75% Moderate	>75% Moderate	1+
1.9	mucinous	G3	N0 (sn)	>75% Moderate	>75% Moderate	Negative
1.8	ductal	G3	N0 (sn)	Negative	Negative	2+
1.5	ductal	G2	N0 (sn)	50-75% Low	50-75% Moderate	2+
0.9	ductal	G2	N0 (sn)	>75% Moderate	>75% Moderate	1+
1.4	ducto-lobular	G2	N1mi (sn)	>75% Moderate	25-50% Moderate	1+
n.e.	ductal	G2	N0 (sn)	25-50% Moderate	Negative	Negative
0.6	ductal	G2	N0 (sn)	>75% Moderate	>75% High	1+
1.5	ductal	G3	N1 mi (sn)	>75% Moderate	25-50% Moderate	1+
2.5	ductal	G3 cit	N0 (sn)	Negative	Negative	3+
0.9	ductal	G3	N0 (sn)	>75% Moderate	10-25% Moderate	2+
2.5	ductal	G2	N0 (sn)	>75% Moderate	25-50% Moderate	Negative
1.2	ductolobular	G2	N0 (sn)	>75% Moderate	>75% Moderate	Negative
1.9	ductal	G3	N0 (sn)	>75% Moderate	>75% Moderate	Negative
1.1	lobular	G2	N1a (2/20)	>75% Moderate	>75% Moderate	Negative
1.2	ductal	G2	N0 (sn)	>75% High	>75% Moderate	2+
1.4	ductolobular	G2	N0 (sn)	>75% Moderate	>75% High	Negative
1.8	lobular	G3	N0 (sn)	>75% Moderate	>75% Moderate	2+
0.3	intraductal	3	3	>75% Moderate	Negative	3+

0.8	ductal	G2	N0 (sn)	>75% Moderate	<10% Moderate	Negative
0.8	ductal	G2	N0 (sn)	>75% High	25-50% Moderate	Negative
1.8	ductal	G3	N0 (sn)	Negative	Negative	Negative
1.0	ductal	G2	NO(sn)	50-75% Moderate	50-75% Moderate	1+
1.3	ductal	G2	N1mi (sn)	50-75% Low	>75% Moderate	Negative

low, moderate and high) is reported
low, moderate and high) is reported