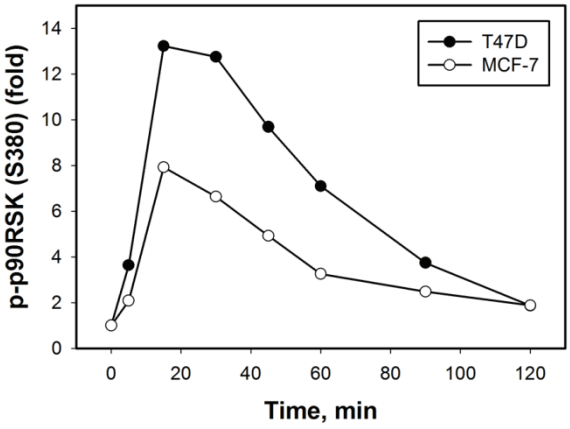
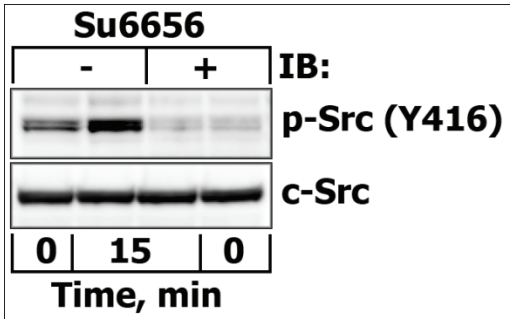
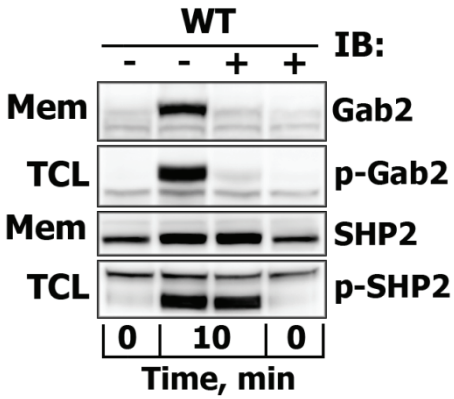
Supplemental Data



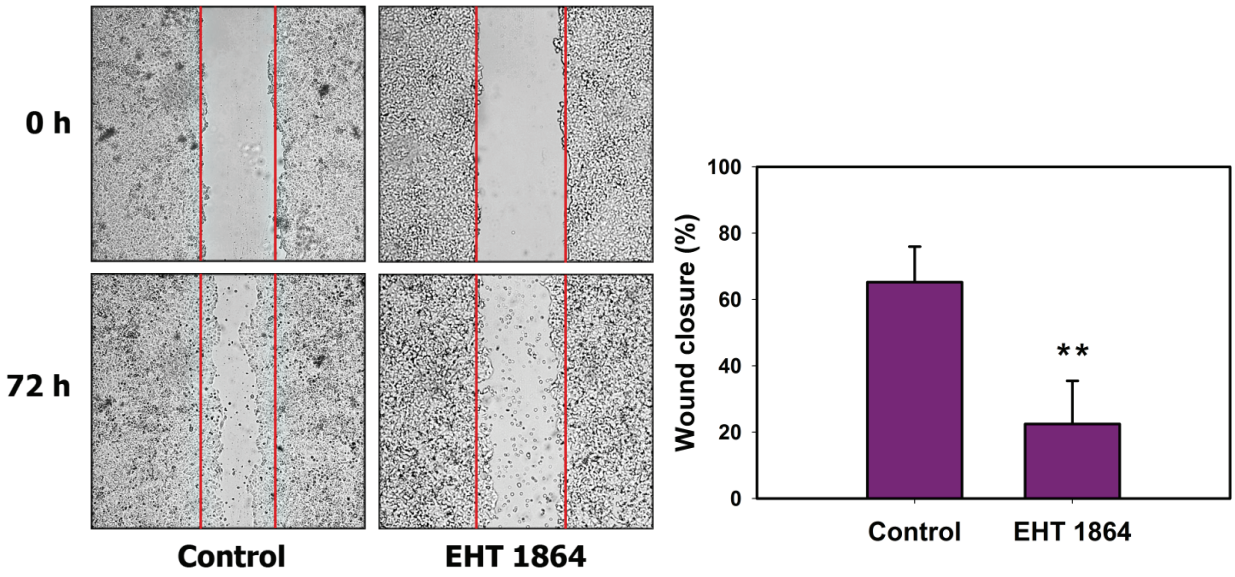
Supplemental Figure 1S. The time-course of PRL-induced p90RSK phosphorylation. Both total and phosphorylated forms of p90RSK proteins of unstimulated and PRL-stimulated (10 nM, for the indicated time intervals) T47D (*black circles*) and MCF7 (*white circles*) cells were detected by immunoblotting (IB) of the total cell lysates (TCL) with anti-p90RSK or anti-phospho-p90RSK (Ser380) antibodies (Abs), respectively. The ratio of phospho-protein:total protein at each time point was expressed as fold changes over basal levels.



Supplemental Figure 2S. The efficacy of SFK inhibition by Su6656. Serum-starved T47D cells were either left untreated (-) or were treated (+) with Su6656 (10 μM, 30min) before stimulation with 10 nM PRL for 15 min. Phosphorylated forms of c-Src (and other SFK) on activating Tyr416 residues and total c-Src proteins were detected by IB of the TCL with anti-phospho-SFK (Tyr416) and anti-c-Src Abs, respectively.

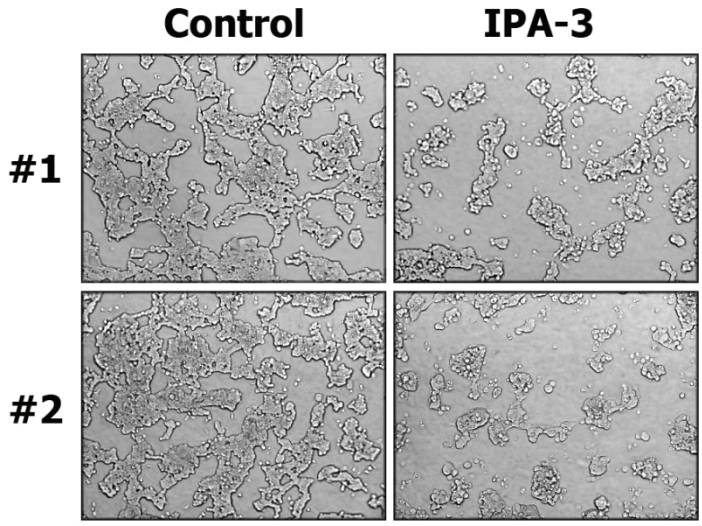


Supplemental Figure 3S. The effects of PI3-kinase inhibition on PRL-induced phosphorylation and recruitment of Gab2 and SHP2. Serum-starved T47D cells were either left untreated (-) or were treated (+) with WT (200 nM, 30min) before stimulation with 10 nM PRL for 10 min. Phosphorylated forms of Gab2 and SHP2 proteins were detected by IB of the TCL with anti-phospho-Gab2 (Tyr452) and anti-phospho-SHP2 (Tyr542) Abs, respectively. Gab2 and SHP2 proteins were detected by IB of the particulate/membrane (Mem) fraction, which was isolated as described under "Materials and Methods".

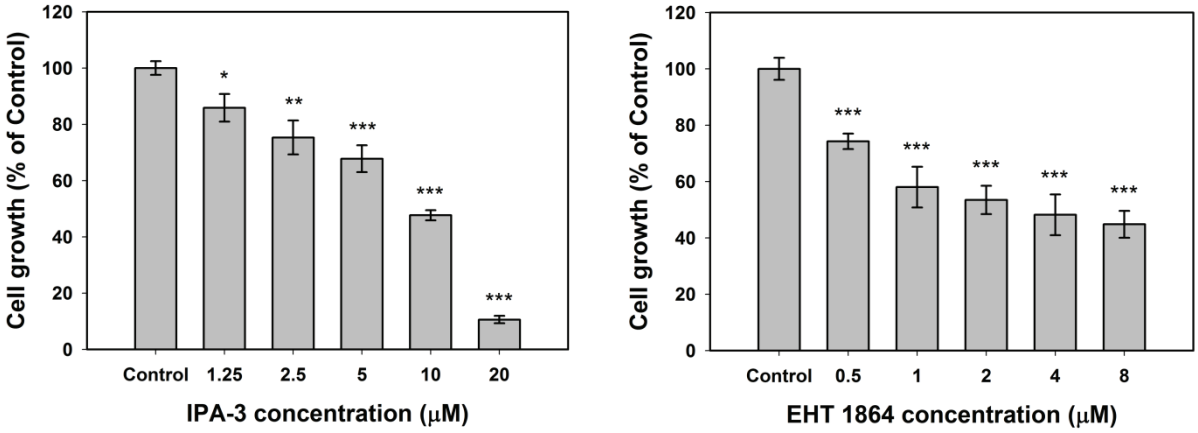


Supplemental Figure 4S. **Effects of Rac inhibition on breast cancer cell migration**. **Left panel**. MCF-7 cells were grown to confluence, scratch wounded, treated with EHT 1864 (2 μM) and stimulated with PRL (10 nM) in serum-free medium. Control cells were cultured in serum-free medium that contained only PRL. Cells were photographed at 0 and 72 hours after wounding with an inverted light microscope at 4× magnification. The red lines denote the original edges of the wound at time 0 h. Experiments were performed in triplicate and representative results are shown. **Right panel**. The *percentage wound* closure (mean ± SD, n=3) was calculated using the equation (S2–S1)/S2*100, where S2 is cell-free scratch area at 0 h after wounding, S1 – cell-free scratch area at 72 hours after wounding. Two asterisks (**) indicate the significance of $P<0.01$ as compared to control.

A.



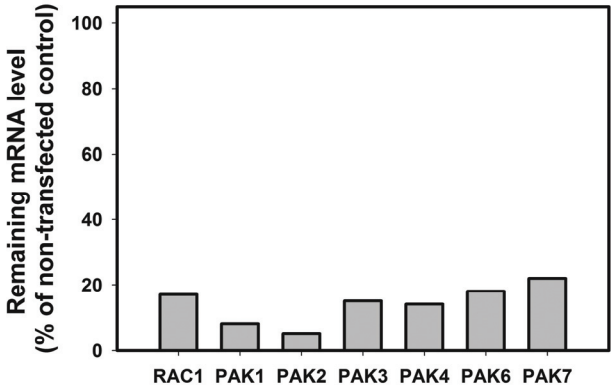
B.



Supplemental Figure 5S. Effects of Rac/PAK inhibition on breast cancer cell growth. A. T47D cells were cultured in 10 nM PRL-containing serum-free medium in the absence (control) or presence of IPA-3 (25 μM) for 4 days. Representative digitized images showing cell densities from two different tissue culture plates were captured by inverted light microscope at magnification of 4×. **B.** T47D cells were plated in quadruplicates into 24-well plates at a density of 20,000 cells/ml. After 24 hours, complete culture medium was changed into fresh low serum-containing medium (1% FBS) supplemented with 10 nM PRL with or without increasing concentrations of IPA-3 (**left panel**) or EHT 1864 (**right panel**). Cell growth was evaluated 72 hours after treatment by AlamarBlue fluorescent assay. Results are expressed as percentages of control and represented as the mean ± SD obtained from three independent experiments.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ as compared to control.

A.



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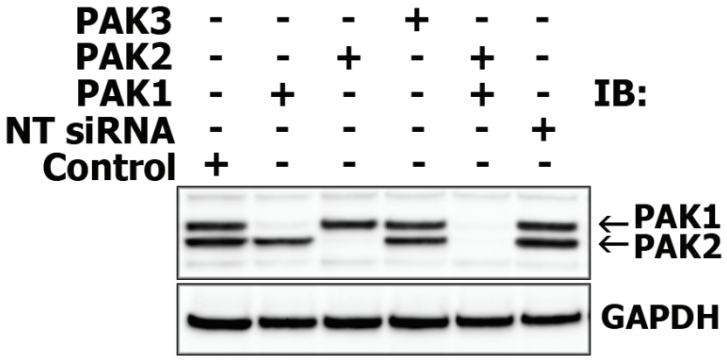
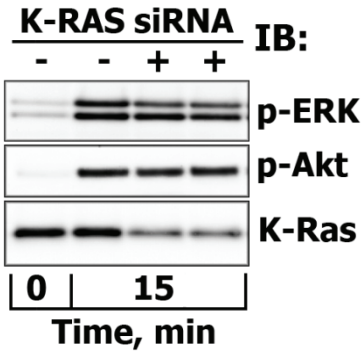
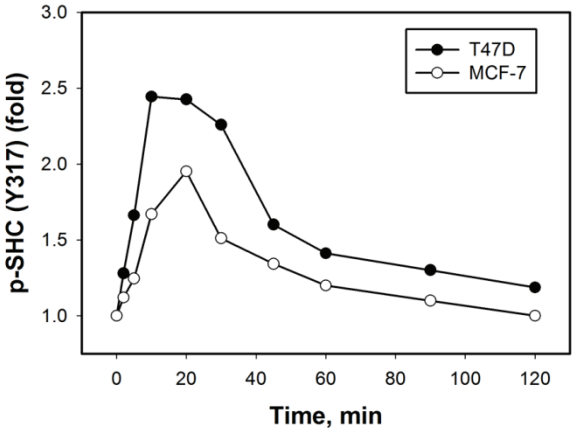


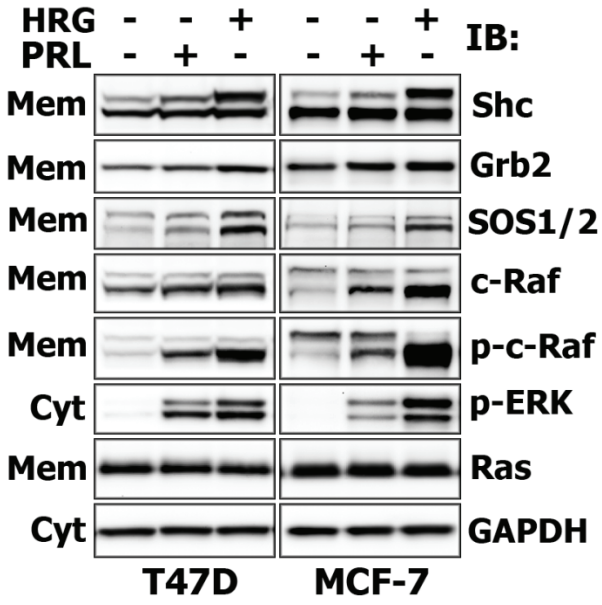
Supplemental Figure 6S. Evaluation of siRNA efficacy and specificity. MCF-7 cells were either left untransfected (Control) or were transiently transfected with non-targeting (NT) negative control siRNA or RAC1, PAK1, PAK2, PAK3, PAK4, PAK6 and PAK7 siRNA duplexes for 72 hours before measurement of relative mRNA levels by quantitative PCR (**A**) or protein detection by IB of the TCL with anti-PAK1/2/3 or anti-GAPDH Abs, respectively (**B**).



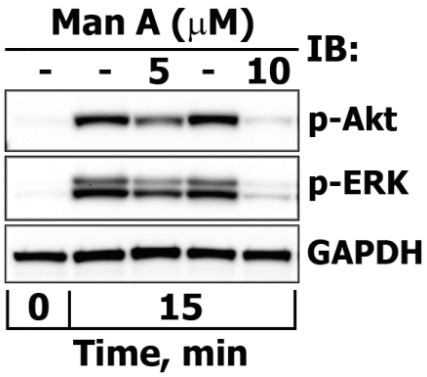
Supplemental Figure 7S. The effects of K-RAS gene silencing on PRL-induced ERK1/2 activation. MCF-7 cells were transiently transfected either with non-targeting (NT) negative control siRNA (-) or with K-RAS siRNA (+) duplexes for 72 hours, and stimulated with 10 nM PRL for 15 min. Phosphorylated forms of ERK1/2, Akt proteins and total K-RAS levels were detected by IB of the TCL with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-phospho-Akt (S473) or anti-K-Ras Abs, respectively.



Supplemental Figure 8S. The time-course of PRL-induced Shc phosphorylation on Grb2 binding site. Both total and phosphorylated forms of Shc proteins of unstimulated and PRL-stimulated (10 nM, for the indicated time intervals) T47D (*black circles*) and MCF7 (*white circles*) cells were detected by IB of the TCL with anti-Shc or anti-Shc (Tyr317) Abs, respectively. The ratio of phospho-protein:total protein at each time point was expressed as fold changes over basal levels.



Supplemental Figure 9S. Comparative ability of PRL and HRG-β to translocate Shc, Grb2, SOS1/2 and c-Raf proteins to the plasma membrane that leads to ERK1/2 activation. Serum-starved T47D or MCF-7 cells were either left unstimulated (-) or were stimulated (+) with PRL (10 nM) or HRG-β (2 nM) for 10 min. p52Shc and p46Shc isoforms**,** Grb2, SOS1 and SOS2 isoforms, c-Raf, Ras, GAPDH and phosphorylated forms of c-Raf (Ser338) and ERK1/2 (Thr202/Tyr204) proteins were detected by IB of the cytosolic (Cyt) or particulate/membrane (Mem) fractions that were isolated as described under "Materials and Methods". Ras and GAPDH levels served as protein loading controls for Mem and Cyt fractions, respectively.



Supplemental Figure 10S. Dose-dependent effects of Manumycin A to inhibit PRL-induced Akt and ERK1/2 activation. Serum-starved T47D cells were either left untreated (-) or were treated with indicated doses of Manumycin A for 7 hours before stimulation with 10 nM PRL for 15 min. Phosphorylated forms of Akt and ERK1/2 proteins were detected by IB of the TCL with anti-phospho-Akt (S473) and anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Abs, respectively. GAPDH levels served as protein loading control.

Supplemental Table 1S. Inhibitors, their IC_{50} values, final concentrations (FC) and preincubation times (PT).

Name	Target(s)	Manufacturer	IC_{50}	FC	PT (min)
Su6656	Src, Yes, Lyn, and Fyn	Cayman Chemicals (Ann Arbor, MI)	280, 20, 130 and 170 nM, respectively	10 μM	30
PF573228	FAK	Tocris Biosciences (Elllsville, MI)	4 nM	0.5 μM	120
AG-490/ Tyrphostin	JAK2/JAK3	Cayman Chemicals	0.1 and 4.3 μM, respectively	50, 100 μM	180
Nicotinoyl hydrazone (NH)	STAT5	EMD Chemicals (Gibbstown, NJ)	47 μM	100-200 μM	60
Nifuroxazide	JAK2/Tyk2		10 μM	100 μM	60
Wortmannin	Class I PI3-kinases	Cayman Chemicals	1-10 nM	200 nM	30
LY 294002	β, α, δ and γ isoforms of PI3-kinase		0.31, 0.73, 1.06, 6.60 μM, respectively	25 μM	30
PI3K-α -2	α, β, γ, and 2Cβ isoforms of PI3-kinase		2, 16, 660, 220 nM, respectively	2 μM	30
Akt-VIII	Akt1, Akt2, Akt3	Sigma-Aldrich (St. Louis, MO)	58, 210, 2120 nM, respectively	10 μM	30
EHT 1864	Rac1/1b/2/3	Tocris Biosciences	40, 50, 60, 250 nM, respectively	10 μM	60
IPA-3	PAK1		2.5 μM	10 μM	30
PAK18	PAK	EMD Chemicals	N/A	10 μM	60
Bisindolylmaleimide I	α, β1, δ, ε and ζ isoforms of PKC		0.0084, 0.0180, 0.210, 0.132, 5.8μM, respectively	5 μM	60
Manumycin A	Ras farnesyltransferase	Cayman Chemicals	K_i = 1.2 μM	2, 5, 10 μM	420
Ras FTase III	Ras farnesyltransferase	EMD Chemicals	12 nM	2 μM	420
OSU-03012	PDK1 and PAK	Echelon Biosciences (Salt Lake City, UT)	5 μM and 1.03± 0.59 μM, respectively	25 μM	30

Supplemental Table 2S. Primary antibodies, hosts and final dilutions (FD) used in this study.

Antibody name (clone)	Host species	FD	Manufacturer
anti-Phosphotyrosine (pY20)	Mouse	1 : 1000	BioLegend (San Diego, CA)
PRL-R	Rabbit	1 : 500	Santa Cruz Biotechnology (Santa Cruz, CA)
p-Jak2 (Tyr1007/1008) (C80C3)		1 : 1000	Cell Signaling Technology (Danvers, MA)
Jak2 (D2E12)			
Jak1 (6G4)			
p-Src (Tyr416) (100F9)			
c-Src	Mouse	1 : 2000	Upstate/Millipore (Billeria, MA)
p-FAK (Tyr925)	Rabbit	1 : 1000	Cell Signaling Technology
p-FAK (Tyr397)			
p-FAK (Tyr576/577)			
FAK			
p-STAT5 (Tyr694)			
STAT5			
p-STAT3 (Tyr705)			
STAT3			
p-STAT1 (Tyr701)			
STAT1			
p-Akt (Ser473) (587F11)	Mouse		
Akt1	Rabbit	1 : 2000	Upstate/Millipore
p-70S6K (Thr389)		1 : 1000	Cell Signaling Technology
p70S6K			
p-S6RP (Ser235/Ser236)			
S6RP			
p-c-Raf (Ser338) (56A6)			
Raf1		1 : 2000	Upstate/Millipore
p-MEK1/2 (Ser217/Ser221)		1 : 1000	Cell Signaling Technology
MEK1/2			
p-ERK1/2 (Thr202/Tyr204)	Mouse		
ERK1/2	Rabbit		
p-p90RSK (Ser380)			
RSK1/RSK/RSK3 (32D7)			
p-SHP2 (Tyr542)			
SHP2		1 : 500	Santa Cruz Biotechnology
p-Gab1 (Tyr627)			
Gab1, CT		1 : 2000	Upstate/Millipore
p-Gab2 (Tyr452)		1 : 1000	Cell Signaling Technology
Gab2		1 : 2000	Upstate/Millipore
GAPDH (6C5)	Mouse		
p-Shc (Tyr317)	Rabbit	1 : 1000	Cell Signaling Technology
Shc		1 : 2000	BD Biosciences (Franklin Lakes, NJ)
Grb2 (C-23)		1 : 1000	Santa Cruz Biotechnology
p-PAK1/2 (Thr423/Thr402)	Rabbit	1 : 1000	Cell Signaling Technology
PAK1/2/3			
p-p38-MAPK (Thr180/Tyr182)			
Rac1	Mouse	1 : 2000	Upstate/Millipore
Ras (pan)			BD Biosciences
K-Ras	Rabbit	1 : 500	Santa Cruz Biotechnology
SOS1/2	Rabbit	1 : 500	Santa Cruz Biotechnology

Supplemental Table 3S. Sequences of siRNA used in the study.

Target	siRNA
PAK1	TCCACTGATTGCTGCAGCTAA
PAK2	CCGGATCATACGAAATCAATT
PAK3	TTCCAGTACTTTGTACAGGAA
PAK4	CGAGAATGTGGTGGAGATGTA
PAK6	CCCAAGTATGCCTGCCACCTA
PAK7	ATGGTGTGCACGTTTCATTAA
K-RAS	AAGGAGAATTTAATAAAGATA
RAC1	ATGCATTTCCTGGAGAATATA

Supplemental Table 4S. Sequences of primers used in the study.

Target	Forward primer	Reverse primer
PAK1	GTGAAGGCTGTGTCTGAGACTC	GGAAGTGGTTCAATCACAGACCG
PAK2	CGACTCCAACACAGTGAAGCAG	TCACTACTGCGGGTGCTTCTGT
PAK3	CGCTGTCTTGAGATGGATGTGG	CAGTCTTAGCGGCTGCTGTTCT
PAK4	GATGATTCGGGACAACCTGCCA	AGGAATGGGTGCTTCAGCAGCT
PAK6	TGAGGAGCAGATTGCCACTGTG	CTGAGCACAGAATCCGAAGTCC
PAK7	AGAAGGTGGTGCCTTGACAGAC	GTCCCTGTGAATCACTCCTTGG
K-RAS	CAGTAGACACAAAACAGGCTCAG	TGTCGGATCTCCCTCACCAATG
RAC1	CGGTGAATCTGGGCTTATGGGA	GGAGGTTATATCCTTACCGTACG