SUPPLEMENTARY MATERIAL

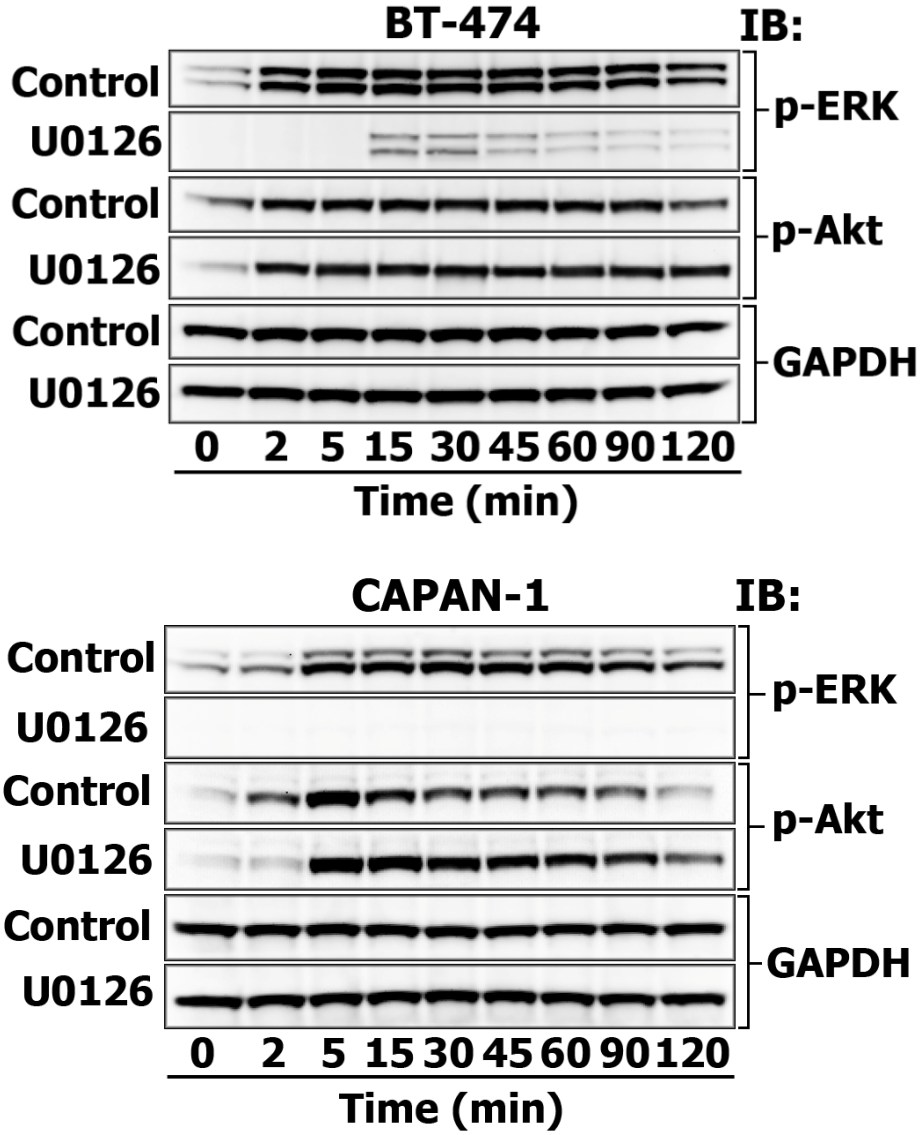

Fig. 1S. Activation of ERK and Akt in the presence of MEK inhibitor U0126 in BT-474 (upper panel) and CAPAN-1 (lower panel) cells. Serum-starved cells were either left untreated or treated with U0126 (10 μM, 30 min) before stimulation with EGF (1 nM) for the indicated time intervals (min). Equal amounts of total cell lysates were resolved by NuPAGE and subjected to Multi-strip Western blotting. Immunoblots (**IB**) were probed with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-phospho-Akt (S473) or anti-GAPDH (loading control) antibodies.

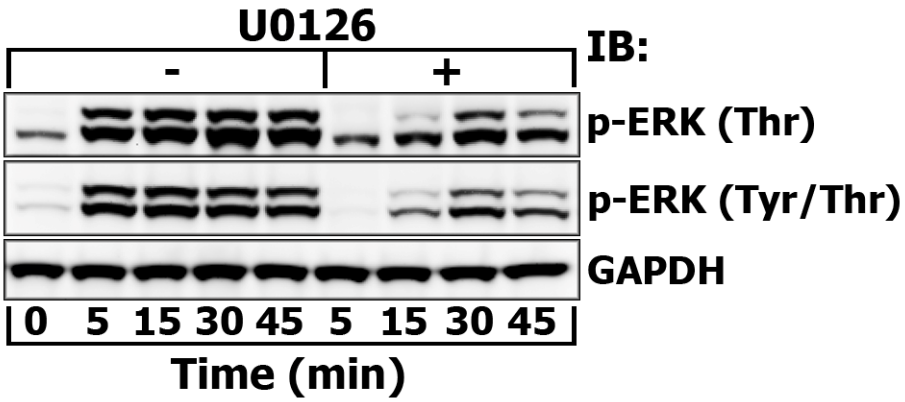

Fig. 2S. ERK phosphorylation on Thr and Tyr residues in the presence of MEK inhibitor U0126. Serum-starved T47D cells were either left untreated or treated with U0126 (10 μM, 30 min) before stimulation with EGF (1 nM) for the indicated time intervals (min). Equal amounts of total cell lysates were resolved by NuPAGE and subjected to Western blotting. Immunoblots (**IB**) were probed with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), anti-phospho-ERK1/2 (Thr202) or anti-GAPDH (loading control) antibodies.

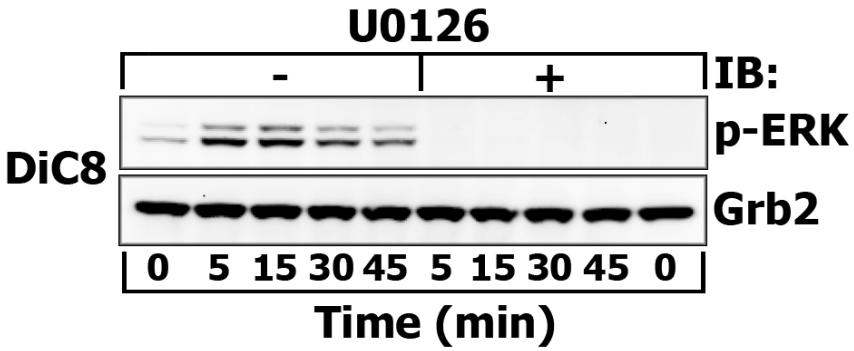

Fig. 3S. PKC-mediated ERK phosphorylation requires MEK activity. Serum-starved T47D cells were either left untreated or treated with U0126 (10 μM, 30 min) before stimulation with 1,2-Dioleoyl-sn-glycerol (DiC8) (100 μM) for the indicated time intervals (min). Equal amounts of total cell lysates were resolved by NuPAGE and subjected to Western blotting. Immunoblots (**IB**) were probed with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) or anti-Grb2 (loading control) antibodies.

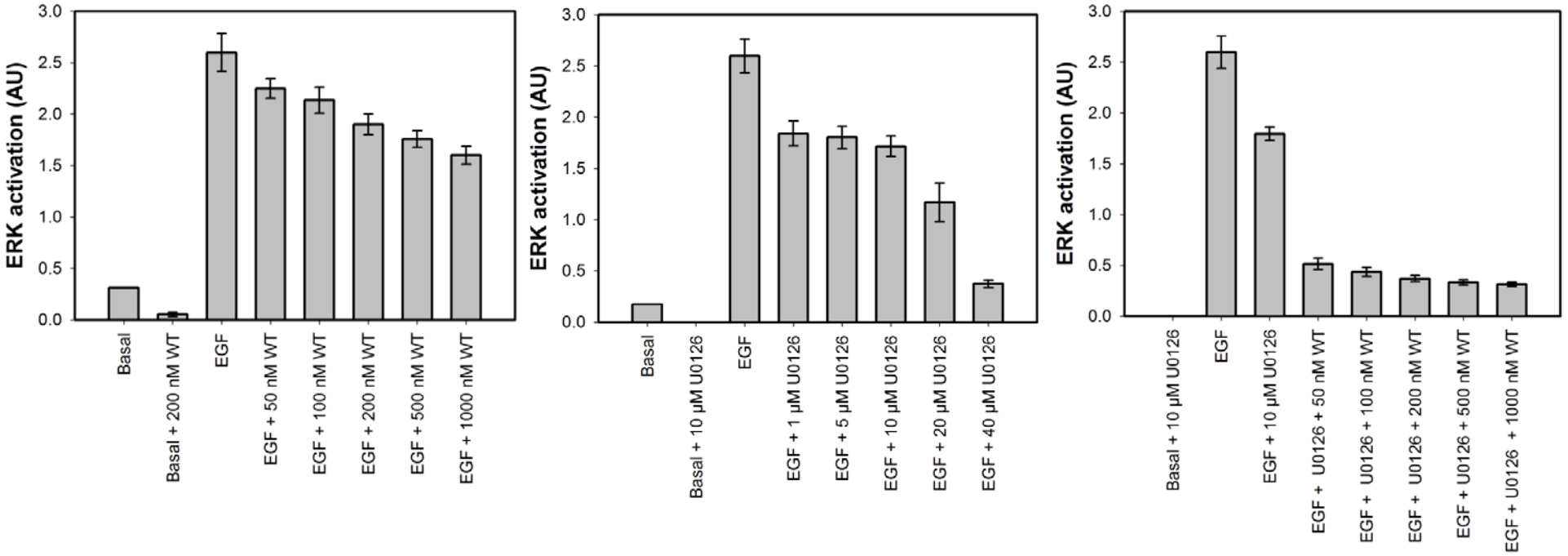

Fig. 4S. Dose-dependence of ERK phosphorylation in the presence of wortmannin (left panel), U0126 (middle panel) or their combination (right panel). Serum-starved T47D cells were either left untreated or treated with indicated concentrations of wortmannin, U0126 or their combination for 30 min before stimulation with EGF (1 nM) for 30 min. Equal amounts of total cell lysates were resolved by NuPAGE and subjected to Western blotting. Immunoblots were probed with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) or anti-ERK1/2 (loading control) antibodies. Bars show the intensities of ERK activation in arbitrary units (AU) plotted as the ratio between phospho-ERK1/2 and total ERK1/2 signal ±SD (error bars) (n=3).

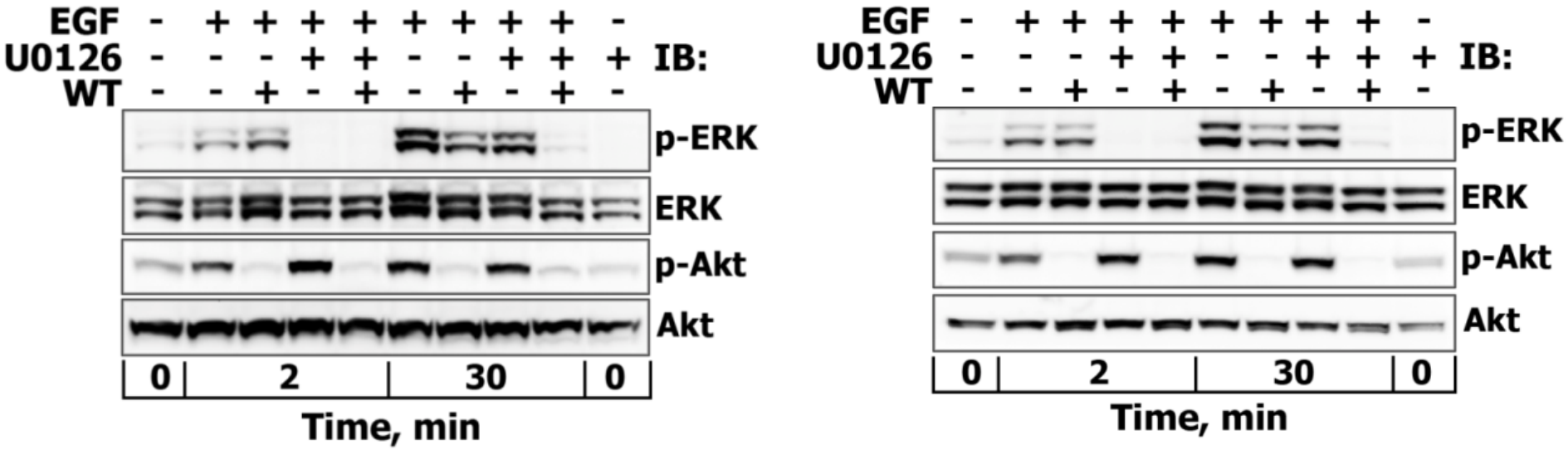

Fig. 5S. **Activation of ERK and Akt in the presence of wortmannin, U0126 and their combination in non-soluble (left panel) and cytosolic (right panel) fractions**. Serum-starved T47D cells were either left untreated or treated with wortmannin (200 nM), U0126 (10 μM) or their combination for 30 min before stimulation with EGF (1 nM) for 2 or 30 min. The subcellular fractions were isolated from digitonin-permeabilized (150 μg/ml, 10 min) T47D cells. Subcellular fractions were resolved by LDS-PAGE and subjected to immunoblotting (IB) with antibodies against phospho-ERK1/2, ERK1/2, phospho-Akt (Ser473) or Akt proteins.

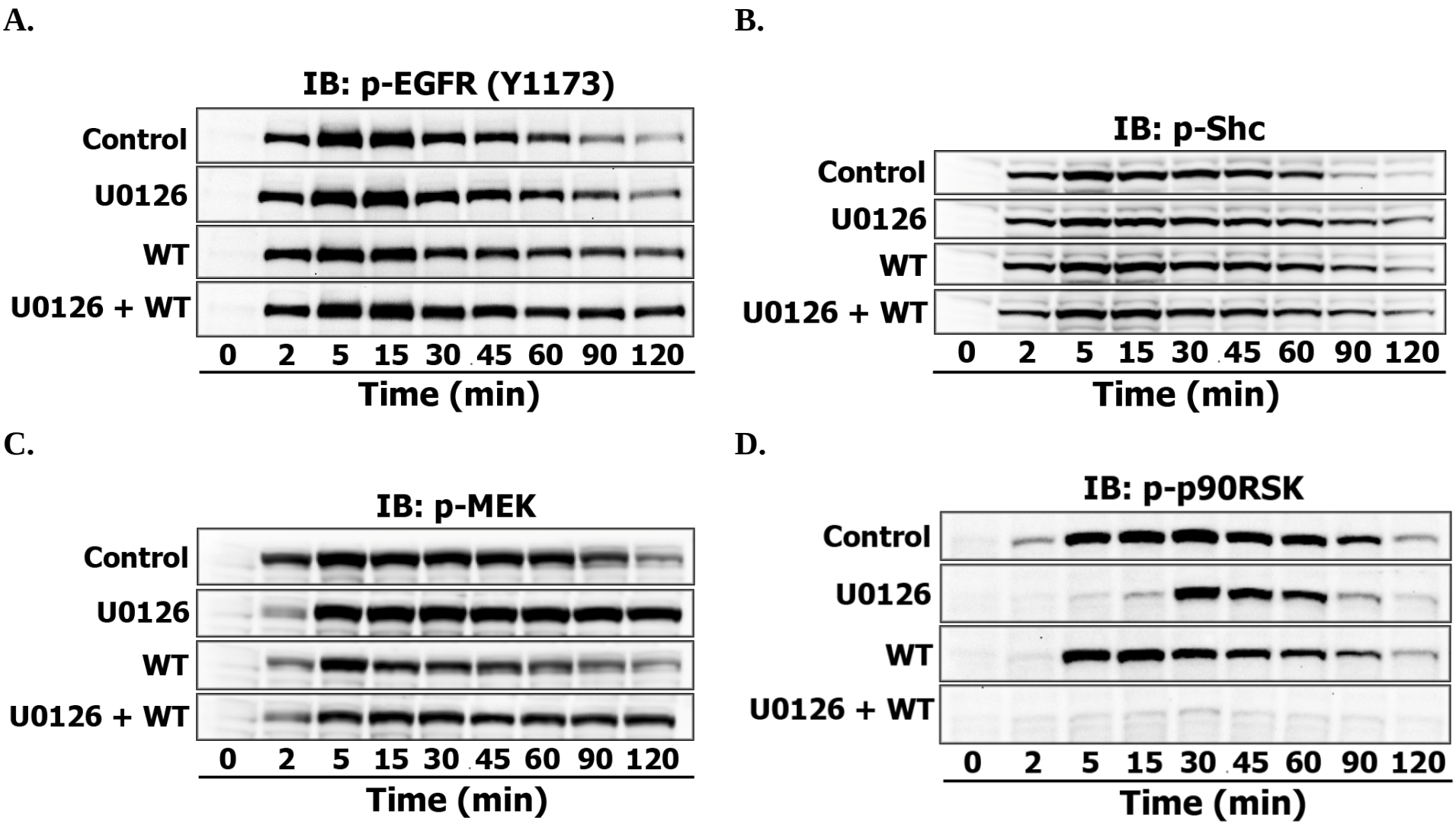

Fig. 6S. Phosphorylation kinetics of EGFR (A), Shc (B), MEK (C) and p90RSK (D) in the presence of wortmannin, U0126 or their combination. Serum-starved T47D cells were either left untreated or treated with wortmannin (200 nM), U0126 (10 μM) or their combination for 30 min before stimulation with EGF (1 nM) for indicated time intervals (min). Equal amounts of total cell lysates were resolved by NuPAGE and subjected to Multi-strip Western blotting. Immunoblots (**IB**) were probed with anti-phospho-EGFR (Tyr1173), anti-phospho-Shc (Tyr317), anti-phospho-MEK1/2 (Ser217/221) or anti-phospho-p90RSK (Ser380) antibodies.

Table S1. Inhibitors, their IC_{50} values, final concentrations and preincubation times used in this study.

Name	Target	Manufacturer	IC_{50} values	Final concentration	Preincubation time (min)
U0126	MEK1/2	Calbiochem/EMD Chemicals (Gibbstown, NJ)	72/58 nM	10 μM	30
Wortmannin	PI3K		1-10 nM	200 nM	
Gö6850	PKC α-, β1-, β2-, γ-, δ-, ε- isozymes		Ki = 10 nM	5 μM	60
Gö6983	PKC α-, β-, γ-, δ-, ζ– isozymes		7/7/6/10/60 nM		
Akt-VIII	Akt1/2/3 isoforms		58/210/2120 nM		
Su6656	Src/Yes/Lyn/Fyn		280/20/130/170 nM	10 μM	
Endothall	PP2A/PP1 phosphatases		90/5000 nM	2 μM	
PD 198306	MEK1/2	Tocris Bioscience (Ellisville, MO)	8 nM	200 nM	30
SQ22536	Adenylyl cyclase		1.4 μM	50 μM	60
NSC 95397	Cdc25A/B/C phosphatases		Ki = 32 nM	5 μM	
PP2	Src-family kinases		4-5 nM		30
OSU-03012	PDK1	Echelon Biosciences (Salt Lake City, UT)	5 μM	50 μM	60
PD 098059	MEK1/2	Cayman Chemical (Ann Arbor, MI)	2-7 μM		30
PD 169316	p38 MAPK		89 nM	5 μM	60
AG-825	ErbB2/EGFR/PDGFR		0.35/19/40 μM		
SB 216763	GSK-3 α/β isoforms		34 nM	7 μM	60

Table S2. Primary and secondary antibodies and their dilutions used in this study.

Antibody name and clone	Referred in figures as	Host species	Dilution	Manufacturer	Catalog No.
Phospho-Shc (Tyr317)	p-Shc	Rabbit	1 : 1000	Cell Signaling Technology (Danvers, MA)	2431
Phospho-EGFR (Tyr1173) (53A5)	p-EGFR				4407
Phospho-p44/42 MAPK (E10) (Thr202/Tyr204 & Thr204/Tyr187)	p-ERK or p-ERK (Tyr/Thr)	Mouse			9106
p44/42 MAPK (ERK1/2)	ERK	Rabbit			9102
Phospho-ERK1/2 (Thr202)	p-ERK (Thr)		1 : 500	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-101760
Phospho-MEK1/2 (Ser217/Ser221)	p-MEK		1 : 1000	Cell Signaling Technology (Danvers, MA)	9121
MEK1/2	MEK				9122
Phospho-Akt1/2/3 (Ser473)	p-Akt				9271
Akt1/2/3	Akt				9272
Phospho-c-Raf (Ser338) (56A6)	p-c-Raf				9427
Raf-1	c-Raf		1 : 2000	Upstate/Millipore Corporation (Billerica, MA)	07-396
Phospho-p90RSK (Ser380) (9D9)	p-p90RSK		1 : 500	Cell Signaling Technology (Danvers, MA)	9335
Phospho-STAT3 (Ser727)	p-STAT3				9134
Phospho-Estrogen receptor α (Ser118)	p-ER-α				2515
GAPDH (6C5)	GAPDH	Mouse	1 : 2000	Chemicon/Millipore Corporation (Billerica, MA)	MAB374
c-Fos (H-125)	c-Fos	Rabbit	1 : 500	Santa Cruz Biotechnology (Santa Cruz, CA)	sc-7202
GRB2 (C-23)	Grb2				sc-255
Phospho-Elk-1 (Ser383)	p-Elk-1			Assay Designs Inc. (Ann Arbor, MI)	KAP-MA035
c-Myc (9E10)	c-Myc	Mouse	1 : 2000	BD Biosciences (San Jose, CA)	551101
Anti-mouse HRP-linked IgG	N/A	Horse	1 : 10000	Cell Signaling Technology (Danvers, MA)	7076
Anti-rabbit HRP-linked IgG	N/A	Goat	1 : 50000	Pierce/Thermo Fisher Scientific (Rockford, IL)	31462