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WHAT IS SIMPLE WESTERN CHARGE?

Simple Western™ Charge is a fully automated immunoassay for the charge heterogeneity characterization of target proteins in complex samples. In the Simple Western Charge assay, proteins are separated by capillary isoelectric focusing (cIEF), followed seamlessly by immunodetection directly in the capillary. Any primary antibody may be used to target a protein of interest, then an HRP-conjugated secondary antibody is used for chemiluminescent detection, with sensitivity down to the low pg range. All steps are performed automatically inside Simple Western instruments like Peggy Sue™ or NanoPro™ 1000, and the results that are generated are reproducible and fully quantitative.

THE SIMPLE WESTERN CHARGE ADVANTAGE

Simple Western Charge has many advantages over traditional methods like slab gel IEF and Western blot. For example, Simple Western Charge is a high-throughput assay, and as many as 96 samples may be analyzed overnight without user intervention. Simple Western Charge uses only a tiny amount of sample; as few as 25 cells may be analyzed. Also, the sample volume requirement is low, only 5-12 µL, and the same sample may be interrogated up to 8 times with different antibodies. Because all steps are automated and precisely executed, Simple Western Charge has excellent reproducibility, with inter- and intra-assay CV<20%. Finally, Simple Western Charge has exquisite resolving power, with a 3-10 pI gradient, providing the ability to detect subtle changes conferred by post-translational modifications like phosphorylation and glycosylation. The ability to detect and quantify nearly all protein isoforms with high sensitivity and resolution makes Simple Western Charge a uniquely powerful method for studying cell signaling networks, cancer, among many other applications.

This eBook provides protocols for the analysis of more than 30 proteins by Simple Western Charge, including common components of signaling pathways, enzymes, membrane proteins, and more.

TABLE OF CONTENTS

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

P-EGFR

SUMMARY

The epidermal growth factor receptor (EGFR) is a transmembrane receptor for the epidermal growth factor family (EGF family) of extracellular protein ligands. Ligand binding activates its intracellular tyrosine kinase domain resulting in auto as well as substrate phosphorylation. Mutation and deregulation of EGFR is implicated in many cancer types. The data shows a time dependent change in anti-phospho EGFR (Y1068) antibody signal in HeLa cells in response to EGF treatment (FIGURE 1,2).

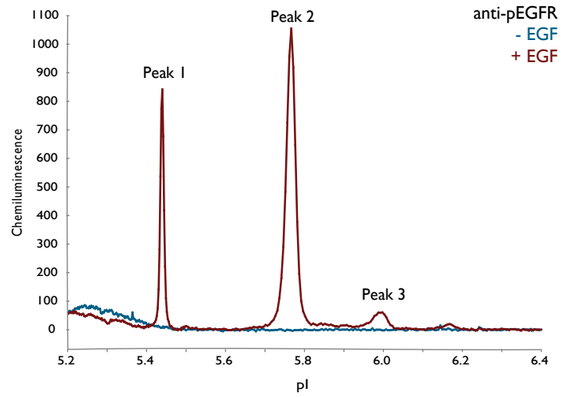


FIGURE 1. Detection of EGFR phosphorylation in response to EGF stimulation. HeLa cells were serum starved overnight and then treated with 50 ng/mL EGF for 5 minutes. The anti-phospho EGFR (Y1068) antibody detected three induced phospho-EGFR peaks, with pI values around 5.4 (Peak 1), 5.8 (Peak 2), and 6.0 (Peak 3). *NOTE: Detection of the chemiluminescent signal produced is relative. Absolute units may vary depending on cell line, treatment, and assay conditions.*

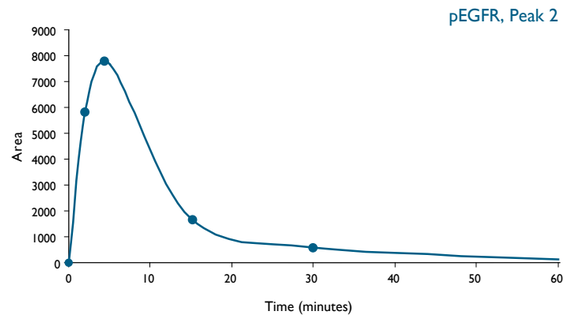


FIGURE 2. Time course of EGF response. Following 50 ng/mL EGF stimulation, peak areas for phospho-EGFR Peak 2 were plotted at time points 0, 2, 5, 15, 30, and 60 minutes.

PROTOCOL

CELL PREPARATION	
Cell culture	HeLa cells (ATCC, PN CCL-2) were cultured in DMEM (ATCC, PN 30-2002) containing 10% FBS (Hyclone, PN SH30070.03) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing DMEM with no additives.
Treatment	50 ng/mL EGF (Millipore, PN 01-107) in DMEM for times indicated in graphs.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standards 4.92, 5.5, 7.0 (ProteinSimple, PN 040-027, PN 040-028, PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-phospho EGFR (Y1068) (Abcam, PN ab40815), 1:200 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	Anolyte Refill (ProteinSimple, PN 040-337)
Catholyte	Catholyte Refill (ProteinSimple, PN 040-338)
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-EGFR (non-pY1173)	Millipore	05-484
Anti-phospho EGFR (Y1068)	Abcam	ab40815

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-phospho EGFR (Y1045), clone 11C2	Millipore	04-284
Anti-p-Tyr (PY99)	Santa Cruz Biotechnology	sc-7020

AKT

SUMMARY

AKT, also referred to as PKB or Rac, plays a critical role in controlling cell survival and apoptosis. This protein kinase is activated by insulin and various growth and survival factors to function in a Wortmannin-sensitive pathway involving PI3 kinase. The main isoforms identified so far are AKT1, 2 and 3. AKT3 is mainly expressed in the brain. AKT1 and 2 play differential roles in glucose homeostasis. Activation at Thr308 and Ser473 are the main activating phosphorylation events for AKT1. Our data show increased phosphorylation of AKT using a phospho-Ser473 specific antibody (FIGURE 3). This antibody is believed to recognize sites corresponding to phospho-Ser473 on all three AKT isoforms (see Cell Signaling Technologies data sheet).

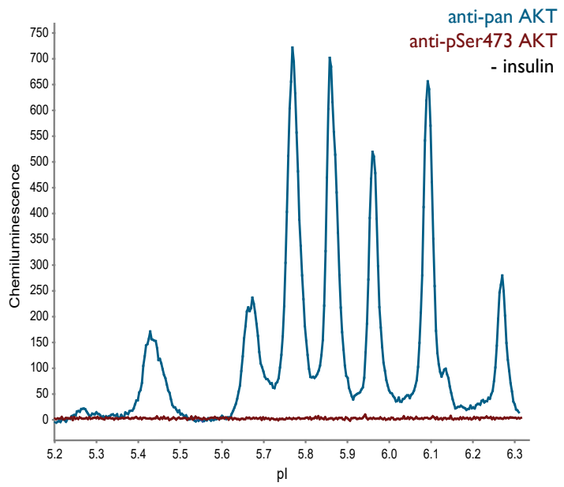


FIGURE 3. Insulin treatment significantly increases AKT phosphorylation at Ser473 in HeLa cells HeLa cells were treated -/+ 200 nM insulin for 5 minutes. In the untreated cells (top panel), a number of discrete peaks were detected using the sc-8312 anti-pan AKT antibody (top panel, blue trace). However, the CST 9271 antibody detected no phospho-Ser473 signal (top panel, red trace). In contrast, three distinct phospho-Ser473 peaks were detected after insulin treatment (bottom panel, red trace). Note that the anti-pan AKT antibody also detected peaks with identical pIs to the three phospho-Ser473 peaks (bottom panel, blue trace). The peaks at pI 5.8 and 5.95 are likely to be AKT2 isoforms, as determined by a specific anti-AKT2 antibody (data not shown).

P-EGFR | AKT | PTEN | ALAS1 | GSK-3A | GSK-3B | TRX1 | HSP70 | HSP70 LC | CASPASE 3 | B2M | P-STAT3 | P-STAT5
PLCG1 | ERK1/2 | MEK1 | MEK2 | SRC | P-JNK | P-P27 | 4E-BP1 | 4E-BP2 | CRK-L | C-MYC | HA | ASNS
LDH | CIAP1 | GFP | EEF2 | MFN1 | MFN2 | SURVIVIN | P-P38

PROTOCOL

CELL PREPARATION	
Cell culture	HeLa cells were cultured in DMEM (ATCC, PN 30-2002) containing 10% FBS (Irvine Scientific, PN 3000-A) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes to dislodge. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in DMEM without serum.
Treatment	200 nM Insulin (Sigma, PN I6634) in DMEM for 5 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Cell culture: Pre-treatment:Treatment: Lysis buffer: Lysis details: Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-AKT1/2 (Santa Cruz Biotechnology, PN SC8312) and Anti-phospho AKT1 (Cell Signaling Technology, PN 9271), both 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-AKT1/2	Santa Cruz Biotechnology	sc-8312
Anti-AKT1	Millipore	05-796
Anti-AKT2	Cell Signaling Technology	2962
Anti-phospho AKT1	Cell Signaling Technology	9271
Anti-phospho AKT1	Epitomics	2118-1

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-AKT1/2	Cell Signaling Technology	9272

PTEN

SUMMARY

Authors: Matthew Riolo Ph.D., Raul Alonso-Sabadell, Gabriela Chiosis Ph.D., Memorial Sloan-Kettering Cancer Center, New York, NY

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumor suppressor protein (**FIGURE 4**) that is frequently mutated or deleted in many tumors. PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PtdInsP3) inhibiting phosphoinositide 3-kinase (PI3K) activation of AKT repressing cell growth, proliferation and survival. Recent studies show that PTEN phosphorylation alters its stability and function.

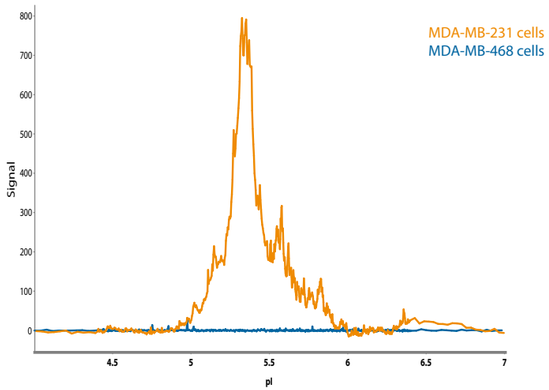


FIGURE 4. Detection of PTEN in triple negative breast cancer cell line MDAMB231. PTEN expression level in untreated MDA-MB-231 cells (orange) compared to untreated MDA-MB-468 (PTEN deficient) cells (blue).

PROTOCOL

CELL PREPARATION	
Cell culture	MDA-MB-468 (PN HTB-132) and MDA-MB-231 cells (PN HTB-26) were obtained from ATCC. Both cell lines were cultured in DMEM/F12 supplemented with non-essential amino acids, 1X Penicillin/Streptomycin and 10% FBS (Gemini Bio-Products, PN 100-106). Cells were cultured in large asks and split 1:5 every third day using 0.25% Trypsin.
Lysis buffer	Bicine/CHAPS Lysis Buer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Media was aspirated, and cells were washed once with 10 mL of ice-cold PBS. Cells were then scraped in 10 mL of ice-cold PBS and collected into cold 15-mL tubes. Tubes were centrifuged at 1250 rpm at 4 °C for 5 minutes. PBS was aspirated and cells were resuspended in 800 L of ice cold PBS and transferred to cold 1.5-mL Eppendorf tubes. Tubes were centrifuged at 1600 rpm at 4 °C for 4 minutes. PBS was aspirated and cells were resuspended in 50 -100 Ls of ice-cold Bicine/CHAPS lysis buer, supplemented with protease and phosphatase inhibitors. Cells were then incubated on ice for at least 30 minutes. Lysates were claried by centrifugation at 13,000 rpm for 20 minutes at 4 °C. Supernatants were stored at -80 °C.

ASSAY REAGENTS	
Protein concentration	0.25 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764), 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510)
Ampholyte premix	50% Premix G2 pH 3-10 (ProteinSimple, PN 040-968), 50% Premix G2 pH 5-8 (ProteinSimple, PN 040-973)
pI standards	pI Standard Ladder 1 (ProteinSimple, PN 040-644), pI standard 4.4 (ProteinSimple, PN 040-026), pI standard 5.5 (Protein Simple, PN 040-028)
Procedure	Step 1) Dilute lysate to 1 mg/mL with sample diluents. Step 2) In a separate tube, mix ampholyte premixes and pI standards. Step 3) Mix step 1 and step 2 at 1:4 to create final protein concentration.
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-PTEN (Cell Signaling Technology, PN 9188, 1:50)
Detection antibody	Amplified Secondary Antibody Detection Kit (ProteinSimple, PN 041-126), 1:100
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide xDR	Mixed 1:1 (ProteinSimple, PN 040-652 and PN 043-379)

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	100 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Detection streptavidin incubation	10 minutes
Chemiluminescence exposure	30, 60, 120, 240, 480 and 960 seconds

ALAS1 LOADING CONTROL

SUMMARY

The human housekeeping protein Delta-aminolevulinate Synthase catalyzes the condensation of glycine with succinyl-CoA to form delta-aminolevulinic acid. It is represented in the NanoPro assay by two peaks around pI 5.6 and 5.9. Our data show its utility as a loading control for EGF stimulation in HeLa (**FIGURE 5**) and MCF10A cells (**FIGURE 6**).

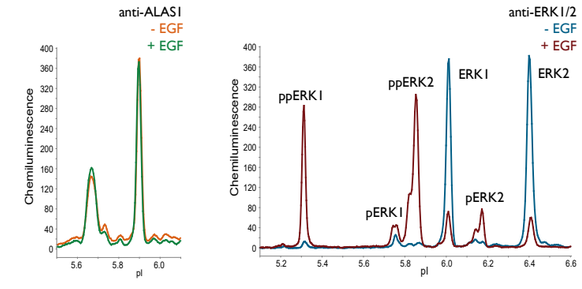


FIGURE 5. ALAS1 serves as a consistent loading control–levels are equivalent in EGF stimulated and non-stimulated HeLa cells. HeLa cells were stimulated with 50 ng/mL epidermal growth factor (EGF) in full media for 15 minutes. Changes in ERK1 and ERK2 were observed in stimulated cells as compared to non-stimulated while ALAS1 remained unchanged.

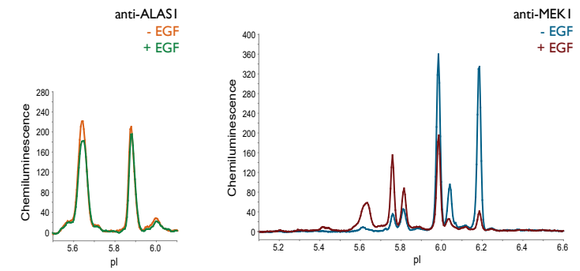


FIGURE 6. ALAS1 serves as a consistent loading control–levels are equivalent in EGF stimulated and non-stimulated MCF10A cells. MCF10A cells were stimulated with 20 ng/mL EGF in full media for 15 minutes. Changes in MEK1 were observed in stimulated cells as compared to non-stimulated while ALAS1 remained unchanged.

PROTOCOL

HELA CELL PREPARATION	
Cell culture	HeLa cells (ATCC, PN CCL-2) were cultured in DMEM (ATCC, PN 30-2002) containing 5% FBS (Irvine Scientific, PN 3000-A) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Mediatech, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing DMEM and 1% FBS.
Treatment	50 ng/mL EGF (Millipore, PN 01-107) in full media (Cellgro, PN 21-031-CV) for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	RIPA Lysis Buffer (ProteinSimple, PN 040-483) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482)
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge.Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.03 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5–8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standards 4.92, 5.5, 7.0 (ProteinSimple, PN 040-027, PN 040-028, PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-ALAS1 (Abcam, PN ab22153), 1:100 in Antibody Diluent (ProteinSimple, PN 040-309
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

MCF10A CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, cat# CRL-10317) were cultured in MEGM (Lonza, cat# CC-3151) containing 5% FBS, 1x Penicillin/Streptomycin/Glutamine, 100 ng/mL Cholera Toxin (Calbiochem, cat# 227035), and MEGM SingleQuot (Lonza, cat# CC-4136) with final concentration of 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 10 µg/mL hEGF, and 5 mg/mL insulin. Cells were split 1:5 every 3 days using 0.25% trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM, 1% FBS, 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 1x Penicillin/Streptomycin/Glutamine.
Treatment	20 ng/mL EGF in full media for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	RIPA Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, cat# 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-Alas1	Abcam	ab22153

GLYCOGEN SYNTHASE KINASE 3α (GSK-3α)

SUMMARY

GSK3 is a critical downstream element of the PI3 kinase/AKT cell survival pathway whose activity can be inhibited by AKT-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3α. While GSK-3α and GSK-3β have high sequence homology, their biological function differs. The data presented shows an increase of peaks detected by anti-phospho GSK-3α antibody as well as an increase of the same peaks detected by the total anti-GSK-3α antibody in response to EGF treatment in MCF10A cells (**FIGURE 7**). At the same time, the peaks not recognized by the anti-phospho Ser21 antibody decrease in size, implying that these peaks represent non-phospho or non-pS21 phospho GSK-3α forms. The position of these peaks around pI 9 is in accordance with the theoretical pI for this sequence. In other cell systems (A549 for example), both the anti-total GSK-3α antibody as well as the anti-phospho GSK-3α antibody also recognize peaks around pI 6. Alignment of the GSKα and GSKβ profiles in addition to use of antibodies recognizing both isoforms confirmed the specificity of the anti-GSK-3α versus the anti-GSK-3β antibodies used (data not shown).

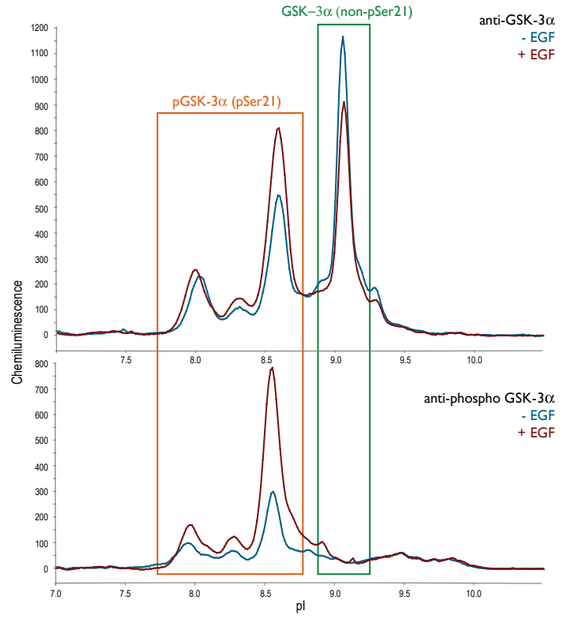


FIGURE 7. EGF stimulation results in increased phosphorylation of GSK-3α at Ser21 MCF10A cells were stimulated with epidermal growth factor (EGF) for 15 minutes. The peaks labeled pSer21 in the figure reproducibly showed increased signal after EGF stimulation, as detected by anti-GSK-3α (upper traces) and antiphospho GSK-3α (Ser21) (lower traces). The peak labeled non-pSer21 in the upper trace showed slightly decreased signal after EGF treatment and was not recognized by anti-phospho GSK-3α (Ser21), which suggests that this peak represents a lower phosphorylation state of GSK-3α with insignificant phosphorylation at Ser21.

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3151) containing 5% FBS (Hyclone, PN 1677-006), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020), 100 ng/mL Cholera Toxin (Calbiochem, PN 227035), and MEGM SingleQuot (Lonza, PN CC-4136) with final concentration of 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 10 µg/mL hEGF, 5 mg/mL insulin. Cells were split 1:5 every 3 days using 0.25% Trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM, 1% FBS, 1x Penicillin/Streptomycin/Glutamine, 100 ng/mL cholera toxin, 13 mg/mL BPE, and 0.5 mg/mL hydrocortisone.
Treatment	20 ng/mL EGF (Millipore, PN 01-107) in full media without MEGM SingleQuot for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	RIPA Lysis Buffer (ProteinSimple, PN 040-483) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Ampholyte-free premix (Premix G2, PN 040-967) with 12% Pharmalyte pI 3–10, 1% TEMED (Sigma, PN T7024)
pI standards	pI Standards 5.5 and 7.0 (ProteinSimple, PN 040-028 and PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-GSK-3α (Cell Signaling Technology, PN 4818) and Anti-phospho GSK-3α (Ser21) (Cell Signaling Technology, PN 9316) in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656) 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240, and 480 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-GSK-3α	Cell Signaling Technology	4818
Anti-phospho GSK-3α (Ser21)	Cell Signaling Technology	9316

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-GSK-3α	Cell Signaling Technology	9338
Anti-GSK-3α/β	Millipore	05-412

GLYCOGEN SYNTHASE KINASE 3β (GSK-3β)

SUMMARY

GSK3 is a critical downstream element of the PI3 kinase/AKT cell survival pathway whose activity can be inhibited by AKT-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β. While GSK-3α and GSK-3β have high sequence homology, their biological function differs. The data presented shows an increase of peaks detected by anti-phospho GSK-3β antibody as well as an increase of the same peaks detected by the total anti-GSK-3β antibody in response to EGF treatment in MCF10A cells (**FIGURE 8**). At the same time, the peaks not recognized by the anti-pS21 antibody decrease in size, implying that these peaks represent non-phospho or non-pS21 phospho GSK-3α forms. The position of these peaks around pI 9 is in accordance with the theoretical pI for this sequence. Alignment of the GSK-3α and GSK-3βprofiles, in addition to use of antibodies recognizing both isoforms, confirmed the specificity of the anti-GSK-3α versus the anti-GSK-3β antibodies used (data not shown).

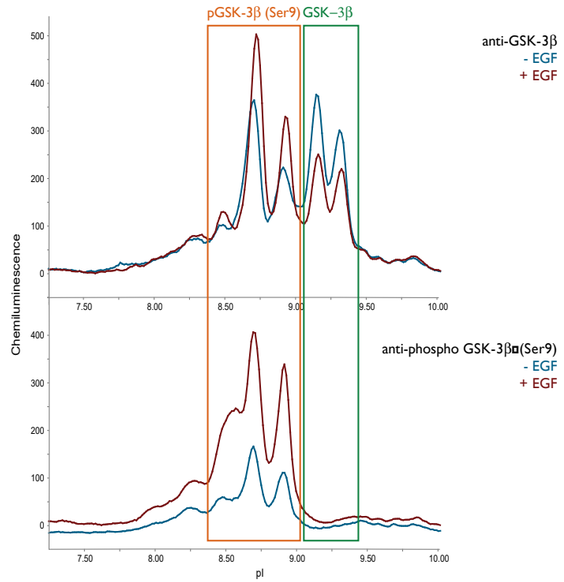


FIGURE 8. EGF stimulation results in increased phosphorylation of GSK-3β at Ser9. MCF10A cells were stimulated with epidermal growth factor (EGF) for 15 minutes. The peaks labeled pGSK-3β (Ser9) in the figure showed increased signal after EGF stimulation, as detected by anti-GSK-3β (upper traces) and anti-phospho GSK-3β (Ser9) (lower traces). The two peaks labeled "GSK-3β" in the upper trace showed decreased signal after EGF treatment and were not recognized by anti-phospho GSK-3β (Ser9), which suggests that these peaks are in lower phosphorylation states of GSK-3β with insignificant phosphorylation at Ser9.

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3151) containing 5% FBS (Hyclone, Scientific, PN 1677-006), 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020), 100 ng/mL Cholera Toxin (Calbiochem, PN 227035), and MEGM SingleQuot (Lonza, PN CC-4136) with final concentration of 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 10 µg/mL hEGF, 5 mg/mL insulin. Cells were split 1:5 every 3 days using 0.25% trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM, 1% FBS, 1x Penicillin/Streptomycin/Glutamine, 100 ng/mL cholera toxin, 13 mg/mL BPE, and 0.5 mg/mL hydrocortisone.
Treatment	20 ng/mL EGF (Millipore, PN 01-107) in full media without MEGM SingleQuot for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	RIPA Lysis Buffer (ProteinSimple, PN 040-483) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Ampholyte-free premix (ProteinSimple Premix G2, PN 040-967) with 12% Pharmalyte pI 3-10, 1% TEMED (Sigma, PN T7024)
pI standards	pI Standards 5.5 and 7.0 (ProteinSimple, PN 040-028 and PN 040-031), 1:100 and pI Standard 9.7 at 2 µM (Anaspec, PN 61750)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-GSK-3β (Abcam, PN ab69739) and Anti-phospho GSK-3β (Ser9) (Cell Signaling Technology, PN 9336), both 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 50 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240, and 480 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-GSK-3β	Abcam	69739
Anti-pGSK-3β	Cell Signaling Technology	9336

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-GSK-3α/β	Millipore	05-412

THIOREDOXIN LOADING CONTROL

SUMMARY

Thioredoxin acts as an antioxidant and is found in nearly all known organisms. It exists in two isoforms and presents a double peak around pI 4.6. The data presented show the utility of Thioredoxin as a loading control for EGF treated A549 and HeLa cells (**FIGURE 9,10**).

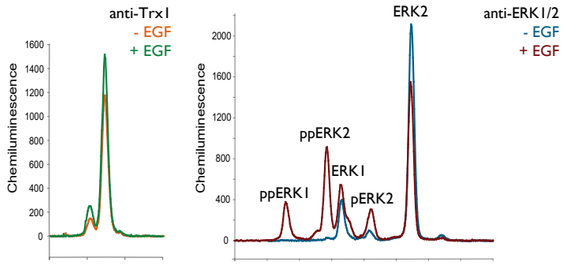


FIGURE 9. Trx1 as a loading control in A549 cells. A549 cells were treated with 30 ng/mL epidermal growth factor (EGF) for 5 minutes. The EGF treatment caused induction of ERK1 and ERK2 phosphorylation (right panel), but did not significantly affect Trx1 signals (left panel).

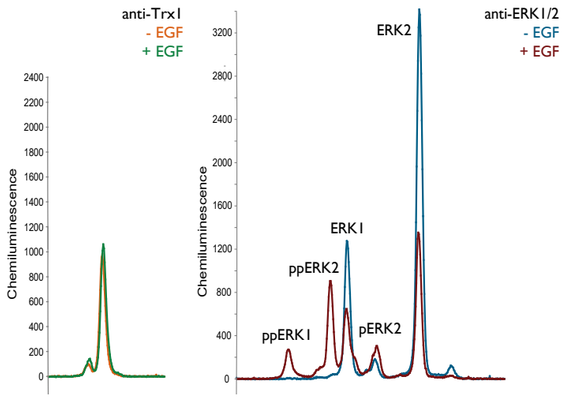


FIGURE 10. Trx1 as a loading control in HeLa cells. HeLa cells were treated with 50 ng/mL EGF for 15 minutes. The EGF treatment caused induction of ERK1 and ERK2 phosphorylation (right panel), but did not significantly affect Trx1 signals (left panel).

PROTOCOL

HELA CELL PREPARATION	
Cell culture	HeLa cells (ATCC, PN CCL-2) were cultured in DMEM (ATCC, PN 30-2002) containing 10% FBS (Hyclone, PN SH30070.03) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Mediatech, PN 25-053-CI) at 37 °C for 3-5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing DMEM without serum.
Treatment	50 ng/mL EGF (Millipore, PN 01-107) in DMEM without serum for 5 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

A549 CELL PREPARATION	
Cell culture	A549 cells (ATCC, PN CCL-185) were cultured in F-12K media (ATCC, PN 30-2004) containing 10% FBS and 1x Penicillin/Streptomycin/Glutamine. Cells were split 1:5 every 3 days using 0.25% trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing F-12K without serum.
Treatment	30 ng/mL EGF in F-12K without serum for 5 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor
Ampholyte premix	80% Premix 5-8 (ProteinSimple Premix G2, PN 040-973) and 20% Premix 4-9 (ProteinSimple Premix G2, PN 040-969)
pI standards	pI Standards 5.5 and 7.0 (ProteinSimple, PN 040-028 and PN 040-031), 1:100 and pI Standard 9.7 at 2 µM (Anaspec, PN 61750)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Trx1 (Cell Signaling Technology, PN 2285), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-Thioredoxin1	Cell Signaling Technology	2285

HEAT SHOCK PROTEIN 70 (HSP70)

SUMMARY

The 70 kDa heat shock proteins (Hsp70) are a family of ubiquitously expressed heat shock proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are an important part of the cell's machinery, and help to protect proteins from misfolding under stress. We show an increase of Hsp-70 expression in response to heat shock at 42 °C in A549 cells (**FIGURE 11**).

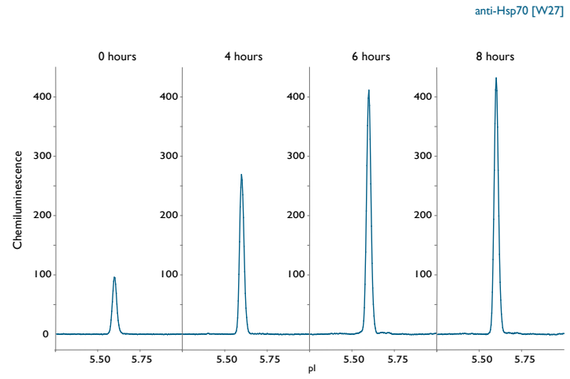


FIGURE 11. Strong increase in expression of Hsp70 after heat shock treatment. A549 cells were placed at 42 °C for 0, 4, 6, or 8 hours. An increase in Hsp70 signal was detected with increasing exposure to elevated temperatures.

PROTOCOL

CELL PREPARATION	
Cell culture	A549 cells (ATCC, PN CCL-185) were cultured in F-12K media (ATCC, PN 30-2004) containing 10% FBS (Hyclone, PN SH30070.03) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Mediatech, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 3.
Treatment	Cells were incubated for 4, 6, or 8 hours at 42 °C, 5% CO_2 while control remained at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.003 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-973)
pI standards	pI Standards 4.92, 5.5, 7.0 (ProteinSimple, PN 040-027, PN 040-028, PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Hsp70 [W27] (Novus Biologicals, PN NB600-571),1:500 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Mouse HRP (ProteinSimple, PN 040-655), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

HEAT SHOCK PROTEIN 70 (HSP70) LOADING CONTROL

SUMMARY

The 70 kilodalton heat shock proteins (Hsp70) are a family of ubiquitously expressed heat shock proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are an important part of the cell's machinery, and help to protect proteins from misfolding under stress. We describe its use as loading control for EGF stimulation in HeLa (**FIGURE 12**) and MCF10A (**FIGURE 13**) cells. In the NanoPro assay, Hsp70 is present as a single peak around pI 5.8 under the conditions described.

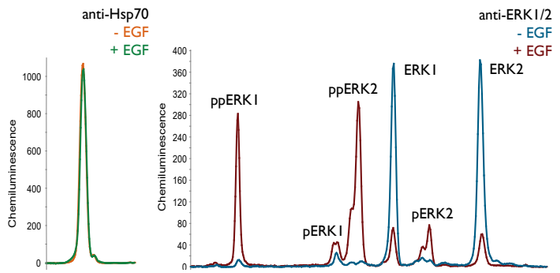


FIGURE 12. Hsp70 serves as a consistent loading control; levels are equivalent in EGF stimulated and nonstimulated HeLa cells. HeLa cells were stimulated with 50 ng/mL epidermal growth factor (EGF) for 15 minutes. Changes in phosphorylation of ERK1 and ERK2 were observed in stimulated cells as compared to non-stimulated while Hsp70 levels remained unchanged.

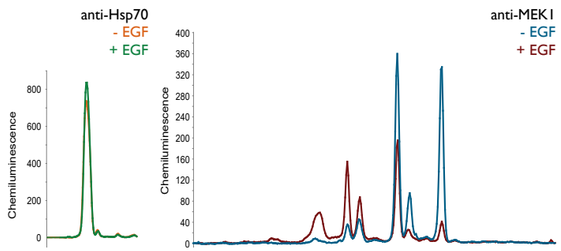


FIGURE 13. Hsp70 serves as a consistent loading control; levels are equivalent in EGF stimulated and nonstimulated MCF10A cells. MCF10A cells were stimulated with 20 ng/mL EGF for 15 minutes. Changes in amounts of MEK1 were observed in stimulated cells as compared to starved cells while Hsp70 levels remained unchanged.

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-HSP-70	Novus Biologicals	NB600-571

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

PROTOCOL

HELA CELL PREPARATION	
Cell culture	HeLa cells (ATCC, PN CCL-2) were cultured in DMEM (ATCC, PN 30-2002) containing 5% FBS (VWR, PN 1677-006) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Mediatech, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 for 14 hours in starvation medium containing DMEM and 1% FBS.
Treatment	50 ng/mL EGF (Millipore, PN 01-107) in starvation medium for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	RIPA Lysis Buffer (ProteinSimple, PN CBS401) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.03 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5–8 (nested), (ProteinSimple Premix G2, PN 040-972)
pI standards	4.92, 5.5, 7.0 (ProteinSimple, PN 040-027, PN 040-028, PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Hsp70 [W27] (Novus Biologicals, PN NB600-571)1:500 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Mouse HRP (ProteinSimple, PN 040-655), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

MCF10A CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3151) containing 5% FBS, 1x Penicillin/Streptomycin/Glutamine, 100 ng/mL Cholera Toxin (Calbiochem, PN 227035), and MEGM SingleQuot (Lonza, PN CC-4136) with final concentration of 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 10 µg/mL hEGF, and 5 mg/mL insulin. Cells were split 1:5 every 3 days using 0.25% trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM, 1% FBS, 13 mg/mL BPE, 0.5 mg/mL hydrocortisone, 100 ng/mL cholera toxin, and 1x Penicillin/Streptomycin/Glutamine.
Treatment	20 ng/mL EGF in full media for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	RIPA Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Wash cells with 10 mL of ice-cold PBS, aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-HSP-70	Novus Biologicals	NB600-571

CASPASE 3

SUMMARY

Caspases 3 exists as an inactive proenzyme that undergoes proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. Its activation is an important apoptosis marker. The data shows increased signal with antibodies specific to the Caspase 3 p17 subunit in response to apoptosis induction through prolonged treatment of K562 cells with Iminatib (aka Gleevec®) at an expected pI of around 6.3–6.5 (**FIGURE 14**).

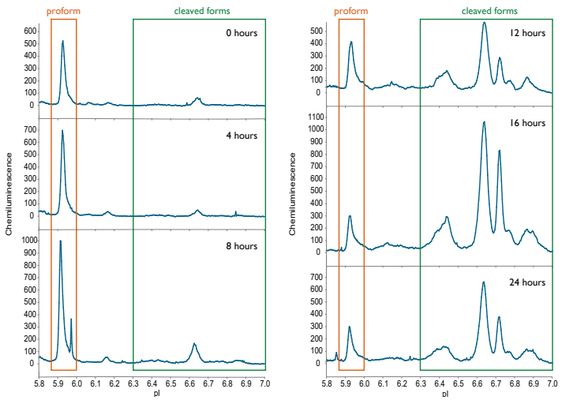


FIGURE 14. Time-dependent cleavage of pro-Caspase 3 in Imatinib-treated K562 cells. K562 cells were treated with 5 µM Imatinib for 0, 4, 8, 12, 16, and 24 hours. The pro-Caspase 3 peak labeled proform at pI 5.95 was identified through specific recognition by pro-Caspase 3 antibodies (Millipore, cat# 04-440 and cat# 05-654, and Cell Signaling Technology, cat# CS9662) (data not shown). The Caspase 3 fragment peaks labeled cleaved forms were induced by Imatinib treatment and recognized by specific anti-p17 antibodies. Interestingly, most antibodies described as anti-p17 specific also cross-react with the proform.

PROTOCOL

CELL PREPARATION	
Cell culture	K562 cells (ATCC, PN CCL-243) were cultured in RPMI 1640 media (Cellgro, PN 10-041-CV) containing 10% FBS (Hyclone, PN SH30070.03) and 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days. Data shown from cells at passage 5.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.
Treatment	5 µM Imatinib Methanesulfonate Salt (LC Laboratories, PN 1-5508) in starvation medium for 0, 4, 8, 12, 16, or 24 hours at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Collect cells by centrifugation (1000 x *g*, 5 minutes). Transfer cells to a 15-mL centrifuge tube, spin (1000 x *g*, 5 minutes) to pellet the cells. Aspirate media. Wash cell pellet with 1 mL of ice-cold PBS (Cellgro, PN 21-031-CV). Transfer cells to a 1.5-mL centrifuge tube, spin (14,000 x *g*, 2 minutes). Aspirate wash. Keeping tube on ice, add 400 µL ice-cold lysis buffer to pellet, pipet up and down to resuspend. Incubate for an additional 30 minutes, rotating. Clarify by centrifugation (14,000 x *g*, 15 minutes). Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.15 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Cleaved Caspase 3 (Asp175) (Cell Signaling Technology, PN 9661), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	120 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	240 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	120, 240, and 480 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-Cleaved Caspase 3 (Asp175)	Cell Signaling Technology	9661
Anti-Cleaved Caspase 3 (Asp175) (5A1E)	Cell Signaling Technology	9664

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-Caspase 3 (pro-form)	Millipore	04-440
Anti-Caspase 3	Abcam	05-412
Anti-Caspase 3	Abcam	ab77973
Anti-Caspase 3	Cell Signaling Technology	9662
Anti-Caspase 3 clone 4-1-18	Millipore	05-654
Anti-Caspase 3 [E83-77]	Abcam	ab32042
Anti-Caspase 3 active (cleaved) form	Millipore	ab3623
Anti-Caspase 3 p17 (T-20)	Santa Cruz Biotechnology	sc-22140

B-2-MICROGLOBULIN LOADING CONTROL

SUMMARY

B-2-Microglobulin, also known as B2M, is a component of MHC class I molecules, which are present on all nucleated cells. In the NanoPro assay, it presents a single peak around pI 6. The data show its application as a loading control for EGF stimulation in HeLa cells (**FIGURE 15**) as well as MCF10A cells (**FIGURE 16**).

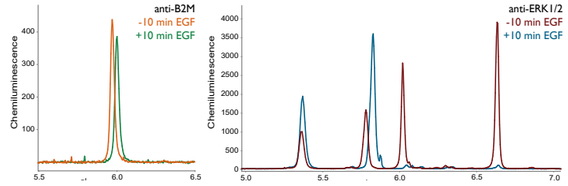


FIGURE 15. B-2-Microglobulin identified as a good loading control candidate for HeLa cells. HeLa cells were stimulated for 10 minutes with 50 ng/mL EGF. While ERK showed a strong response to treatment, B-2-Microglobulin did not change, identifying it as a good candidate for use as a loading control.

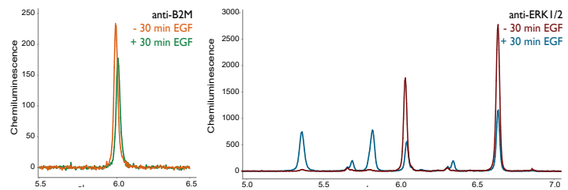


FIGURE 16. B-2-Microglobulin identified as a good loading control candidate for MCF10A cells. MCF10A cells were stimulated for 30 minutes with 100 nM EGF. While ERK showed a strong response to treatment, B-2-Microglobulin did not change, identifying it as a good loading control.

PROTOCOL

HELA CELL PREPARATION	
Cell culture	HeLa cells (ATCC, PN CCL-2) were cultured in DMEM (ATCC, PN 30-2002) and 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing DMEM.
Treatment	50 ng/mL EGF (Sigma, PN E1257) in DMEM for 10 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80°C

ASSAY REAGENTS	
Protein concentration	0.03 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (Premix G2, PN 040-972)
pI standards	4.92, 5.5, 7.0 (ProteinSimple, PN 040-027, PN 040-028, PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-B-2-Microglobulin (Abcam, PN ab75853), 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

MCF10A CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CCL-10317) were cultured in MEGM (Lonza, PN CC-3151) containing 10% FBS (Hyclone, PN1677-006), 100 ng/mL Cholera Toxin (CalBiochem, PN 227035), 1x Penicillin/Streptomycin/Glutamine, and MEGM SingleQuot Lonza, PN CC4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM.
Treatment	600 ng/mL EGF (Sigma, PN E1257) in MEGM for 30 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-B-2-Microglobulin	Abcam	ab75853

P-STAT3

SUMMARY

The Signal Transducer and Activator of Transcription (STAT) proteins regulate many aspects of cell growth, survival and differentiation. The transcription factors in this family are activated by Janus Kinase (JAK). Dysregulation of this pathway is frequently observed in primary tumors and leads to increased angiogenesis, enhanced survival of tumors and immunosuppression. STAT3 is constitutively active in overexpressing BCR-ABL K562 myelogenous leukaemia cells. Imatinib (also known as Gleevec®), a BCR-ABL inhibitor, reduces STAT3 phosphorylation in these cells (**FIGURE 17**). STAT3 is also part of the Epidermal Growth Factor (EGF) signaling cascade as shown in MCF10A cells (**FIGURE 18**).

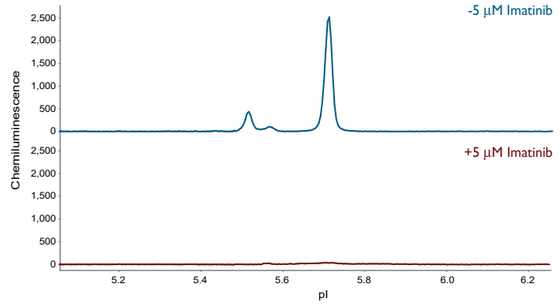


FIGURE 17. Imatinib treatment reduces STAT3 phosphorylation in K562 cells. K562 cells were treated -/+ 5 µM imatinib for 24 hours. The CST 9131 anti-phospho STAT3 antibody recognized three peaks in the control cells (blue trace). These peaks were strongly inhibited by imatinib treatment (red trace). A similar phospho-STAT3 signature was detected in EGF-treated MCF10A cells using Abcam 30646 (see figure below).

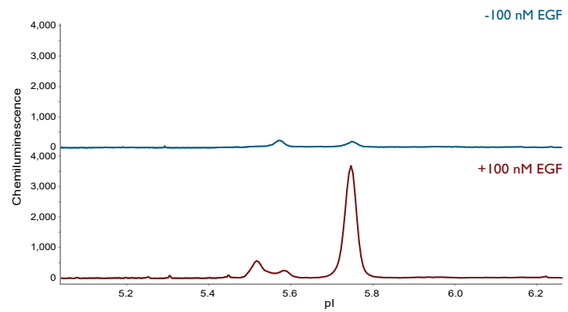


FIGURE 18. EGF treatment increases STAT3 phosphorylation in MCF10A cells. MCF10A cells were treated -/+100 nM EGF for 5 minutes. The Abcam 30646 anti-phospho STAT3 antibody detected little phospho- STAT3 signal in the untreated MCF10A cells (blue trace). Exposure to EGF significant increase phospho- STAT3 signal in these cells (red trace).

PROTOCOL

K562 CELL PREPARATION	
Cell culture	K562 cells (ATCC, PN CCL-243) were cultured in RPMI 1640 media (Cellgro, PN10-041-CV) containing 10% FBS (Irvine Scientific, PN 3000-A) and 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days by removing an aliquot of cells and transferring them to fresh media. Data shown from cells at passage 5.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.
Treatment	5 M Imatinib Methanesulfonate Salt (LC laboratories, PN 1-5508) in starvation medium for 24 hours at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Collect cells by centrifugation (1000 x *g*, 5 minutes). Transfer cells to a 15-mL centrifuge tube, spin (1000 x *g*, 5 minutes) to pellet the cells. Aspirate media. Wash cell pellet with 1 mL of ice-cold PBS (Cellgro, PN 21-031-CV). Transfer cells to a 1.5-mL centrifuge tube, spin (14,000 x *g*, 2 minutes). Aspirate wash. Keeping tube on ice, add 400 µL ice-cold lysis buffer to pellet, pipet up and down to resuspend. Incubate for an additional 30 minutes, rotating. Clarify by centrifugation (14,000 x *g*, 15 minutes). Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

MCF10A CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3151) containing 5% FBS, 1x Penicillin/Streptomycin/Glutamine and MEGM SingleQuot (Lonza, PN CC-4136). Cells were split 1:5 every 3 days using 0.25% Trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 4.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 for 20 hours in starvation medium.
Treatment	600 ng/mL EGF in starvation medium for 5 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Lysis details: Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-phospho STAT3 (Cell Signaling Technology, cat # 9131 and Abcam, PN ab30646), 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240 and 480 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-phospho STAT3	Abcam	ab30646
Anti-phospho STAT3	Cell Signaling Technology	9131

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-STAT3	Santa Cruz Biotechnology	sc-483
Anti-STAT3	Genscript	A00276
Anti-STAT3	Cell Signaling Technology	4904 and 9132
Anti-phospho STAT3	Genscript	A00251

P-STAT5

SUMMARY

The Signal Transducer and Activator of Transcription (STAT) proteins regulate many aspects of cell growth, survival and differentiation. The transcription factors in this family are activated by Janus Kinase (JAK). Dysregulation of this pathway is frequently observed in primary tumors and leads to increased angiogenesis, enhanced survival of tumors and immunosuppression. STAT5 is constitutively active in the overexpressing BCR-ABL K562 myelogenous leukaemia cell line. Imatinib (also known as Gleevec®), a BCR-ABL inhibitor, reduces STAT5 phosphorylation in these cells (**FIGURE 19**). STAT5 is also part of the Epidermal Growth Factor (EGF) signaling cascade as shown in MCF10A cells (**FIGURE 20**).

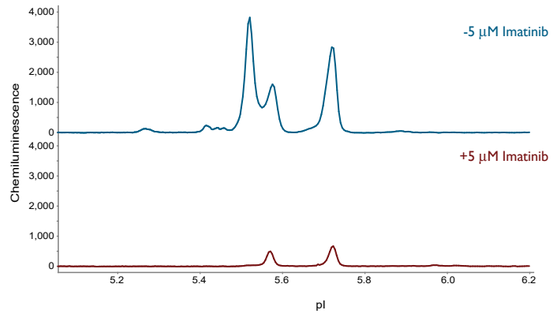


FIGURE 19. Imatinib treatment reduces STAT5 phosphorylation in K562 cells. K562 cells were treated -/+ 5 µM imatinib for 24 hours. The Abcam ab30648 anti-phospho STAT5a antibody recognized several peaks in the control cells (blue trace). These peaks were strongly inhibited by imatinib treatment (red trace). Peaks with similar pIs were detected in EGF-treated MCF10A cells (see figure below).

P-EGFR	AKT	PTEN	ALAS1	GSK-3A	GSK-3B	TRX1	HSP70	HSP70 LC	CASPASE 3	B2M	P-STAT3	P-STAT5
PLCG1	ERK1/2	MEK1	MEK2	SRC	P-JNK	P-P27	4E-BP1	4E-BP2	CRK-L	C-MYC	HA	ASNS
LDH	CIAP1	GFP	EEF2	MFN1	MFN2	SURVIVIN	P-P38					

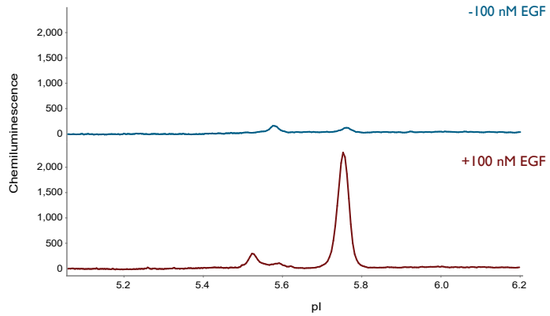


FIGURE 20. EGF treatment increases STAT5 phosphorylation in MCF10A cells. MCF10A cells were treated -/+ 100 nM EGF for 5 minutes. The Abcam ab30648 anti-phospho STAT5a antibody detected weak phospho STAT5 signals in the untreated MCF10A cells (blue trace). Exposure to EGF strongly induced phospho STAT5 signal (red trace).

PROTOCOL

MCF10A CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3150) containing 10% FBS, 1x Penicillin/Streptomycin/Glutamine, and MEGM Single-Quots. Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 4.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing MEGM.
Treatment	600 ng/mL EGF in starvation medium for 5 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

K562 CELL PREPARATION	
Cell culture	K562 cells (ATCC, PN CCL-243) were cultured in RPMI 1640 media (Cellgro, PN 10-041-CV) containing 10% FBS (Irvine Scientific, PN 3000-A) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days by removing an aliquot of cells and transferring them to fresh media. Data shown from cells at passage 5.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.
Treatment	5 µM Imatinib Methanesulfonate Salt (LC laboratories, PN 1-5508) in starvation medium for 24 hours at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Collect cells by centrifugation (1000 x *g*, 5 minutes). Transfer cells to a 15-mL centrifuge tube, spin (1000 x *g*, 5 minutes) to pellet the cells. Aspirate media. Wash cell pellet with 1 mL of ice-cold PBS (Cellgro, PN 21-031-CV). Transfer cells to a 1.5-mL centrifuge tube, spin (14,000 x *g*, 2 minutes). Aspirate wash. Keeping tube on ice, add 400 µL ice-cold lysis buffer to pellet, pipet up and down to resuspend. Incubate for an additional 30 minutes, rotating. Clarify by centrifugation (14,000 x *g*, 15 minutes). Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-phospho STAT5a (Abcam, cat # ab30648), 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240 and 480 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-phospho STAT5a	Abcam	ab30648

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-STAT5	Abcam	68465
Anti-STAT5	Santa Cruz Biotechnology	sc-28685
Anti-STAT5	Genscript	A00253
Anti-phospho STAT5a/b	Cell Signaling Technology	9351

PHOSPHOLIPASE C GAMMA 1 (PLCγ1)

SUMMARY

Phospholipase C (PLC) catalyzes the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) to produce the metabolite second messenger molecules inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). Increase of IP3 results in elevated intracellular free Ca^{2+}. PLC's are activated through G-protein coupled receptor stimulation as well as tyrosine receptor kinase activation and therefore bridge both important signaling pathways. The PLC family consists of 12 isoforms with different roles in signaling. For example, PLCγ1 forms a complex with activated EGF receptors, which leads to the phosphorylation of PLCγ at Tyr771, 783 and 1245. Here we detect the phosphorylation of PLCγ1 in HEK293 cells in response to EGF treatment (FIGURE 21).

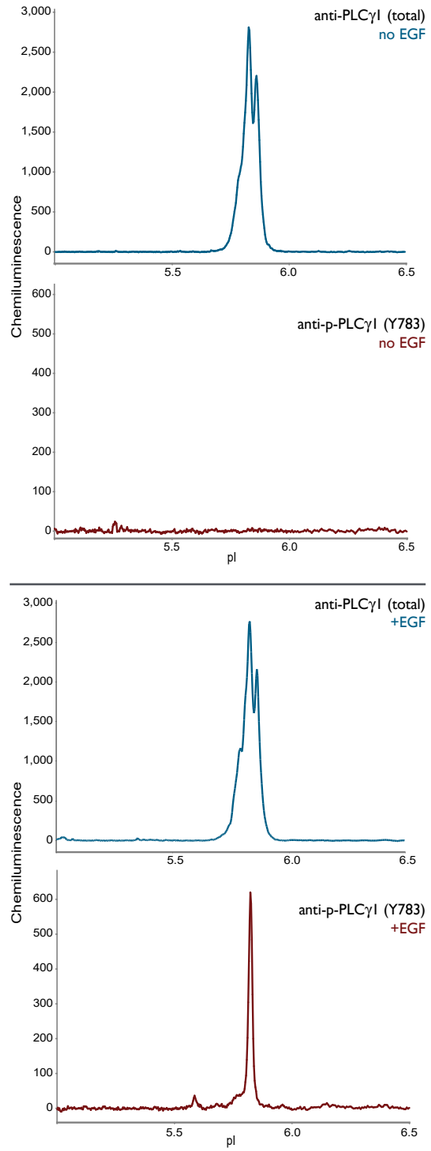


FIGURE 21. EGF treatment results in increased PLCγ1 phosphorylation in HEK293 cells. HEK293 cells were treated -/+ 50 ng/mL EGF for 15 minutes. EGF treatment resulted in a dramatic increase in a pI 5.8 peak detected with the anti-phospho (Y783) PLCγ1 antibody (bottom panel, lower trace). Additionally, a slight shoulder in the total PLCγ1 peak profile near pI 5.8 was detected with the anti-PLCγ1 (total) antibody (bottom panel, upper trace). Similar peak profiles have been generated in EGF-treated HeLa cells and serum-treated U937 cells (data not shown).

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

PROTOCOL

CELL PREPARATION	
Cell culture	HEK293 cells (ATCC, PN CRL-1573) were cultured in EMEM (ATCC, PN 30-2003) containing 10% FBS (Irvine Scientific, PN 3000-A) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEM without serum.
Treatment	50 ng/mL EGF in MEM without serum for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.08 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-PLCγ1 (Cell Signaling Technology, PN 2822) and anti-phospho PLC1 (Abcam, PN ab53125), both 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-PLCγ1	Cell Signaling Technology	2822
Anti-phospho PLCγ1	Abcam	ab53125

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-PLCγ1	Abcam	ab52200
Anti-phospho PLCγ1	Millipore	072134

ERK1/2

SUMMARY

Extracellular signal-regulated kinases (ERK) or classical MAP kinases are widely expressed intracellular signaling molecules involved in regulation of meiosis, mitosis, and post-mitotic functions in differentiated cells. Many different stimuli including growth factors, cytokines, virus infection, ligands for heterotrimeric *G* protein-coupled receptors, transforming agents, and carcinogens activate the ERK pathway. We show an exampleof ERK phosphorylation in MCF10A cells in response to treatment with epidermal growth factor (EGF) (**FIGURE 22**).

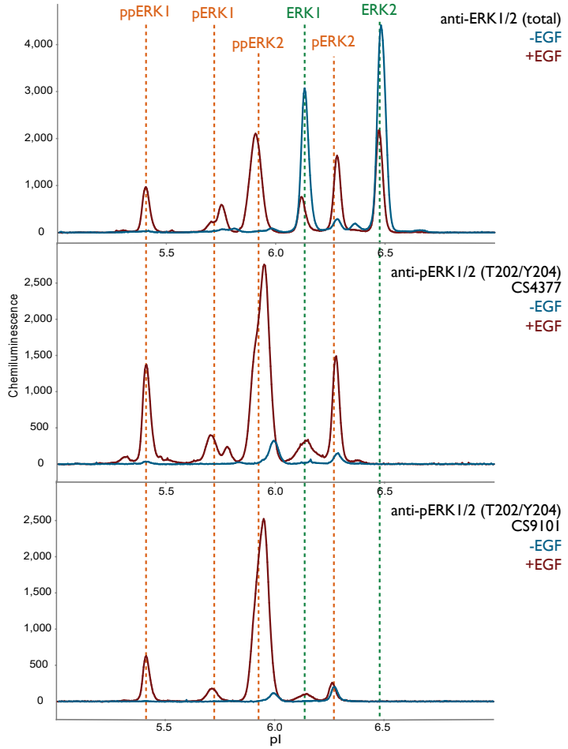


FIGURE 22. EGF stimulation results in increased ERK1/2 phosphorylation in MCF10A cells. MCF10A cells were treated -/+ 600 ng/mL EGF for 10 minutes. Non-phosphorylated ERK1 and ERK2 were the dominant ERK species present in untreated MCF10A cells, as detected using the Millipore 06-182 anti-ERK1/2 (total) antibody (upper panel, blue trace). All three antibodies detected increased signal from phosphorylated ERK1 and phosphorylated ERK2 isoforms after EGF stimulation (red traces in upper, middle, and lower panels). However, the two anti-pERK1/2 antibodies shown in the middle and lower traces exhibited markedly different selectivities. Whereas the CS4377 recognized both the single- and dual-phosphorylated forms of ERK1/2, the CS9101 antibody preferentially recognized the dualphosphorylated species. This type of detailed characterization of antibody selectivity is made possible by the IEF separation step in NanoPro assays.

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC3151) containing 5% FBS (Hyclone, PN 1677-006), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020) and MEGM SingleQuot (Lonza, PN CC4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM minus FBS and growth supplements.
Treatment	600 ng/mL EGF (Sigma, PN E1257) in MEGM for 10 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Sample diluent: Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-973)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-ERK1/2 (Millipore, PN 06-182), 1:200 and Anti-phospho ERK (Cell Signaling Technology, PN 9101 or PN 4377), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

P-EGFR | AKT | PTEN | ALAS1 | GSK-3A | GSK-3B | TRX1 | HSP70 | HSP70 LC | CASPASE 3 | B2M | P-STAT3 | P-STAT5
PLCG1 | ERK1/2 | MEK1 | MEK2 | SRC | P-JNK | P-P27 | 4E-BP1 | 4E-BP2 | CRK-L | C-MYC | HA | ASNS
LDH | CIAP1 | GFP | EEF2 | MFN1 | MFN2 | SURVIVIN | P-P38

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-ERK1/2	Millipore	06-182
Anti-phospho ERK	Cell Signaling Technology	9101
Anti-phospho ERK	Cell Signaling Technology	4377

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-ERK1	Millipore	05-754
Anti-ERK2	Biolegend	624202
Anti-ERK2	Millipore	06-333

MEK1

SUMMARY

Dual-specificity mitogen-activated protein kinases (MEK) are members of the dual-specificity protein kinase family, which act upstream from the classical MAP kinases through phosphorylation and thus activation of ERK1 and ERK2 in response to a wide variety of extra- and intracellular signals. While the functions of MEK1 and MEK2 are very similar, these kinases differ significantly in the way they are regulated. For example, serum addition can specifically induce MEK1 activity in CHO cells. By contrast, MEK2 appears to be the functionally predominant isoform in formyl-methionyl-leucyl-phenylalanine treated neutrophils. Here we show MEK1 activation in MCF10A cells treated with EGF (**FIGURE 23**).

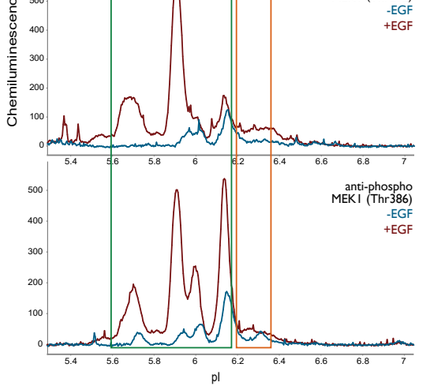


FIGURE 23. EGF stimulation results in increased MEK1 phosphorylation in MCF10A cells. MCF10A cells were treated -/+ EGF (600 ng/mL, 10 minutes). Several phospho MEK1 peaks (green box) increased with EGF treatment, as detected by an anti-phospho MEK1 antibody (bottom two traces) and anti-total MEK1 antibody (top trace). The non-phospho peaks at pI 6.25 decreased dramatically after EGF treatment (orange box).

P-EGFR	AKT	PTEN	ALAS1	GSK-3A	GSK-3B	TRX1	HSP70	HSP70 LC	CASPASE 3	B2M	P-STAT3	P-STAT5
PLCG1	ERK1/2	MEK1	MEK2	SRC	P-JNK	P-P27	4E-BP1	4E-BP2	CRK-L	C-MYC	HA	ASNS
LDH	CIAP1	GFP	EEF2	MFN1	MFN2	SURVIVIN	P-P38					

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC3151) containing 10% FBS (Hyclone, PN 1677-006), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020), and MEGM SingleQuots (Lonza, PN CC4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM.
Treatment	600 ng/mL EGF (Sigma, cat# E1257) in MEGM for 10 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-MEK1	Millipore	07-641
Anti-phospho MEK1(Thr292)	Millipore	07-852
Anti-phospho MEK1 (Thr386)	Phospho Solution	p180-386
Anti-phospho MEK1 (S298)	Cell Signaling Technology	CS9128

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-phospho MEK1	Abcam	32088

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-973)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-MEK1 (Millipore, PN 07-641), 1:100, anti-phospho MEK1 (Thr292, Millipore, PN 07-852), 1:50, anti-phospho MEK1 (Thr386) (Phospho Solution, PN p180-386), 1:50 and anti-phospho MEK1 (S298) (Cell Signaling Technology, PN CS9128), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

MEK2

SUMMARY

Dual-specificity mitogen-activated protein kinases (MEK) are members of the dual-specificity protein kinase family, which act upstream from the classical MAP kinases through phosphorylation and thus activation of ERK1 and ERK2 in response to a wide variety of extra- and intracellular signals. While the functions of MEK1 and MEK2 are very similar, theses kinases differ significantly in the way they are regulated. For example, serum addition can specifically induce MEK1 activity in CHO cells. By contrast, MEK2 appears to be the functionally predominant isoform in formyl-methionyl-leucyl-phenylalanine treated neutrophils. Here we show MEK2 activation in MCF10A cells in response to EGF stimulation (**FIGURE 24**).

P-EGFR	AKT	PTEN	ALAS1	GSK-3A	GSK-3B	TRX1	HSP70	HSP70 LC	CASPASE 3	B2M	P-STAT3	P-STAT5
PLCG1	ERK1/2	MEK1	MEK2	SRC	P-JNK	P-P27	4E-BP1	4E-BP2	CRK-L	C-MYC	HA	ASNS
LDH	CIAP1	GFP	EEF2	MFN1	MFN2	SURVIVIN	P-P38					

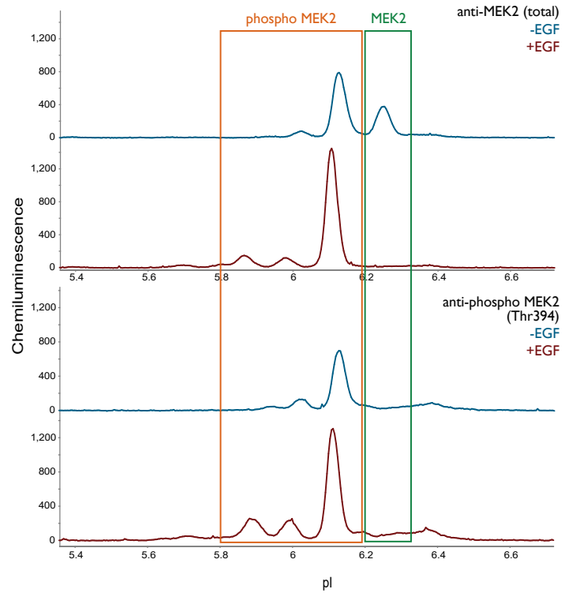


FIGURE 24. EGF stimulation results in increased MEK2 phosphorylation in MCF10A cells. MCF10A cells were treated -/+ EGF (600 ng/mL,10 minutes). Several phospho-MEK2 peaks (orange box) increased with EGF treatment, as detected by an anti-phospho MEK2 antibody (bottom two traces) and an anti-total MEK2 antibody (top two traces). The non-phospho peak at pI 6.25 (green box) decreased dramatically after EGF treatment (compare two top traces).

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC3151) containing 10% FBS (Hyclone, PN 1677-006), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020), and MEGM SingleQuots (Lonza, PN CC4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM.
Treatment	600 ng/mL EGF (Sigma, PN E1257) in MEGM for 10 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-973)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-total MEK2 (Cell Signaling Technology, PN 9125) and anti-phospho MEK2 (Thr394) (Millipore, PN 07-854), both 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-total MEK2	Cell Signaling Technology	9125
Anti-phospho MEK2 (Thr394)	Millipore	07-854

SRC

SUMMARY

Src is involved in regulating growth and differentiation in eukaryotic cells. Src activity is regulated by tyrosine phosphorylation at two sites, but with opposing effects. Phosphorylation of Tyr416 in the activation loop of the kinase domain by Csk upregulates enzyme activity, whereas phosphorylation of Tyr529 in the carboxy-terminal tail renders the enzyme less active. We evaluated Src response to EGF in A431 cells (**FIGURE 25**).

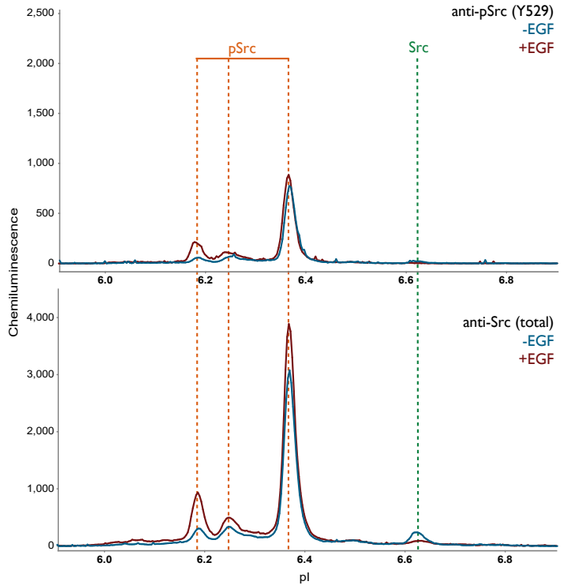


FIGURE 25. EGF treatment of A431 cells results in a small, but reproducible, increase in Src phosphorylation. A431 cells were treated -/+ 100 ng/mL EGF for 10 minutes. The profiles generated with the Anti-phospho Src (Y529) Antibody (Cell Signaling Technology, cat# 2105) showed an increase in the acidic peaks between pI 6.2 and 6.4 (upper traces, labeled pSrc). The Anti-Src (total) Antibody (Abcam, cat# ab47405) also detected peaks between pI 6.2 and 6.4 that increased upon EGF treatment (lower traces, labeled pSrc). In addition, the anti-Src (total) antibody detected a peak at pI 6.6 which decreased after EGF treatment (lower traces, labeled Src). These antibodies have been used to generate similar profiles in HEK293 cells (data not shown).

PROTOCOL

CELL PREPARATION	
Cell culture	A431 cells (ATCC, PN CCL-1555) were cultured in DMEM (Irvine Scientific, PN30-2002) containing 10% FBS (Hyclone, PN 1677-006) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes to dislodge. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing DMEM with no additives.
Treatment	100 ng/mL EGF (Sigma, PN E1257) in DMEM for 10 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.06 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (ProteinSimple Premix G2, PN 040-973)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646) plus pI Standard 5.5 (ProteinSimple, PN 040-028)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Src (Abcam, PN ab47405) and Anti-phospho Src (Tyr529, Cell Signaling Technology, PN 2105), both 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	120 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-Src	Abcam	ab47405
Anti-phospho Src (Tyr529)	Cell Signaling Technology	2105

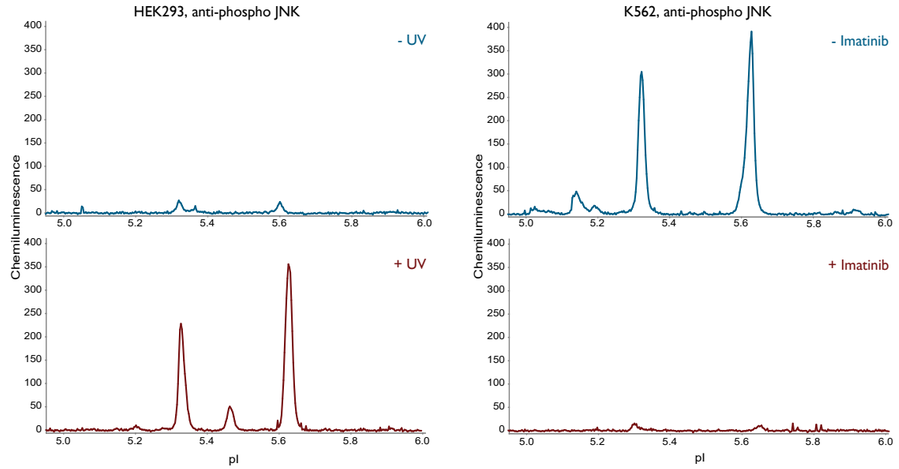
OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-Src	Assay Design	905-678
Anti-Src	Cell Signaling Technology	2108
Anti-Src	Cell Signaling Technology	2109
Anti-phospho Src (Tyr529)	Abcam	ab4817
Anti-phospho Src (Tyr418)	Cell Signaling Technology	2101
Anti-phospho Src	Abcam	ab4816

P-JNK

SUMMARY

c-Jun N-terminal kinases (JNK), originally identified as kinases that bind and phosphorylate c-Jun on Ser63 and Ser73, are mitogen-activated protein kinases which are responsive to stress stimuli, such as cytokines, UV-irradiation, heat shock, and osmotic shock, and are involved in T cell differentiation and apoptosis. JNK1, 2 and 3 share a total of 10 isoforms in the pI range of 5.4–7.6. All three JNK kinases share a similar Thr/Tyr phosphorylation site (T183/Y185). We evaluate the change in JNK phosphorylation in UV-treated HEK293 cells and imatinib-treated K562 cells using a dual phospho-antibody against that site (**FIGURE 26**).

FIGURE 26. Detection of JNK phosphorylation UV-treated HEK293 cells and imatinib-treated K562 cells. HEK293 cells (left panel) were treated with -/+ UV-C exposure for 1 minute. The anti-phospho (T183/Y185) JNK antibody revealed a low level of JNK T183/Y185 phosphorylation in the untreated HEK293 cells (left panel, blue trace). After UV treatment, this antibody recognized three strongly-induced peaks (left panel, red trace). In contrast, untreated K562 cells showed significant levels of JNK T183/Y185 phosphorylation (right panel, blue trace). Treatment with imatinib drastically reduced the anti-phospho JNK signal (right panel, red trace).



PROTOCOL

HEK293 CELL PREPARATION	
Cell culture	HEK293 cells (ATCC, PN CRL-1573) were cultured in EMEM (ATCC, PN 30-2003) containing 10% FBS (Hyclone, PN SH30070.03) and 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 8.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.
Treatment	Cells were washed with PBS (Mediatech, PN 21-031-CV) and aspirated. Plate was exposed 6 inches from a UV-C lamp (30 mW/cm2, warmed for 5 minutes) for 0 and 60 seconds at room temperature.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer with 1x DMSO Inhibitor Mix
Ampholyte premix	80% premix 5-8 (ProteinSimple Premix G2, PN 040-973) and 20% Ampholyte-free Premix premix (ProteinSimple Premix G2, PN 040-967) with 12% Pharmalyte pI 3-10
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-phospho JNK (T183/Y185) (Cell Signaling, PN 9251), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

K562 CELL PREPARATION	
Cell culture	K562 cells (ATCC, PN CCL-243) were cultured in RPMI 1640 media (Cellgro, PN10-041-CV) containing 10% FBS and 1x Penicillin/Streptomycin/Glutamine. Cells were split 1:5 every 3 days by transferring an aliquot of cells to fresh media. Data shown from cells at passage 5.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.
Treatment	5 M Imatinib Methanesulfonate Salt (LC laboratories, PN 1-5508) in starvation medium for 24 hours at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Collect cells by centrifugation (1000 x *g*, 5 minutes). Transfer cells to a 15-mL centrifuge tube, spin (1000 x *g*, 5 minutes) to pellet the cells. Aspirate media. Wash cell pellet with 1 mL of ice-cold PBS (Cellgro, PN 21-031-CV). Transfer cells to a 1.5-mL centrifuge tube, spin (14,000 x *g*, 2 minutes). Aspirate wash. Keeping tube on ice, add 400 µL ice-cold lysis buffer to pellet, pipet up and down to resuspend. Incubate for an additional 30 minutes, rotating. Clarify by centrifugation (14,000 x *g*, 15 minutes). Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	240 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240, and 480 seconds

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-phospho JNK (T183/Y185)	(Cell Signaling	9251

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-phospho JNK (T183/Y185, T221/Y223)	Millipore	07-175
Anti-JNK/SAPK1	Millipore	06-748
Anti-JNK1 (F-3)	Santa Cruz Biotechnology	sc-1648

p-p27 / Kipl

SUMMARY

p27, also known as Kip1, is a cell cycle regulatory/inhibitory protein. It is similar to other members of the Cip/Kip family which includes the p21Cip1/Waf1 and p57Kip2 genes. p27 shares its functional characteristic of being able to bind several different classes of Cyclin and CDK molecules, acting as a CDK inhibitor. We show the response of p27 to EGF treatment in MCF10A cells (**FIGURE 27**).

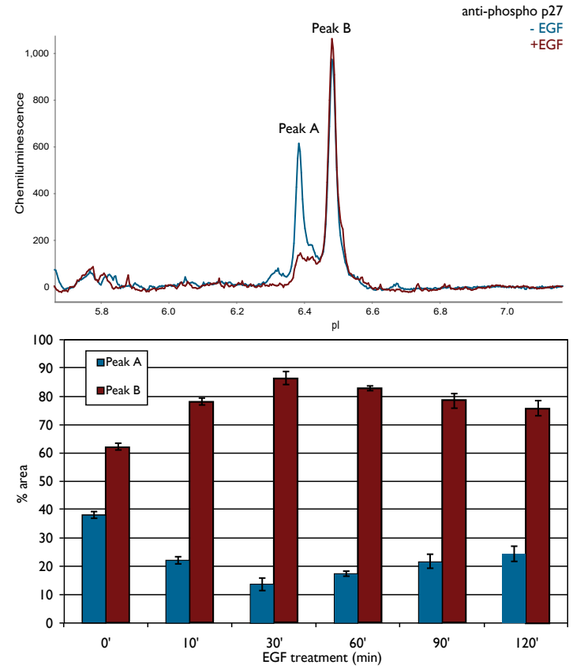


FIGURE 27. Specific p27 phosphorylation is reduced in a time-dependent manner upon EGF treatment in MCF10A cells. The figure at left shows representative profiles generated using an anti-phospho p27 antibody with untreated (blue trace) and maximally-stimulated (600 ng/mL EGF for 30 minutes, red trace) MCF10A cells. The bar graphs quantify relative peak area changes for phospho peaks A (blue) and B (red) over time (n=4). Peaks A and B both collapse upon phosphatase treatment, confirming their phospho-peak identities (data not shown). In addition, a third peak around pI 6.8 appears after phosphatase treatment. This peak has been putatively identified as non-phospho p27 by detection with Anti-total p27 (Santa Cruz Biotechnology, cat# sc-528; data not shown).

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC3151) containing 10% FBS (Irvine Scientific, PN 3000-A), 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020), and MEGM SingleQuots (Lonza, PN CC4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 8.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 overnight in starvation medium containing MEGM without serum.
Treatment	600 ng/mL EGF in starvation medium for 30 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-phospho p27 (Abcam, PN ab60019), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-p27 (total)	Santa Cruz Biotechnology	sc-528
Anti-p27 (total)	Abcam	ab7961
Anti-phospho p27	Abcam	ab60019 and ab62364

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-p27 (total)	Millipore	06-445
Anti-p27 (total)	Rockland	100-401-172

4E-BP1

SUMMARY

Translation repressor protein 4E-BP inhibits cap-dependent translation by binding to the eIF4E translation initiation factor. Hyperphosphorylation of 4E-BP disrupts this interaction and results in activation of cap-dependent translation. Both the PI3 kinase/ Akt pathway and FRAP/mTOR kinase regulate 4E-BP activity. 4E-BP1 has been implicated as a biomarker for several cancer types, while 4E-BP2 has been shown to potentially play a role in energy homeostasis. We show 4E-BP1 activation in MCF10A cells in response to EGF using total and anti-phospho 4E-BP1 antibodies that enable distinction between phospho and non-phospho peaks (FIGURE 28).

PROTOCOL

CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3150) containing 10% FBS (Irvine Scientific, PN 3000-A), 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020), and MEGM SingleQuots (Lonza, PN CC-4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 4.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing MEGM.
Treatment	600 ng/mL EGF (Sigma, PN E1257) in starvation medium for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

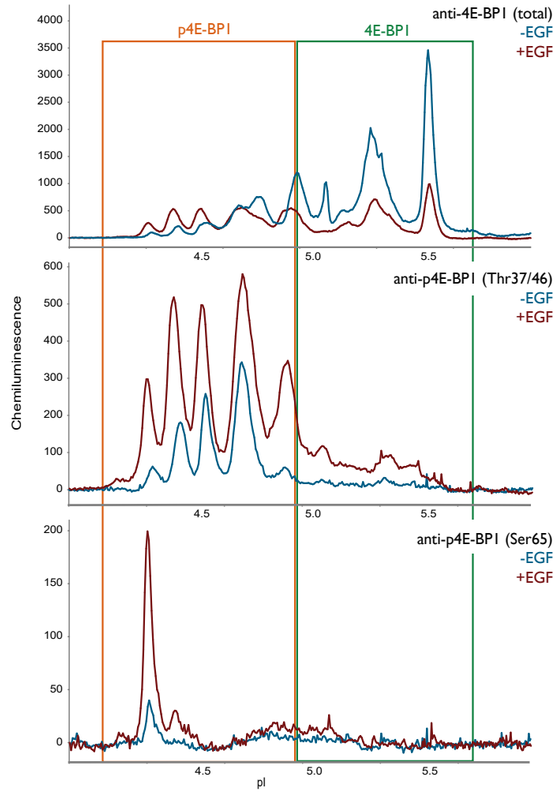


FIGURE 28. EGF-induced phosphorylation of 4E-BP1 in MCF10A cells. MCF10A cells were stimulated with 600 ng/mL EGF for 15 minutes. Treated and untreated lysates were probed with anti-4E-BP1 (total), (top traces), anti-p4E-BP1 (Thr37/46), (middle traces), and anti-p4E-BP1 (Ser65), (lower traces). In the top traces (total 4E-BP1 antibody), EGF treatment caused decreased signals for several basic peaks (green box, non-phospho 4E-BP1), and increased signals for several acidic peaks (orange box, phospho 4E-BP1). These acidic peaks were all detected by anti-p4E-BP1 (Thr37/46), (middle traces), identifying them as Thr37/46 phosphorylated isoforms. As expected, anti-p4E-BP1 (Thr37/46) detected no peaks in the non-phospho 4E-BP1 region (green box). Somewhat surprisingly, the anti-p4E-BP1 (Ser65) antibody (lower traces) recognized only the most acidic peak in the EGF-response profile. Based on these data, one might speculate that phosphorylation at Thr36/46 is a required prerequisite for Ser65 phosphorylation.

ASSAY REAGENTS	
Protein concentration	0.2 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-4E-BP1 (Cell Signaling Technology, PN 9644), Anti-phospho 4E-BP1 (Thr37/46) (Cell Signaling Technology, PN 9459) and Anti-phospho 4E-BP1 (Ser65) (Cell Signaling Technology, PN 9451) all 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-4E-BP1	Cell Signaling Technology	9644
Anti-phospho 4E-BP1 (Thr370/46)	Cell Signaling Technology	9459
Anti-phospho 4E-BP1 (Ser65)	Cell Signaling Technology	9451
Anti-non-phospho 4E-BP1 (Thr46)	Cell Signaling Technology	4923
Anti-phospho 4E-BP1/2/3 (Thr45)	Epitomics	2334-1
Anti-4E-BP1 (cross-reacts with 4E-BP2)	Millipore	07-1416

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-4E-BP1	Epitomics	1557-1
Anti-phospho 4E-BP1	Cell Signaling Technology	2855
Anti-phospho 4E-BP1 (Ser65)	Cell Signaling Technology	9456
Anti-phospho 4E-BP1 (Thr46)	Abcam	ab27792

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	100 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

4E-BP2

SUMMARY

Translation repressor protein 4E-BP inhibits cap-dependent translation by binding to the eIF4E translation initiation factor. Hyperphosphorylation of 4E-BP disrupts this interaction and results in activation of cap-dependent translation. Both the PI3 kinase/AKT pathway and FRAP/mTOR kinase regulate 4E-BP activity. 4E-BP1 has been implicated as a biomarker for several cancer types, while 4E-BP2 has been shown to potentially play a role in energy homeostasis. We show 4E-BP2 activation in MCF10A cells in response to EGF (**FIGURE 29**) and 4E-BP2 inhibition in MCF7 cells with LY294002 (PI3 kinase inhibitor) (**FIGURE 30**).

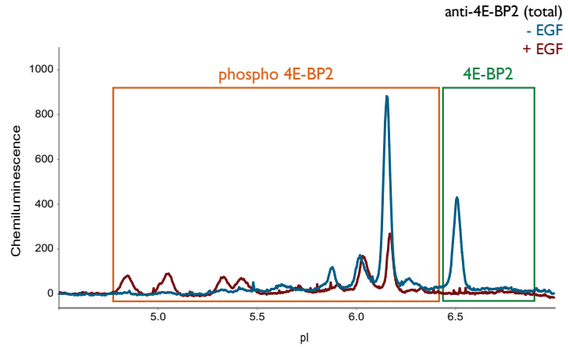


FIGURE 29. 4E-BP2 phosphorylation is induced by EGF stimulation in MCF10A cells. MCF10A cells were stimulated with 600 ng/mL EGF for 15 minutes. Treated (red trace) and untreated (blue trace) lysates were probed with anti-4E-BP2 (total). EGF treatment resulted in reduced signal for the basic peaks near pI 6.5 and 6.7 (non-phospho 4E-BP2 isoforms) and increased signal for several more acidic peaks.

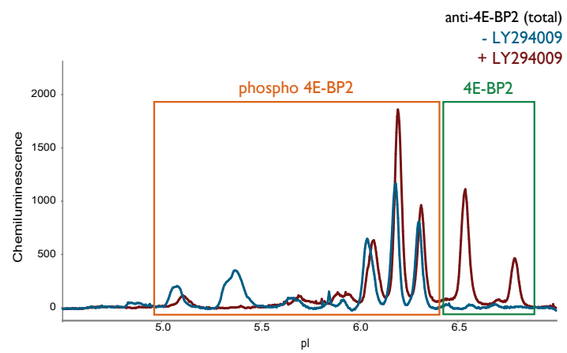


FIGURE 30. The PI3 kinase inhibitor LY294002 inhibits 4E-BP2 phosphorylation in IGF-1-stimulated MCF7 cells. The MCF7 cells treated with 100 ng/mL IGF-1 for 30 minutes (blue trace) showed high levels of 4E-BP2 phosphorylation (orange box), with no significant signal from non-phospho 4E-BP2 (green box). Signal from non-phospho 4E-BP2 increased dramatically when IGF-1 treatment was followed by a 60 minute incubation with 50 mM LY294002 (red trace).

PROTOCOL

MCF10A CELL PREPARATION	
Cell culture	MCF10A cells (ATCC, PN CRL-10317) were cultured in MEGM (Lonza, PN CC-3150) containing 10% FBS (Irvine Scientific, PN 3000-A), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020), and MEGM SingleQuots (Lonza, PN CC-4136). Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3–5 minutes. Data shown from cells at passage 4.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing MEGM.
Treatment	600 ng/mL EGF (Sigma, PN E1257) in starvation medium for 15 minutes at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

MCF7 CELL PREPARATION	
Cell culture	MCF7 cells (ATCC, PN HTB-22) were cultured in EMEM (ATCC, PN 30-2003) containing 10% FBS. Cells were split 1:5 every 3 days using 0.25% trypsin at 37 °C for 3–5 minutes. Data shown from cells at passage 5.
Pre-treatment	Before EGF stimulation, cells were placed at 37 °C, 5% CO_2 for 16 hours in starvation medium containing EMEM without FBS.
Treatment	100 ng/mL IGF-1 (Sigma, PN I-3769) in starvation medium for 30 minutes at 37 °C, 5% CO_2. Add 50 µM LY294002 (LC Laboratories, PN L-7962) and incubate at 37 °C, 5% CO_2 for an additional 60 minutes.
Lysis buffer	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix and 1x Aqueous Inhibitor Mix.
Lysis details	Wash cells with 10 mL of ice-cold PBS (Cellgro, PN 21-031-CV), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 30 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

CRK-L

SUMMARY

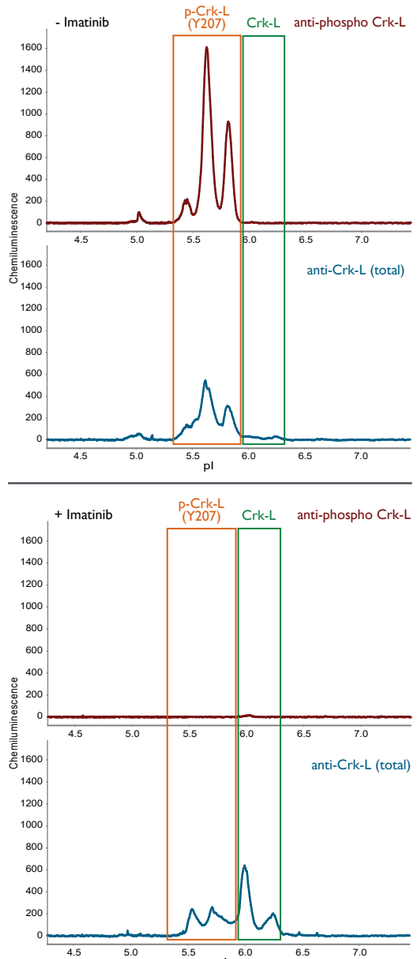
Crk-like protein (Crk-L) is an adapter protein and phosphotyrosine-containing substrate implicated in transformation by the bcr-abl oncogene and in signaling by cytokines. It has been shown to activate the RAS and JUN kinase signaling pathways and transformed fibroblasts in a RAS-dependent fashion. Crk-L is a substrate of the BCR-ABL tyrosine kinase and plays a role in fibroblast transformation by BCR-ABL. We show that Crk-L phosphorylation is reduced in response to Imatinib (commonly known as Gleevec®) treatment in K562 cells (**FIGURE 31**).

ASSAY REAGENTS	
Protein concentration	0.2 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-4E-BP2 (Cell Signaling Technology, PN 2845), 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	100 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-4E-BP2	Cell Signaling Technology	2845

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-phospho 4E-BP1 (cross-reacts with phospho-4E-BP2)	Millipore	07-1416



P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

FIGURE 31. Detection of decreased Crk-L phosphorylation in K562 cell upon Imatinib treatment. K562 cells were treated -/+ 5 µM Imatinib for 1 hour and lysed. Traces from untreated controls are shown in the top panel and traces from Imatinib-treated cells are shown on the bottom. For the untreated cells, the anti-phospho (Y207) Crk-L antibody detected three discrete peaks in the pI 5.5–5.9 region (top panel, orange box, upper trace). The anti-total Crk-L antibody detected peaks at similar pIs (top panel, orange box, lower trace), putatively identified as phospho-Crk-L isoforms. Treatment with Imatinib (bottom panel) completely suppressed signal from phospho-Crk-L peaks as detected by the anti-phospho (Y207) Crk-L antibody (bottom panel, upper trace). The Imatinib treatment concurrently caused the appearance of two new peaks in the anti-total Crk-L antibody trace, putatively identified as non-phospho (Y207) isoforms.

PROTOCOL

CELL PREPARATION	
Cell culture	K562 cells (ATCC, PN CCL-243) were cultured in RPMI 1640 media (Cellgro, PN 10-041-CV) containing 10% FBS (Irvine Scientific, PN 3000-A) and 1x Penicillin/Streptomycin/ Glutamine (JRS Scientific, PN 20020). Cells were split 1:5 every 3 days by removing an aliquot of the cells and transferring them to fresh media. Data shown from cells at passage 5.
Pre-treatment	Cells were starved for 20 hours before stimulation at 37 °C, 5% CO_2 in starvation medium containing RPMI 1640 without serum.
Treatment	5 M Imatinib Methanesulfonate Salt (LC laboratories, PN 1-5508) in starvation medium for 1 hour at 37 °C, 5% CO_2.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Collect cells by centrifugation (1000 x *g*, 5 minutes). Transfer cells to a 15-mL centrifuge tube, spin (1000 x *g*, 5 minutes) to pellet the cells. Aspirate media. Wash cell pellet with 1 mL of ice-cold PBS (Cellgro, PN 21-031-CV). Transfer cells to a 1.5-mL centrifuge tube, spin (14,000 x *g*, 2 minutes). Aspirate wash. Keeping tube on ice, add 400 µL ice-cold lysis buffer to pellet, pipet up and down to resuspend. Incubate for an additional 30 minutes, rotating. Clarify by centrifugation (14,000 x *g*, 15 minutes). Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-8 (nested) (ProteinSimple Premix G2, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Crk-L (Cell Signaling Technology, PN 3182) and Anti-phospho Crk-L (Abcam, PN ab52908) both 1:50 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656) and Anti-Mouse HRP (ProteinSimple, PN 040-655), both 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	100 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-Crk-L	Cell Signaling Technology	3182
Anti-Crk-L	Millipore	05-414
Anti-phospho Crk-L	Millipore	09-466
Anti-phospho Crk-L	Abcam	ab52908

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-Crk-L	Abcam	63491 and 8578
Anti-phospho Crk-L	Cell Signaling Technology	3181

C-MYC EPITOPE TAG ON EGFP

SUMMARY

Author: Thomas Frogne PhD, Hagedorn Research Institute, Denmark

Epitope tags are widely used for affinity purification as well as for highly sensitive detection of recombinant proteins. The c-Myc-tag consists of a short peptide sequence (MEEQKLISEEDLLM). EGFP was expressed with a c-Myc-tag at either the C- or N-terminus of the protein. As expected from the amino acid composition of this tag, a slight acidic shift in the pI of the tagged protein can be observed (FIGURE 32).

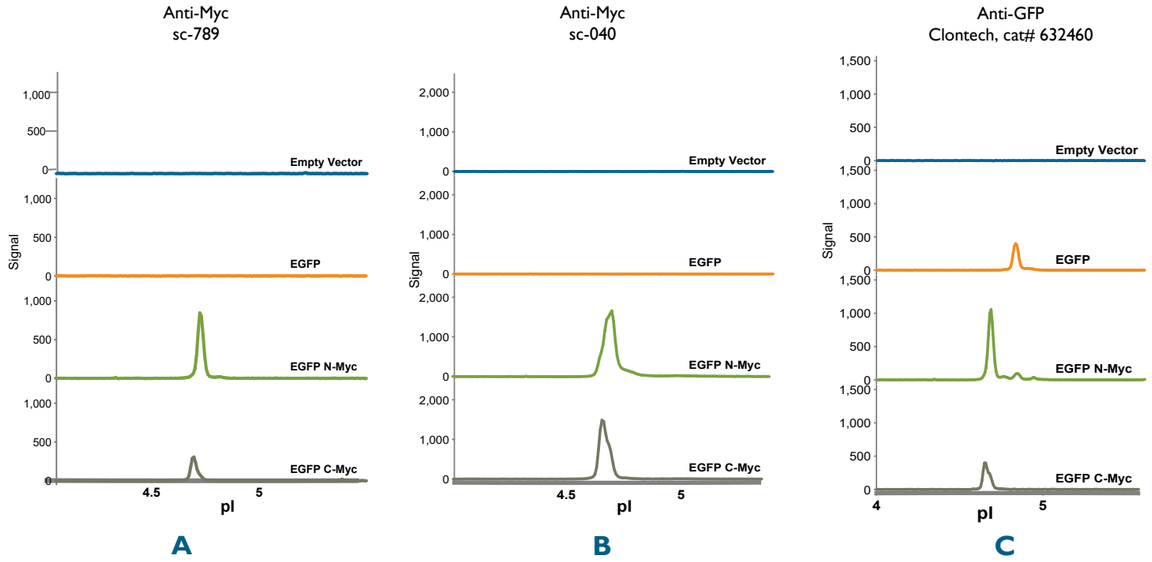


FIGURE 32. c-Myc-tagged EGFP expressed in mouse L-cells. c-Myc-tagged EGFP expressed in mouse L-cells was detected specifically by two anti-Myc antibodies (panels A, Santa Cruz Biotechnology, #sc-789; and B, Santa Cruz Biotechnology, #sc-040). Specificity was confirmed by vector-only and EGFP-only transfections, which did not result in any signal with the anti-Myc antibodies. As expected, the anti-GFP antibody (panel C, Clontech, # 632460) resulted in a significant peak in the GFP-only transfection. The anti-GFP antibody recognized also as expected both c-Myc-tagged EGFP constructs.

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

PROTOCOL

CELL PREPARATION	
Cell culture	LTK also known as L-cells, (ATCC, PN CRL-2648), a mouse fibroblast cell line, similar to 3T3 cells, were cultured in DMEM containing 10% FBS and Penicillin/Streptomycin. Cells were split 1:100 every week using trypsin.
Constructs	Expression from EGFP vector backbone (Clontech, PN 6084-1 and 6085-1) was driven by a CMV promoter. PCR reaction was run from this backbone to generate plasmids expressing EGFP with either an N or C-terminal tag. All clones were verified by sequencing.
Tag sequence	c-Myc: MEEQKLISEEDLLM
Transfection	Cells were cultured in 6-well plates and transfected with Lipofectamine 2000, (Invitrogen, PN 11668019), according to manufactures instructions. The applied constructs were Empty vector, EGFP or EGFP with an N- or C-terminal c-Myc tag. The transfection efficiency was between 20% and 30%, judged by EGFP fluorescence.
Lysis buffer	Tissue Reagent I (Invitrogen, PN FNN0071) with the addition of protease inhibitors (Roche, PN 1 836153).
Lysis details	Wash cells once with ice-cold PBS, aspirate well. Add 100 µL of ice-cold lysis buffer per well. Swirl around to ensure good coverage. Incubate for 10 minutes on ice. Scrape off cells, transfer to a microfuge tube and clarify by centrifugation (17,000 x *g*, 30 minutes) in a cooled centrifuge, transfer supernatant to a fresh microfuge tube. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	10, 60, 120, 240, 600 and 1200 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-Myc	Santa Cruz Biotechnology	sc-789
Anti-Myc	Santa Cruz Biotechnology	sc-040

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 4-9 (ProteinSimple Premix G2, PN 040-969)
pI standards	pI Standard Ladder 1 (ProteinSimple, PN 040-644)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Myc (Santa Cruz Biotechnology, PN sc-789, clone A14 and sc-040, clone 9E10), 1:400 and Anti-GFP (Clontech, PN 632460),1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Detection antibody: Anti-Rabbit HRP (ProteinSimple, PN 040-656) and Anti-Mouse HRP (ProteinSimple, PN 040-655), both 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

HA EPITOPE TAG ON EGFP

SUMMARY

Author: Thomas Frogne PhD, Hagedorn Research Institute, Denmark

Epitope tags are widely used for affinity purification as well as for highly sensitive detection of recombinant proteins. The HA-tag consists of a short peptide sequence (MAYPYDVPDYASM). Here, EGFP was expressed with a HA-tag at both the C- and N-terminal of the protein. As expected from the amino acid composition of this tag, a slight acidic shift in the pI of the tagged protein can be observed (**FIGURE 33**).

PROTOCOL

CELL PREPARATION	
Cell culture	LTK, also known as L-cells (ATCC, PN CRL-2648), a mouse fibroblast cell line, similar to 3T3 cells, were cultured in DMEM containing 10% FBS and Penicillin/Streptomycin. Cells were split 1:100 every week using trypsin.
Constructs	Expression from EGFP vector backbone (Clontech, PN 6084-1 and 6085-1) was driven by a CMV promoter. PCR reaction was run from this backbone to generate plasmids expressing EGFP with either an N- or C-terminal tag. All clones were verified by sequencing.
Tag sequence	HA: MAYPYDVPDYASM
Transfection	Cells were cultured in 6-well plates and transfected with Lipofectamine 2000 (Invitrogen, PN 11668019), according to manufacturer's instructions. The applied constructs were Empty vector, EGFP or EGFP with an N- or C-terminal HA tag. The transfection efficiency was between 20% and 30%, judged by EGFP fluorescence.
Lysis buffer	Tissue Reagent I (Invitrogen, PN FNN0071) with the addition of protease inhibitors (Roche, PN 1 836153).
Lysis details	Wash cells once with ice-cold PBS, aspirate well. Add 100 µL of ice-cold lysis buffer per well. Swirl around to ensure good coverage. Incubate for 10 minutes on ice. Scrape off cells, transfer to a microfuge tube and clarify by centrifugation (17,000 x *g*, 30 minutes) in a cooled centrifuge, transfer supernatant to a fresh microfuge tube. Storage: -80 °C

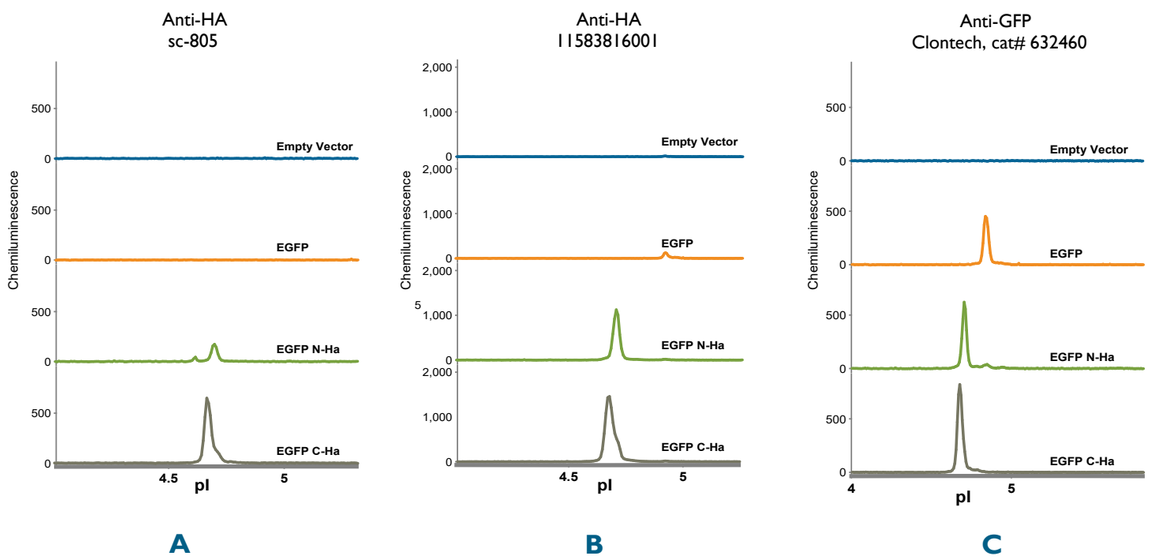


FIGURE 33. HA-tagged EGFP expressed in mouse L-cells. HA-tagged EGFP expressed in mouse L-cells was detected specifically by two anti-HA antibodies (panels A, Santa Cruz Biotechnology, cat# sc-805 and B, Roche Applied Sciences, cat# 11583816001). Specificity was confirmed by vector-only and GFP-only transfections, which did not result in any signal with the HA antibodies. As expected, the anti-GFP antibody (panel C, Clontech, cat# 632460) resulted in a significant peak in the GFP-only transfection. The anti-GFP antibody recognized, also as expected, both HA-tagged GFP constructs.

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 4-9 (ProteinSimple Premix G2, PN 040-969)
pI standards	pI Standard Ladder 1 (ProteinSimple, PN 040-644)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-HA (Santa Cruz Biotechnology, PN sc-805 and Roche Applied Sciences, PN 11583816001) and Anti-GFP (Clontech, PN 632460), all 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656) and Anti-Mouse HRP (ProteinSimple, PN 040-655), both 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	10, 60, 120, 240, 600 and 1200 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-HA	Santa Cruz Biotechnology	sc-805
Anti-HA	Roche Applied Sciences	11583816001

ASPARAGINE SYNTHETASE (ASNS)

SUMMARY

Author: Philip L Lorenzi PhD, MD Anderson Research Institute, Houston, TX

The enzyme-drug L-asparaginase has been used since the 1970s to treat acute lymphoblastic leukemia. Asparagine synthetase (ASNS) expression has been found to be correlated with L-asparaginase efficacy in leukemia cell lines, in leukemia primary tumor samples, and more recently in cancer cell lines from other tissues of origin. Silencing ASNS expression by RNAi has indicated the L-asparaginase/ASNS relationship is causal and suggests that ASNS expression may be useful as a predictive clinical biomarker of L-asparaginase efficacy. ASNS presents as a single peak in the NanoPro assay (**FIGURE 34**). Expected changes of expression are observed upon siRNA as well as L-asparaginase treatment.

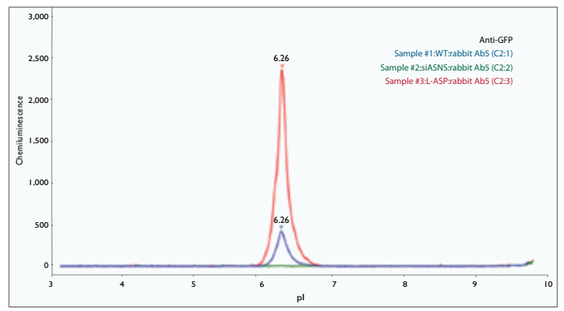


FIGURE 34. Asparagine synthetase (ASNS) in OVCAR-8 cells. ASNS in OVCAR-8 cells (US National Cancer Institute) presents in the NanoPro assay as a single peak around pI 6.3 using the protocol described. ASNS expression was stimulated by cell treatment with L-asparaginase as well as inhibited by specific siRNA treatment.

PROTOCOL

CELL PREPARATION	
Cell culture	OVCAR-8 cells (US National Cancer Institute) were cultured in RPMI-1640 (Thermo/Hyclone, PN SH30096FS) containing 5% FBS (Thermo/Hyclone, PNSH30070.01) and 2 mM Glutamine (Thermo/Hyclone, PN SH3003401). Cells were split 1:10 every 3 days using 0.05% Trypsin (Thermo/Hyclone #SH3023601) at 37 °C for 3–5 minutes. Data shown from cells at passage 7.
Pre-treat-ment:	Cells were seeded with either 5 nM ASNS siRNA complexed with INTERFERin transfection reagent (PolyPlus Transfection, VWR, PN 89129-930) or corresponding negative control siRNA (QIAGEN, PN1027281).
Treatment	Cells were fed with fresh medium containing 0.1 U/mL of L-asparaginase (Sigma, PN A3809) or vehicle control at 48 hours after seeding for a duration of 48 hours.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash cells with 10 mL of ice-cold Wash Buffer (ProteinSimple, PN# 040-313), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 20 minutes on ice. Clarify by centrifugation (20,000 x *g*, 10 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10–30 µL) on ice and snap freeze on liquid nitrogen. Storage: -80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	40 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODY		
NAME	VENDOR	PART #
Anti-ASNS	Sigma	HPA029318

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 5-7 (PGM) (Serva, PN 42936.01) at 12% in ampholyte-free Premix G2 (ProteinSimple, PN 040-967)
pI standards	Custom ladder containing pI Standards 4.9, 5.5, 6.0, 6.4, and 7.0 (ProteinSimple, PN 040-027, PN 040-028, PN 040-031), 1:100
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-ASNS (Sigma PN HPA029318) at final 5 µg/mL in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656), 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

LACTATE DEHYDROGENASE (LDH)

SUMMARY

Author: Huifang (Tracy) Guo PhD and Jennifer Dennison PhD, Department of Systems Biology, MD Anderson Cancer Center, Houston, TX

Lactate dehydrogenase (LDH) is a reversible enzyme that catalyzes the reduction of pyruvate to lactate or the oxidation of lactate to pyruvate. In most human cells, LDH in its native form is a tetramer with 5 possible isoforms (LDH1-5), combinations of LDHA (calculated pI: 8.4) and/or LDHB (calculated pI: 5.7) (**FIGURE 35**). LDH overexpression and differential expression of LDH isoforms have been implicated in the pathogenesis and progression of many cancers.

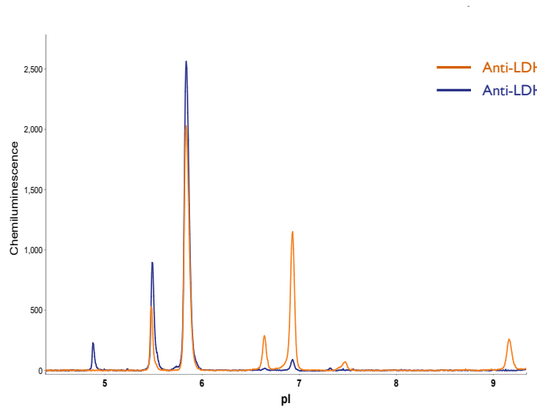


FIGURE 35. LDHA and LDHB in WM35 cells. The NanoPro technology using native conditions allows investigation of complexes, as shown by the examples of LDHA and LDHB. The peaks at pI 9.2 and 4.9 were recognized only by the LDHA (Cell Signaling Technology, cat# 3582) or LDHB (Abcam, cat# ab85319) antibody and consequently were identified as LDH5 (LDHAx4) and LDH1 (LDHBx4), respectively. Peak identification was also supported by LDH standards and denaturing experiments (data not shown). The peaks with intermediate pI values were recognized by both LDHA and LDHB antibodies, indicating that these complexes were composed of both LDHA and LDHB proteins.

PROTOCOL

CELL PREPARATION	
Cell culture	WM35 cells (MD Anderson Cancer Center Core Facility) were cultured in RPMI 1640 (Mediatech, Inc, PN 10-040-CV) containing 5% FBS (Gibco, PN 10437). Cells were split 1:10 every 5 days using 1x Trypsin EDTA (Mediatech, Inc, PN25-053-CI) at 37 °C for 3-5 minutes. Data shown from cells at passage 7.
Lysis buffer	Lysis buffer: Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 2 mM EDTA.
Lysis details	Wash cells with 10 mL of ice-cold PBS (HyClone, PN SH30256.01), aspirate well. Add 400 µL ice-cold lysis buffer to 10-cm plate on ice, swirl around to ensure good coverage, and incubate 10 minutes on ice. Scrape plate, pipet up and down to mix. Transfer lysate to microfuge tube, lyse for an additional 20 minutes on ice. Clarify by centrifugation (14,000 x *g*, 15 minutes) in a cooled centrifuge. Transfer supernatant to a fresh microfuge tube. Immediately aliquot supernatant (10-30 µL) on ice and snap freeze on dry ice. Storage: -80 °C

ASSAY REAGENTS	
Protein concentration	0.02 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 3-10 (ProteinSimple Premix G2, PN 040-968)
pI standards	pI Standard Ladder 1 (ProteinSimple, PN 040-644), spike in pI standard 9.7 (ProteinSimple, PN 040-790)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-LDHA (Cell Signaling Technology, PN 3582), 1:50 and Anti-LDHB (Abcam, PN 85319), 1:500 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656) and Anti-Mouse HRP (ProteinSimple, PN 040-655) in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	Anti-LDHA: 240 minutes, Anti-LDHB: 60 minutes
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	60 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, and 240 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-LDHA	Cell Signaling Technology	3582
Anti-LDHB	Abcam	ab85319

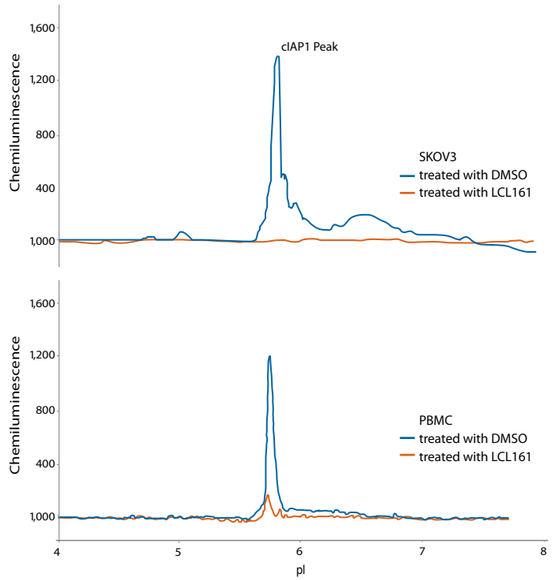


FIGURE 36. SMAC-mimetic inhibitor, LCL161 treatment inhibits cIAP1 levels in SKOV3 and PBMC cells. SKOV3 cells (4 x 10^5/mL) and blood from healthy donors (8 mL) were treated with 2 µM LCL161 or DMSO for 2 hours. The anti-cIAP1 antibody recognized a peak at pI 5.77 in DMSO (blue trace). This peak was inhibited by LCL161 treatment (orange trace).

cIAP1

SUMMARY

Authors: N. S. Lakshmi Yeleswarapu, Novartis Pharmaceuticals, East Hanover, NJ 07936

cIAP1 is a member of the inhibitor of apoptosis (IAPs) family of proteins. It is up-regulated in several human cancers and plays an important role in tumor survival. cIAP1 functions to prevent cellular apoptosis by preventing the activation and/or inhibiting the function of dierent caspases (FIGURE 36).

PROTOCOL

CELL PREPARATION	
Cell culture	SKOV3 cells are maintained in a humidified 5% CO_2 incubator at 37 °C. Cell lines are grown in RPMI1640 medium, penicillin/streptomycin and 10% fetal bovine serum.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Cells are washed with ice cold 1x PBS and lysed with 150 µL of Bicine CHAPs Lysis Buffer with Aqueous Inhibitor Mix and DMSO Inhibitor Mix for 30 min on ice. Lysates are cleared by centrifugation at 13,000 x *g* for 15 minutes at 4 °C and protein concentrations are determined by the Lowry method (Bio-Rad). Storage: –80 °C

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

PBMC PREPARATION	
Sample collection	Collect 8 mL of blood from healthy donors in CPT tubes (BD Vacutainer, Ref# 362753) and invert 8 – 10 times. Centrifuge the blood sample at room temperature in a horizontal rotor (swing-out head) at 1500 to 1800 RCF (Relative Centrifugal Force) for 20 minutes. After centrifugation, mononuclear cells and platelets will be in a whitish layer just under the plasma layer. Immediately after centrifugation, use a serological pipette to remove and discard as much of the plasma layer as possible without disturbing the white layer. Next, collect the white layer and transfer to a 15 mL conical tube with cap (BD PN 352096). Add 10 mL of ice cold PBS and centrifuge at 1000 RCF for 10 minutes at 4 °C. Aspirate and discard the supernatant without disturbing the cell pellet. Add 0.5 mL of ice cold PBS to the pellet to resuspend and then transfer to a 1.5 mL Eppendorf tube. Centrifuge the tube at 13000 x *g* for 5 minutes in a microfuge at 4 °C. Discard the supernatant without disturbing the pellet.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Add 100-150 µL of Bicine CHAPs Lysis Buffer with Aqueous Inhibitor Mix and DMSO Inhibitor Mix to the PBMC pellet. Pipette up and down until the pellet is resuspended. Incubate for 30 minutes on ice. Lysates are cleared by centrifugation at 13,000 x *g* for 15 minutes at 4 °C and protein concentrations are determined by the Lowry method (Bio-Rad). Storage: –80 °C

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL for cell lysates and 0.25 mg/mL for PBMCs (final concentration in capillary by Lowry method).
Sample diluent	Bicine/CHAPS Lysis Buffer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Premix 3 – 10 (ProteinSimple Premix G2, PN 040-968)
pI standards	pI Standard Ladder 1 (ProteinSimple, PN 040-644)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Goat Anti-cIAP1 (R&D systems, PN AF8181), 5µg/mL
Detection antibody	Mouse Anti-Goat HRP (Thermo Fisher Scientific, PN 31400), 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	40 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	30 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240, 480 and 960 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Goat Anti – cIAP1	R&D Systems	AF8181

GREEN FLUORESCENT PROTEIN (GFP)

SUMMARY

Authors: Paul Goldsmith, Ph.D., Jessie Chen, Ph.D., and Michelle Herrmann, National Cancer Institute, Bethesda, MD

Green Fluorescent Protein (GFP) was originally isolated from jellyfish and exhibits a bright green fluorescence when exposed to blue light. In cell and molecular biology, GFP is commonly utilized as a reporter of protein expression. GFP can be introduced and its expression maintained in a wide range of cell lines and organisms. Thus, assays suitable for GFP detection for useful tools for following the expression of proteins for which high affinity antibodies are lacking. The data demonstrates identification of a highly sensitive antibody for GFP detection via NanoPro assay (**FIGURE 37**).

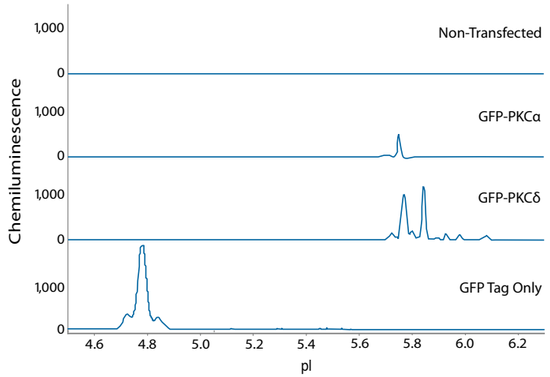


FIGURE 37. Detection of GFP in Transfected LNCap Cells. LNCap cells were transfected with 4 *g* plasmid encoding GFP-PKCα, GFP-PKC, or GFP only. The anti-GFP antibody detects GFP peaks corresponding to expression of protein encoded by the plasmid utilized for transfection. GFP-PKC peaks also detected at identical pI by an anti-PKC-specific antibody (data not shown).

PROTOCOL

CELL PREPARATION	
Cell culture	LNCaP human prostate cells (ATCC, PN CRL-1740) were cultured in RPMI-1640 (ATCC, PN 30-2001) and supplemented with 10% FBS (ATCC, PN 30-2020) and 2 mM glutamine (ATCC, PN 30-2214) in a humidified incubator in 5% CO_2. Cells were split 1:4 every 3 days using 0.25% Trypsin (Invitrogen, PN 25-200) at 37 °C for 3 – 5 minutes.
Transfection	Two days after plating into 6 cm plates, cells were transfected with 4 µg of plasmid using Lipofectamine and Plus reagent (Invitrogen, PN 18324-012 and PN 11514-015 respectively) following the manufacturer's instructions. The transfected plasmids were pEGFN1 for GFP (Clontech, discontinued), GFP-PKCα (bovine) and GFP-PKCdelta (mouse) (Kedei *et al*., 2004*). 4 µg DNA was mixed with 8 µL Plus reagent in 250 µL OPTI-MEM (Invitrogen, PN 31985), 12 µL Lipofectamine was diluted in 250 µL OPTI-MEM, combined after 15 minutes, and added to the cells containing 2 mL of OPTI-MEM (3 mL cell culture medium was replaced with 2 mL OPTI-MEM). After 3 – 4 hrs incubation, the transfection mixture was replaced with normal cell culture medium.
Treatment	48 hours after transfection, cell culture medium was removed, cells were washed once in 3 mL PBS (Cellgro, PN 21-031-CV) and collected in 1 mL PBS using a cell scraper (Sarstedt, PN 83.1830). Cells were centrifuged at 4000 RPM for 5 minutes using a cooled-table Eppendorf centrifuge (5417R). Supernatant was removed using vacuum, and the pellet was kept at – 80 °C until further use.
Lysis buffer	RIPA Lysis Buffer (ProteinSimple, PN 040-483) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	200 µL ice-cold lysis buffer (containing protease and phophatase inhibitors) was added to 3 x 106 cells, resuspended and incubated 20 minutes by rotating in a cold room. Lysates were clarified in a chilled centrifuge (14,000 x *g*, 15–20 minutes). Supernatants were aliquoted for storage. Storage: –80 °C

* Kedei N, Lundberg DJ, Toth A, Welburn P, Garfield SH, Blumberg PM. Characterization of the interaction of ingenol 3-angelate with protein kinase C. Cancer Res. 2004 May 1;64(9):3243-55.

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

ASSAY REAGENTS	
Protein concentration	0.01 – 0.1 mg/mL final in capillary by Pierce 660 nM Protein Assay
Sample diluent	M-Per Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, PN 78501) plus 4x DMSO Inhibitor Mix (comparable to Bicine/CHAPS Lysis Buffer, ProteinSimple, PN 040-764)
Ampholyte premix	Ampholyte Premix: G2 Premix 5 – 8 (nested) (ProteinSimple, PN 040-972)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646), pI Standard 5.5 (ProteinSimple PN 040-028)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-GFP (Roche Applied Science, PN 11814460001), 1:100 in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Mouse HRP (ProteinSimple, PN 040-655) 1:100 in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

SAMPLE PREPARATION	
Step 1	Dilute lysate with sample diluents to 0.2 mg/mL.
Step 2	In a separate tube, mix Premix G2 and pI standards.
Step 3	Mix 1 part diluted lysate prepared in Step 1 with 3 parts Premix G2 + pI Standards prepared in Step 2 (1:3 ratio) to create final protein concentration of 0.05 mg/mL

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	90 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	60, 120, 240 and 480 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-GFP	Roche Applied Science	11814460001

OTHER ANTIBODY SUGGESTIONS		
NAME	VENDOR	PART #
Anti-GFP	Invitrogen	A11122

EEF2

SUMMARY

Authors: Leslee Tworek, and Peter Zahradka. University of Manitoba, Winnipeg, Canada

Eukaryotic elongation factor-2 (eEF2) catalyzes ribosome translocation during translation of mRNA. Phosphorylation of eEF2 by eEF2 kinase (also known as CaM kinase III) inactivates the protein and can block protein synthesis. Stimulation of growth is associated with a decrease in eEF2 phosphorylation. Inhibition of eEF2 phosphorylation in the hippocampus has recently been associated with an anti-depressant effect. We show that insulin treatment reduces phosphorylation of eEF2 in H4IIE cells. Inhibition of phosphorylation of eEF2 is restored in the presence of Nh125 (**FIGURE 38**).

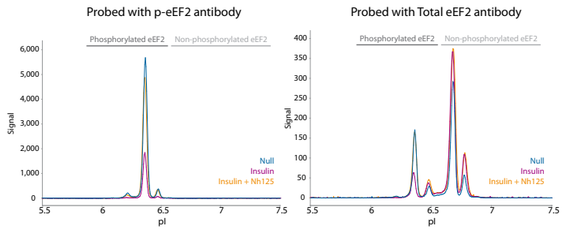


FIGURE 38. Detection of EEF2 Phosphorylation in H4IIE Cells Upon Treatment. H4IIE cells were treated 250 nM Insulin for 10 minutes and lysed. Lysates from null (control), insulin treated and insulin + Nh125 were probed with p-eEF2 (left) and Total eEF2 (right) antibodies. Phospho peaks were detected with the phosphorylated and total eEF2 antibodies at pI 6.21, 6.35, 6.48. Non phosphorylated peaks were detected with the total antibody at pI 6.68 and 6.78. Treatment with Insulin suppressed eEF2 phosphorylation (purple traces) compared to the null samples (blue traces). In presence of 30 nM Nh125 (orange traces), insulin-mediated suppression of p-eEF2 was restored.

PROTOCOL

CELL PREPARATION	
Cell culture	H4IIE cells (ATCC, PN CRL-1548) were cultured in αMEM (Invitrogen, PN12000-063) containing 10% FBS (Invitrogen, PN124083-020) and 1× Penicillin/Streptomycin (Invitrogen, PN15140-122). Cells were split 1:6 every 3 days using 1.8% Trypsin (Invitrogen, PN15090-046) at 37 °C for 3-5 minutes.
Pre-treatment	Before treatment with Insulin cells were place at 37°C, 5% CO_2 for 72 hours in starvation medium containing αMEM with no additives.
Treatment	250 nM Insulin for 10 minutes.
Lysis buffer	Bicine/CHAPS Lysis Buer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash 2× with Cell Wash Buer (20 mM Bicine, 250 mM Sucrose, pH 7.5) and aspirate well. Add ice-cold lysis buer to plate (400 µL/10-cm dish), swirl and incubate 5 minutes on ice. Scrape plate, pipette up-and-down to mix and transfer to a pre-chilled 1.5-mL microfuge tube. Incubate 30 minutes on ice, vortexing briefly every 5 minutes. Clarify lysate by centrifuging for 15 minutes at 14,000 × *g* and immediately collect supernatant. Aliquot supernatant (10 µL) on ice and snap freeze in liquid nitrogen. Storage: –80 °C

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	120 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	30, 60, 120, 240, 480 and 960 seconds (240 seconds usually being sufficient)

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510)
Ampholyte premix	Ampholyte Premix: G2 Premix 5 – 8 (nested) (ProteinSimple, PN 040-972)
pI standards	pI Standard Ladder 1: 4.0, 4.9, 6.0, 6.4, 7.3 (ProteinSimple, PN 040-644) Procedure: Step 1) Dilute lysate to 0.2 mg/mL with sample diluents. Step 2) In a separate tube mix ampholyte premix G2 and pI standards. Step 3) Mix step 1 and step 2 at 1:4 to create final protein concentration.
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-eEF2 (Cell Signaling, PN 2332, 1:100), Anti-Phospho-eEF2 (T56) (Cell Signaling, cat #2331, 1:100) in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656)
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

MITOFUSIN 1 (MFN1)

SUMMARY

Authors: Mary Nivison, Philip J. Horner lab, University of Washington, Seattle, WA (m nivison@uw.edu)

Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) are two highly homologous mitochondrial outer membrane proteins necessary for mitochondrial fusion. Mitochondrial fusion is necessary to maintain mitochondrial health and function, and mitochondrial dysfunction is implicated in diseases such as Charcot-Marie-Tooth 2A and dominant optic atrophy, as well as in multiple tissue types. Phosphorylation of these proteins has not been widely studied. Our data show detection of Mfn1 protein in three neuronal tissues: retina, optic nerve, and the superior colliculus region of the brain in a mouse model, DBA/2J, a widely accepted model for glaucoma (FIGURE 39).

P-EGFR	AKT	PTEN	ALAS1	GSK-3A	GSK-3B	TRX1	HSP70	HSP70 LC	CASPASE 3	B2M	P-STAT3	P-STAT5
PLCG1	ERK1/2	MEK1	MEK2	SRC	P-JNK	P-P27	4E-BP1	4E-BP2	CRK-L	C-MYC	HA	ASNS
LDH	CIAP1	GFP	EEF2	MFN1	MFN2	SURVIVIN	P-P38					

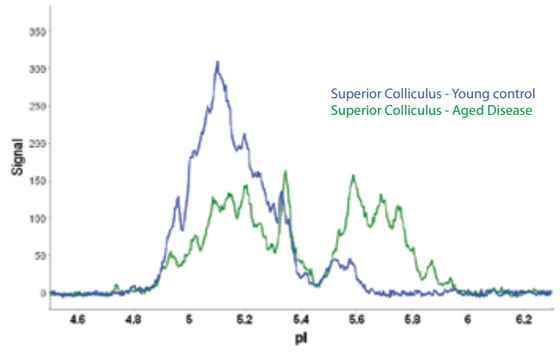


FIGURE 39. Detection of MFN1 in superior colliculus tissue samples. Mfn1's two major peak clusters show a shift occurring over age and disease in the superior colliculus. The shift of peak area to the right is present in young diseased animals (green e-gram trace), while the size of the peak area to the left decreases with age (blue e-gram trace). Similar types of peak shifts occur in retinal and optic nerve tissue. Data shown for superior colliculus. Other tissue results not shown. Please contact author for details.

PROTOCOL

CELL PREPARATION	
Cell harvest	Retina, optic nerve and superior colliculus were removed from mice that had been lethally anesthetized. Tissue samples were immediately placed into tubes being stored on dry ice. Tubes were weighed prior to and after the addition of the tissues to determine tissue weight, inhibitors and DMSO added) is added to the tube. *NOTE: for tissue harvest details, please contact author at mnivison@uw.edu* Determine lysate concentration using BCA assay. Snap freeze on dry ice. Storage: -80 °C
Animal age and control definitions	Young animals are 3 months old, with no outward disease manifestation; old animals are 9-12 months of age, by which age disease will have manifest itself in the DBA/2J strain, but not in the controls. This allows age-matched comparisons to differentiate changes due to aging vs. changes due to disease. The DBA/2J strain (diseased) have two naturally occurring mutations that cause them to develop glaucoma naturally as they age, while the control animals have only one mutation and do not develop disease.

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	240 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	120 minutes
Chemiluminescence exposure	120, 240, 480 and 960 seconds

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Ampholyte premix: Ampholyte-free Premix G2 (ProteinSimple, PN 040-967) containing 8% v/v of pH 4-7 Servalyt Ampholyte (Helix Technologies, PN 42948.01)
pI standards	pI Standards 4.4, 6.4 and 7.0 (ProteinSimple, PN 040-026, 040-030 and 040-031)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Mfn1 (Santa Cruz Biotechnology, PN sc50330), 1:300
Detection antibody	Amplified Secondary Antibody Detection Kit (ProteinSimple, PN 041-126), 1:100
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

MITOFUSIN 2 (MFN2)

SUMMARY

Authors: Mary Nivison, Philip J. Horner lab, University of Washington, Seattle, WA (mnivison@uw.edu)

Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) are two highly homologous mitochondrial outer membrane proteins necessary for mitochondrial fusion. Mitochondrial fusion is necessary to maintain mitochondrial health and function, and mitochondrial dysfunction is implicated in diseases such as Charcot-Marie-Tooth 2A and dominant optic atrophy, as well as in multiple tissue types. Phosphorylation of these proteins has not been widely studied. Our data show detection of Mfn2 protein in three neuronal tissues: retina, optic nerve, and the superior colliculus region of the brain in a mouse model, DBA/2J, a widely accepted model for glaucoma (FIGURE 40).

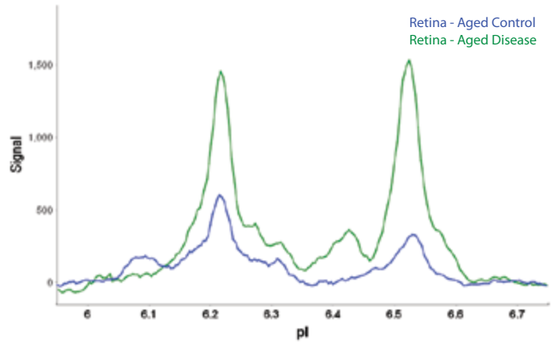


FIGURE 40. Detection of Mfn2 in retina samples. Mfn2 consistently displays two peaks, which change in detected signal intensity and prominence dependent on tissue type, age and disease state. In the retina, aged, diseased animals show a 2.5-fold increase in Mfn2 expression (blue e-gram trace) when compared to age-matched control animals (green e-gram trace). In the optic nerve, Mfn2 expression decreases with age in diseased animals, while only shifting peak expression in controls. In the superior colliculus, there are subtle, but not significant, changes in expression levels (data not shown).

PROTOCOL

TISSUE PREPARATION	
Tissue harvest	Retina, optic nerve and superior colliculus were removed from mice that had been lethally anesthetized. Tissue samples were immediately placed into tubes being stored on dry ice. Tubes were weighed prior to and after the addition of the tissues to determine tissue weight, protease inhibitors and DMSO is added to the tube. *NOTE: for tissue harvest details, please contact author at mnivison@uw.edu* Determine lysate concentration using BCA assay. Snap freeze on dry ice. Storage: -80 °C
Animal age and control definitions	Young animals are 3 months old, with no outward disease manifestation; old animals are 9-12 months of age, by which age disease will have manifest itself in the DBA/2J strain, but not changes due to disease. The DBA/2J strain (diseased) have two naturally occurring mutations that cause them to develop glaucoma naturally as they age, while the control animals have only one mutation and do not develop disease.

ASSAY REAGENTS	
Protein concentration	0.05 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buer plus 1x DMSO Inhibitor Mix
Ampholyte premix	Ampholyte-free Premix G2 (ProteinSimple, PN 040-967) containing 8% v/v of pH 4-7 Servalyt Ampholyte (Helix Technologies, PN 42948.01)
pI standards	pI Standards 4.4, 6.4 and 7.0 (ProteinSimple, PN 040-026, 040-030 and 040-031)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Mfn2 (Sigma, PN M6319), 1:25
Detection antibody	Amplified Secondary Antibody Detection Kit (ProteinSimple, PN 041-126), 1:100
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Chemiluminescence exposure	120, 240, 480 and 960 seconds

SURVIVIN

SUMMARY

Authors: Ashraf Khalil MD, PhD; Linnea E. Taniguchi MSc.; Mark Jameson MD, PhD, FACS; University of Virginia, Charlottesville VA (ak8wr@hscmail.mcc.virginia.edu).

Survivin, an inhibitor of apoptosis protein (IAP) is a pro-survival molecule that is increased in nearly every human tumor studied. In head and neck squamous cell carcinoma, Survivin levels are significantly greater than in normal upper aerodigestive mucosa. High Survivin levels in these tissues correlate with a higher probability of nodal metastasis and loco-regional recurrence, but may also indicate higher radiosensitivity. Survivin includes multiple sites for post-translational modification, including 4-5 major phosphorylation sites (FIGURE 41).

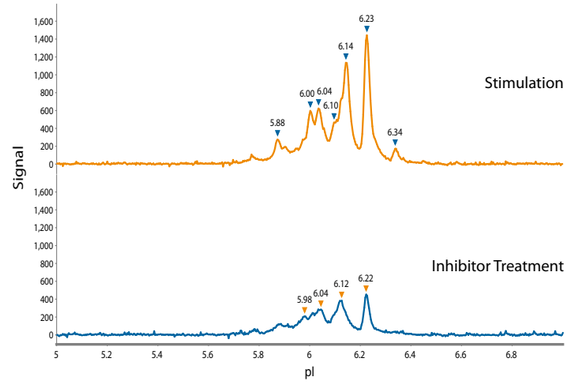


FIGURE 41. Detection of survivin in OSC19 head and neck squamous cell carcinoma cell line. OSC19 cells were treated with either 150 µM OSI906 for 2 hours followed by stimulation with des [1-3] IGF for 15 minutes (upper panel) or treated with 100 µM of the Hif-1A inhibitor YC-1 for 24 hours (lower panel). Impact on Survivin expression in response to stimulation or inhibitor treatment correlated with band intensities detected by Western blot (data not shown).

PROTOCOL

CELL PREPARATION	
Cell culture	OSC19 human tongue squamous cell carcinoma cells were obtained from the laboratory of Jerey N Myers, MD, PhD (M. D. Anderson). Cells were cultured in DMEM/F12 (Gibco, PN 11330) containing 4% FBS (Gibco, PN 16000), 1x Penicillin/Streptomycin/Glutamine (JRS Scientific, PN 20020) in 6 cm dishes. Cells were split 1:5 every 3 days using 0.25% Trypsin (Cellgro, PN 25-053-CI) at 37 °C for 3-5 minutes. Data shown from cells at passage 5.
Treatment	12 hours before treatment with OSI906, media was aspirated and replaced with DMEM/F12 containing 0.5% FBS. Cells were exposed to 150 µM OSI906 (dissolved in DMSO) for 2 hours before stimulation with 10 nM des [1-3] IGF for 15 minutes, (des [1-3] IGF dissolved in 10 mM HCL and diluted in 0.5% BSA). Inhibitor treated cells were exposed to 100 µM YC-1 in DMSO for 24 hours.
Lysis buffer	Bicine/CHAPS Lysis Buffer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Media was aspirated, and cells were washed once with 2 mL ice-cold PBS. 100 µL ice-cold Bicine/CHAPS lysis buer, supplemented with protease and phosphatase inhibitors, was added to cells before scraping into a pre-chilled microfuge tube. Cells incubated for 30 minutes in cold room on rotary shaker. Lysates clarified in centrifuge (16,100 x *g* for 15 minutes at 4 °C). Supernatants aliquoted (5 µL) for storage at -80 °C.

ASSAY REAGENTS	
Protein concentration	0.2 mg/mL final in capillary by BCA assay
Sample diluent	Bicine/CHAPS Lysis Buer (ProteinSimple, PN 040-764), 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510), 40 mM DTT
Ampholyte premix	Premix G2 pH 5-8 (ProteinSimple, PN 040-973)
pI standards	pI Standard Ladder 3 (ProteinSimple, PN 040-646)
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-Survivin (Cell Signaling Technology, PN cs-2806, 1:50) in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (GE Healthcare, Product Code NA934, 1:100) in Antibody Diluent
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide	Mixed 1:1 (ProteinSimple, PN 043-379 and PN 040-652)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	80 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	480 and 960 seconds

OUR FAVORITE ANTIBODIES		
NAME	VENDOR	PART #
Anti-Survivin	Cell Signaling Technology	cs-2806

PHOSPHO-P38

SUMMARY

Authors: Joshua Hillman, Michael Rosenbach, and David Boyle, University of California, San Diego.

The Mitogen Activated Protein Kinase p38 is activated by stress stimuli such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis. We show that Interleukin-1 (IL-1) treatment induces phosphorylation of p38 in broblasts (FIGURE 42). In addition, two independent antibodies recognize peaks at pIs 5.4 and 5.8 for phosphorylated p38 (FIGURE 43).

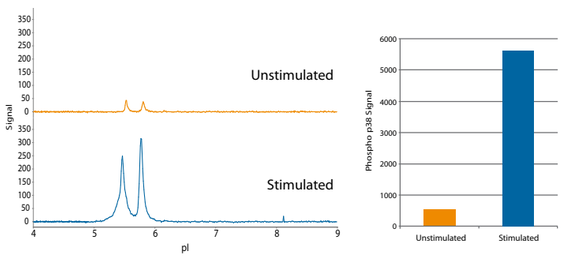


FIGURE 42. Detection of P38 phosphorylation in fibroblasts upon treatment. Fibroblasts were treated with IL-1 for 15 minutes and lysed. Lysates from unstimulated (orange trace) and IL-1 treated cells (blue trace) were probed with Anti-Phospho-p38 antibody (Cell Signaling, cat# 9211). Phospho peaks were detected at pIs 5.4 and 5.8. Quantitation of the detected peaks shows 10-fold induction of phosphorylated p38 upon stimulation.

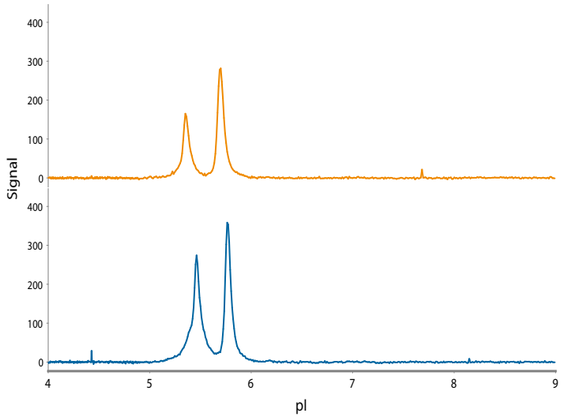


FIGURE 43. Recognition of P38 phosphorylation in fibroblast by 2 independent P-P38 antibodies. IL-1 stimulated fibroblasts lysates were probed with Anti-Phospho-p38 antibody from Cell Signaling (cat# 9211, orange trace) and Millipore (cat# 09-272, blue trace). Phospho peaks were detected at pIs 5.4 and 5.8 by the two antibodies.

PROTOCOL

CELL PREPARATION	
Cell culture	Fibroblast-like synoviocytes (FLS) obtained from the joints (synovial tissue) of arthritis patients undergoing joint replacement surgery were cultured in DMEM containing 10% FBS, L-glutamine, penicillin, streptomycin and gentamicin in a humidified chamber containing an atmosphere of 5% CO_2. After 4 passages cells reached 99% homogeneity.
Pre-treatment	Before treatment with Interleukin-1 cultured broblasts were placed at 37 °C, 5% CO_2 for 48 hours in starvation medium containing DMEM with no additives
Treatment	Interleukin-1 for 15 minutes at a final concentration of 2 ng/mL.
Lysis buffer	Bicine/CHAPS Lysis Buer (ProteinSimple, PN 040-764) plus 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510) and 1x Aqueous Inhibitor Mix (ProteinSimple, PN 040-482).
Lysis details	Wash 2× with Cell Wash Buer (20 mM Bicine, 250 mM Sucrose, pH 7.5) and aspirate well. Add ice-cold lysis buer to plate (400 µL/10 cm dish), swirl and incubate 5 minutes on ice. Scrape plate, pipette up-and-down to mix and transfer to a pre-chilled 1.5-mL microfuge tube. Incubate 30 minutes on ice, vortexing briey every 5 minutes. Clarify lysate by centrifuging for 15 minutes at 14,000 × *g* and immediately collect supernatant. Aliquot supernatant (10 µL) on ice and snap freeze in liquid nitrogen. Storage: –80 °C

P-EGFR AKT PTEN ALAS1 GSK-3A GSK-3B TRX1 HSP70 HSP70 LC CASPASE 3 B2M P-STAT3 P-STAT5
PLCG1 ERK1/2 MEK1 MEK2 SRC P-JNK P-P27 4E-BP1 4E-BP2 CRK-L C-MYC HA ASNS
LDH CIAP1 GFP EEF2 MFN1 MFN2 SURVIVIN P-P38

ASSAY REAGENTS	
Protein concentration	0.1 mg/mL final in capillary by BCA assay
Sample diluent	Sample Diluent (ProteinSimple, PN 040-649), 1x DMSO Inhibitor Mix (ProteinSimple, PN 040-510)
Ampholyte premix	Premix G2 pH 3-10 (ProteinSimple, PN 040-968)
pI standards	pI Standard Ladder 1: 4.0, 4.9, 6.0, 6.4, 7.3 (ProteinSimple, PN 040-644)
Procedure	Step 1) Dilute lysate to 0.4 mg/mL with sample diluents. Step 2) In a separate tube mix ampholyte premix G2 and pI standards. Step 3) Mix step 1 and step 2 at 1:4 to create final protein concentration.
Wash	Wash Buffer (ProteinSimple, PN 041-108)
Primary antibody	Anti-p38 (Millipore, cat # 09-272, 1:50), Anti-Phospho-p38 (T180/182) (Cell Signaling, PN 9211, 1:50) in Antibody Diluent (ProteinSimple, PN 040-309)
Detection antibody	Anti-Rabbit HRP (ProteinSimple, PN 040-656)
Anolyte	ProteinSimple, PN 040-337
Catholyte	ProteinSimple, PN 040-338
Luminol/Peroxide xDR	Mixed 1:1 (ProteinSimple, PN 040-652 and PN 043-379)

ASSAY CONDITIONS	
System	NanoPro 1000
Sample loading time	25 seconds
Focus conditions	21000 µW, 40 minutes
Immobilization	120 seconds
Wash 1	2 x 150 seconds (default)
Primary antibody incubation	120 minutes
Wash 2	2 x 150 seconds (default)
Detection antibody incubation	60 minutes
Wash 3	2 x 150 seconds (default)
Chemiluminescence exposure	30, 60, 120, 240, 480 and 960 seconds

P-EGFR	AKT	PTEN	ALAS1	GSK-3A	GSK-3B	TRX1	HSP70	HSP70 LC	CASPASE 3	B2M	P-STAT3	P-STAT5
PLCG1	ERK1/2	MEK1	MEK2	SRC	P-JNK	P-P27	4E-BP1	4E-BP2	CRK-L	C-MYC	HA	ASNS
LDH	CIAP1	GFP	EEF2	MFN1	MFN2	SURVIVIN	P-P38					

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P-EGFR	AKT	PTEN	ALAS1	GSK-3A	GSK-3B	TRX1	HSP70	HSP70 LC	CASPASE 3	B2M	P-STAT3	P-STAT5
PLCG1	ERK1/2	MEK1	MEK2	SRC	P-JNK	P-P27	4E-BP1	4E-BP2	CRK-L	C-MYC	HA	ASNS
LDH	CIAP1	GFP	EEF2	MFN1	MFN2	SURVIVIN	P-P38					

NOTES