Abel et al

INVENTORY OF SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Comparisons between ChIP binding peaks and differential gene expression

RefSeq transcript annotations were used to overlay promoter occupancy rate estimations, for both the 3kb upstream and downstream regions, with the set of differentially expressed genes. Genes were defined as upregulated, upregulated and bound, downregulated, and downregulated and bound with promoter occupancy rates 0.25 and above considered as bound by FOXD3. Each of these four gene subsets, in addition to supersets of all differentially expressed and all bound promoters, were analyzed for enrichment of Gene Ontology Biological Process terms (1). Additionally, the four gene subsets were analyzed for KEGG pathway enrichment (2). Enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (3, 4). For selected enriched KEGG pathways, the KEGG pathway coloring tool was used to highlight the pathway nodes for genes either bound by FOXD3, differentially regulated, or both.

Microarrays

For FOXD3 knockdown experiments, total RNA was collected using the 5 Prime PerfectPure RNA isolation kit (5 Prime, Inc). Quality of RNA samples was assessed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA). Microarrays and analyses were performed by the Kimmel Cancer Center Genomics facility. RNA labeling and hybridization was conducted using the Human Exon 1.0ST GeneChip (Affymetrix, Santa Clara, CA). Probe signals were normalized and corrected according to background signal. Adjusted signal strength was used to generate quantitative raw values, which were log-transformed for all subsequent analyses.

Antibodies

Primary antibodies used were: ERK1/2 (K-23), ERK2 (D-2), phospho-ERBB3 Y1328 and BRAF (F-7) from Santa Cruz Biotech. Inc. (Santa Cruz, CA); ERBB3 (1B2E), phospho-ERBB3 Y1197 (C56E4), phospho-ERBB3 Y1289 (21D3), ERBB2 (D8F12), phospho-ERBB2 Y1196 (D66B7), phospho-MET Y1234/1235 (D26), phospho-PDGFRβ Y751 (C63G6), AKT, phospho-AKT T308 (C31E5E), phospho-AKT S473 (D9E), phospho-p70 S6-kinase T389 (108D2), phospho-S6 ribosomal protein S235/236 (91B2), NRG1 (Heregulin, 2573), and phospho-ERK1/2 (D13.14.4E) from Cell Signaling Technology Inc.; FOXD3 (Poly6317) from BioLegend (San Diego CA) and V5 epitope (46-0705) from Invitrogen.

Additional cell lines and cell culture

Sbcl2, WM35, WM3211, WM9, WM278, WM793, WM48, WM983A, WM983B, and WM983C were kindly donated by Dr. Meenhard Herlyn (Wistar Institute, Philadelphia). SK-MEL3, SK-MEL5, SK-MEL24, SK-MEL28, SK-MEL32, and SK-MEL207 were purchased from ATCC (Manassas, VA). All cells were cultured in MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% fetal bovine serum, 0.2% sodium bicarbonate, and 5μg/ml insulin. To generate NRG1 positive control lysate, 293FT cells were transfected overnight with pBabe-hygro-NRG1α kindly provided by Dr. Joyce Liu (Dana-Farber Cancer Institute, Boston) (5).

Additional inhibitors and growth factors

Gefitinib and erlotinib were generously provided by Dr. Ulrich Rodeck (Department of Dermatology and Cutaneous Biology, Thomas Jefferson University). MK2206 was purchased from Selleck Chemicals (Houston, TX). NRG1α and neuroglycan C were purchased from R&D Systems (Minneapolis, MN).

Short-interfering RNA (siRNA)

The siRNA sequences for BRAF #1, FOXD3#1, FOXD3 #3, FOXD3 #18, ERBB3, and ERBB4 were ACAGAGACCUCAAGAGUAAUU, ACGACGGGCUGGAAGAGAA, UGGAAGAGAAGGACAGCGA, GCAAUAGGGACGCGCCAAU, GCAGUGGAUUCGAGAAGUG, and GCAGGAAACAUCUAUAUUA, respectively. The non-targeting siRNA, UAGCGACUAAACACAUCAAUU, was used as a control. SMARTpool ERBB2 siRNA contained 4 different siRNA with the sequences GGACGAAUUCUGCACAAUG, GACGAAUUCUGCACAAUGG, CUACAACACAGACACGUUU, and AGACGAAGCAUACGUGAUG.

Lentiviruses and shRNA

The sequences for shRNA oligonucleotides used in this study are: LacZ2.1 5'-CACCAAATCGCTGATTTGTGTAGTCGTTCAAGAGACGACTACACAAATCAGCGA-3'and 5'-AAAATCGCTGATTTGTGTAGTCGTCTCTTGAACGACTACACAAATCAGCGATTT-3', ERBB3#10 5'-CACCGCCTACCAGTTGGAACACTTATTCAAGAGATAAGTGTTCCAACTGGTAGGC-3' and 5'-AAAAGCCTACCAGTTGGAACACTTATCTCTTGAATAAGTGTTCCAACTGGTAGGC-3', and ERBB3#12 5'-CACCGTATATGAATCGGCAACGAGATTTCAAGAGAATCTCGTTGCCGATTCATATA-3' and 5'-AAAATATATGAATCGGCAACGAGATTCTCTTGAAATCTCGTTGCCGATTCATATAC-3'. Oligonucleotides were annealed and ligated into pENTR™/H1/TO vector (Invitrogen) following the BLOCK-iT™ Inducible H1 RNAi Entry Vector Kit manual. Resulting shRNA

constructs were recombined into pLentipuro/BLOCK-iT-DEST using Gateway® LR Clonase II® (Invitrogen). pLentipuro/BLOCK-iT-DEST is a modification of pLenti4/BLOCK-iT-DEST (Invitrogen) wherein the SV40 promoter/zeocin resistance cassette was replaced with the human PGK promoter/puromycin resistance gene. Recombinant lentiviruses were packaged in 293FT cells (Invitrogen) by co-transfecting $6x10^6$ cells with 4 μg each of lentivirus plasmid, and packaging plasmids pLP1, pLP2, and pLP/VSV-G (Invitrogen), using 36μl FuGENE® HD (Promega, Fitchburg, Wisconsin) as a transfection reagent. Viral supernatants were collected after 72 hours of transfection. 1205LuTR cells were transduced with viral supernatant for 72 hours, followed by selection with puromycin (5μg/ml) for at least 2 weeks.

Primers

Primers used for ChIP experiments were as follows: ERBB3 Intron 1 forward 5'-ATCCCACCCTCAGTAGACAC-3' and reverse 5'-CAACTTTGATTACCCTCCTC-3', ACTB promoter forward 5'-AGTGTGGTCCTGCGACTTCTAAG-3' and reverse 5'-CCTGGGCTTGAGAGGTAGAGTGT-3'. Primers used for quantitative RT-PCR were: ERBB3 forward 5'-GAATGGCCTGAGTGTGACCG-3'and reverse 5'-GTTGGGCAATGGTAGAGTAG-3', and EEF1A1 forward CGTTACAACGGAAGTAAAATC-3'and reverse 5'-CAGGATAATCACCTGAGC.

SUPPLEMENTAL REFERENCES

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4. Huang da, W., Sherman, B.T., and Lempicki, R.A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4:44-57.
5. Sheng Q, L.X., Fleming E, Yuan K, Piao H, Chen J, Moustafa Z, Thomas RK, Greulich H, Schinzel A, Zaghlul S, Batt D, Ettenberg S, Meyerson M, Schoeberl B, Kung AL, Hahn WC, Drapkin R, Livingston DM, Liu JF. 2010. An activated ErbB3/NRG1 autocrine loop supports in vivo proliferation in ovarian cancer cells. *Cancer Cell* 17:298–310.

SUPPLEMENTAL FIGURE LEGENDS AND FIGURES

Supplemental Figure 1 Pathway analysis of genes regulated by FOXD3 in melanoma.
KEGG pathway analysis was performed for genes regulated by FOXD3. Dark blue represents genes that are downregulated FOXD3 without binding of FOXD3 in the -3kbp or +3kbp (<0.25 occupancy), light blue represents downregulated genes with FOXD3 binding (≥0.25 occupancy), red represents upregulated genes without binding, and orange represents upregulation of genes with binding of FOXD3.

Supplemental Figure 2 Genes affected by BRAF inhibition and knockdown of endogenous FOXD3.
(A) Heatmap showing microarray results for WM115 cells transfected with no siRNA, non-targeting control (Cnt), BRAF-targeting, or FOXD3-targeting siRNAs alone or in combination with BRAF targeting siRNA for 96 hours. Upregulated genes are shown in red and downregulated genes are shown in green. A red arrow indicates the position of ERBB3.
(B) Heatmap showing microarray results for 1205Lu cells transfected with no siRNA, non-targeting control, or two distinct FOXD3-targeting siRNAs for 72 hours followed by 24 hour treatment with DMSO or PLX4720 (5 μM). Upregulated genes are shown in red and downregulated genes are shown in blue. A red arrow indicates the position of ERBB3.

Supplemental Figure 2 PLX4032 and AZD6244 enhance ERBB3 signaling in melanoma cells.

(A) A375 cells were treated with DMSO or PLX4032 (1 μM) overnight, followed by a 1 hour treatment with increasing doses of NRG1β. Cell lysates were immunoblotted for the targets indicated.

(B) A375 cells were treated with DMSO or PLX4032 (1 μM) overnight, followed by a stimulation by NRG1β (10 ng/ml) for 0, 5, 15, 30 or 60 minutes. Cell were then lysed and immunoblotted as indicated.

(C & D) A375 (C) and WM266-4 (D) cells were treated overnight with DMSO, PLX4032 (1 μM), or AZD6244 (3.3 μM), followed by one additional hour with or without NRG1β (10 ng/ml). Cells were lysed and immunoblotted as indicated.

(E) 1205Lu cells were transfected with either control siRNA, or two distinct FOXD3-targeting siRNAs for 72 hours. Cells were then treated for an additional 24 hours with PLX4032 (1 μM) or DMSO, after which NRG1β (10 ng/ml) was added for an additional hour to activate ERBB3. Cells were lysed and immunoblotted as indicated.

Supplemental Figure 4 Enhanced ERBB3 signaling with multiple ligands.

A375 cells were treated overnight with either DMSO or PLX4032 (1 μM). Cells were then stimulated with 50 ng/ml NRG1α or neuroglycan C (NGC) for 0, 15, 30, or 60 minutes, lysed, and immunoblotted as indicated.

Supplemental Figure 5 EGFR and ERBB4 are not responsible for NRG1β-mediated ERBB3 phosphorylation and signaling.

(A) WM115 cells were pretreated for 24 hours with AZD6244 (3.3 μM) and then treated with or without NRG1β and a dose-range of lapatinib for 1 hour. Cell lysates were immunoblotted as indicated.

(B) WM115 cells were treated overnight with PLX4032 or DMSO, then treated with either gefitinib (1 μM) or erlotinib (1 μM) for 1 hour before stimulation with NRG1β (10 ng/ml) for an additional hour. Cells were lysed and immunoblotted as indicated.

(C) WM115 cells were transfected with non-targeting control siRNA as well as siRNA targeting ERBB3 and ERBB4 for 72 hours. Cells were treated with PLX4032 (1 μM) or DMSO for 24 hours, followed stimulation with NRG1β (10 ng/ml) for an additional hour before lysis. Lysates were immunoblotted as indicated.

Supplemental Figure 6 Effects of Lapatinib on colony formation and cell viability.

(A) A375 cells were plated at clonal density in the presence of DMSO or lapatinib (1 μM), either with or without NRG1β (10 ng/ml). Media and additives were replaced every 3 days. Cells were allowed to grow for 7 days and were then fixed and stained with crystal violet.

(B) WM115 cells were grown in the presence of DMSO, lapatinib (1 μM), PLX4032 (1 μM), AZD6244 (3.3 μM), or combinations of PLX4032 or AZD6244 with lapatinib in the presence or absence of NRG1β (10 ng/ml) for 72 hours. Cell viability was determined by AlamarBlue® assay and normalized to DMSO treated cells (considered 100% viable). The means ± s.e.m. (n=3) are shown. p-values are indicated.

(C) As for B, except 1205Lu cells were analyzed.

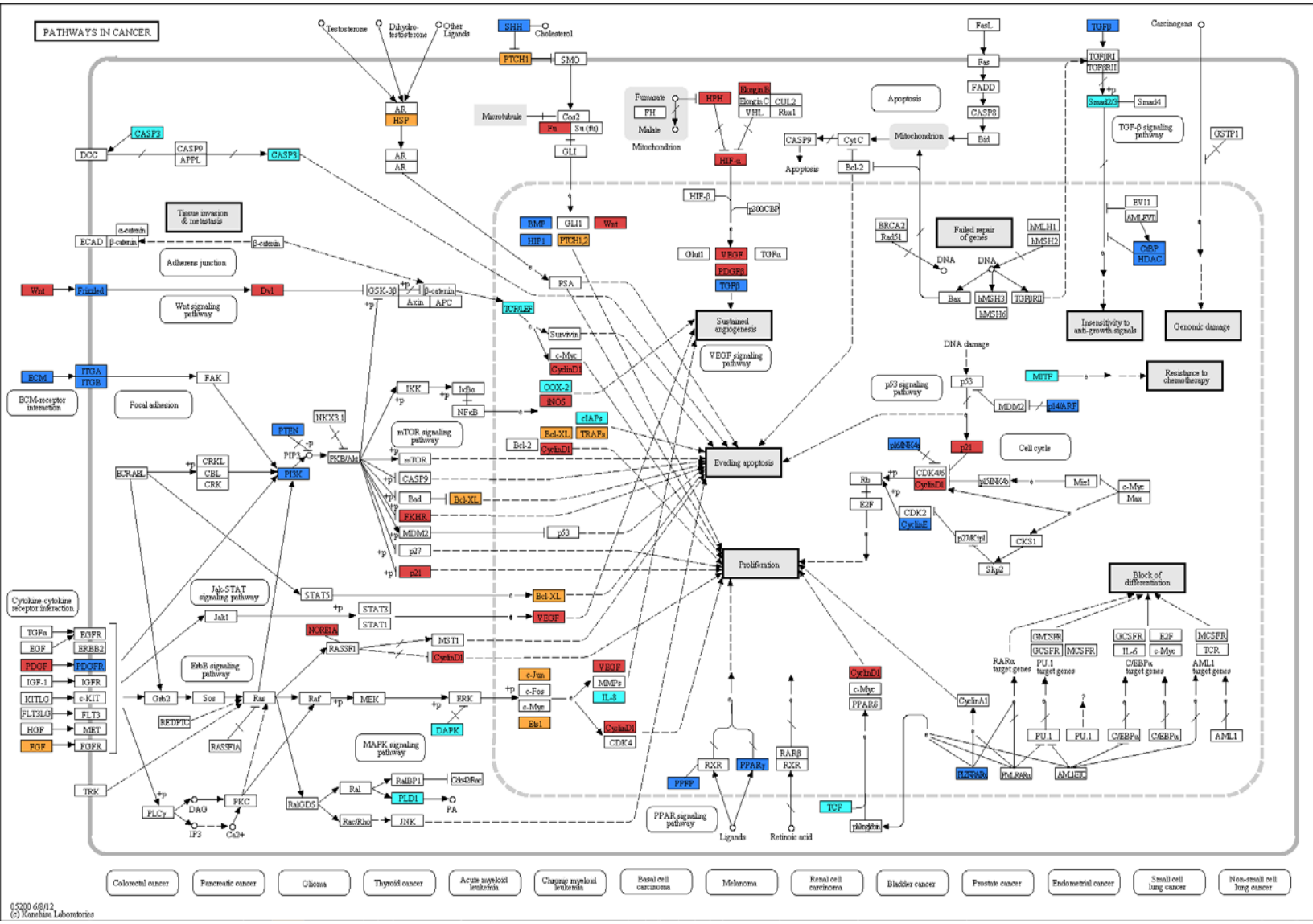
Supplemental Figure 7 AKT inhibitors overcome NRG1β-ERBB3 signaling.

(A) 1205Lu cells were treated overnight with either vehicle, PLX4032 (1 μM), MK2206 (1 μM), MK2206 (5 μM), or a combination of PLX4032 and MK2206 (1 and 5 μM). Cells were stimulated with 10 ng/ml NRG1β for 1 hour, and lysates were immunoblotted, as indicated.
(B and C) 1205Lu (B) and WM115 (C) cells were grown in the presence of either vehicle, PLX4032 (1 μM), MK2206 (1 μM), MK2206 (5 μM), or a combination of PLX4032 and MK2206 (1 and 5 μM), with or without NRG1β (10 ng/ml). After 72 hours, cell viability was determined by AlamarBlue® assay and normalized to DMSO treated cells (considered 100% viable). The means ± s.e.m. (n=4) are shown.

Supplemental Figure 8 ERBB3 and NRG1 expression in human melanoma cell lines.
(A) A panel of human melanoma cell lines was immunoblotted for ERBB3, NRG1, and an actin loading control. NRG1-transfected 293 cell lysate was run as a positive control. The upper panel represents primary (radial growth phase/vertical growth phase) melanoma cell lines. The lower panel contains cells lines isolated from metastatic melanoma.
(B) Melanoma cell lines were treated overnight with either vehicle or PLX4032 (1 μM). Harvested lysates were analyzed for NRG1 expression.

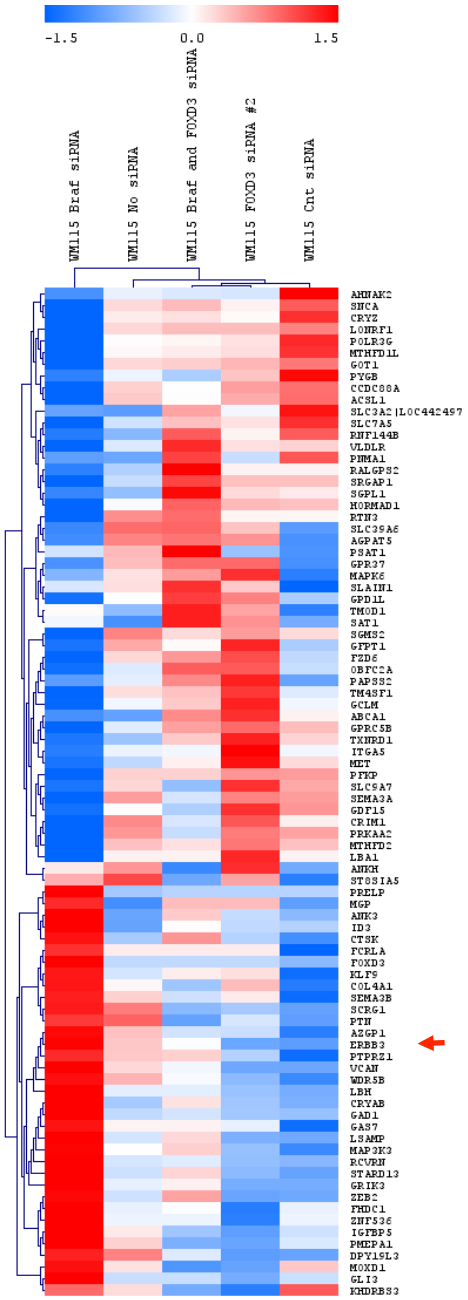
Supplemental Table 1 Genes regulated by FOXD3 in melanoma cells (<25% false discovery rate).

Abel et al, Supplemental Figure 1



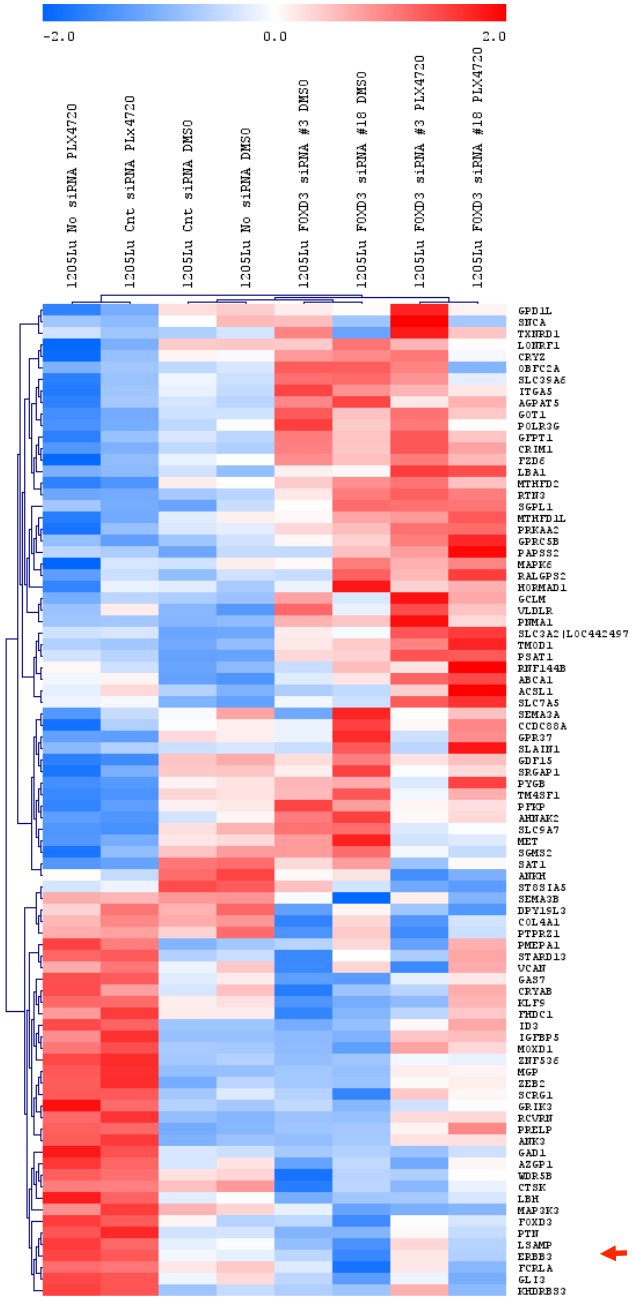
A

WM115

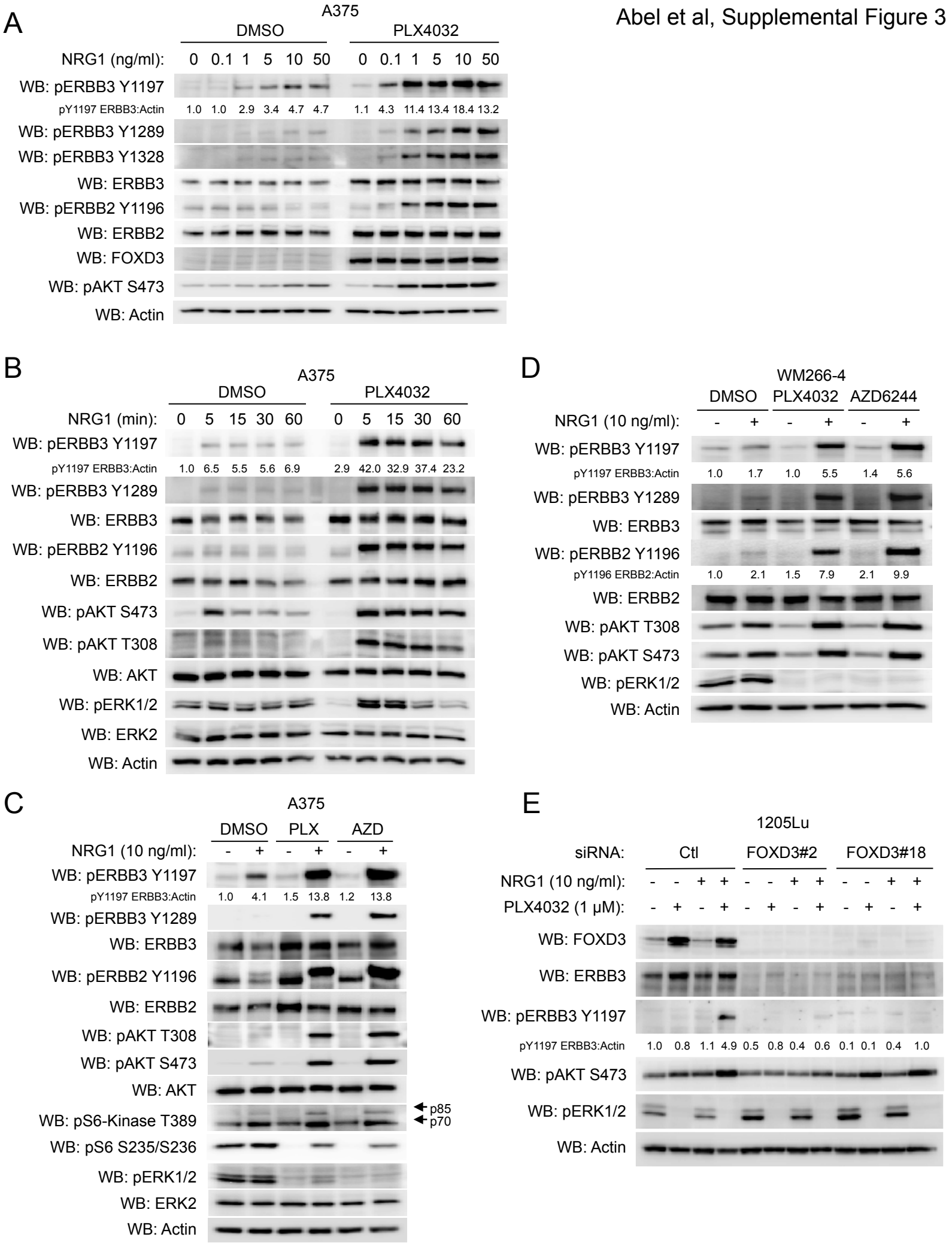


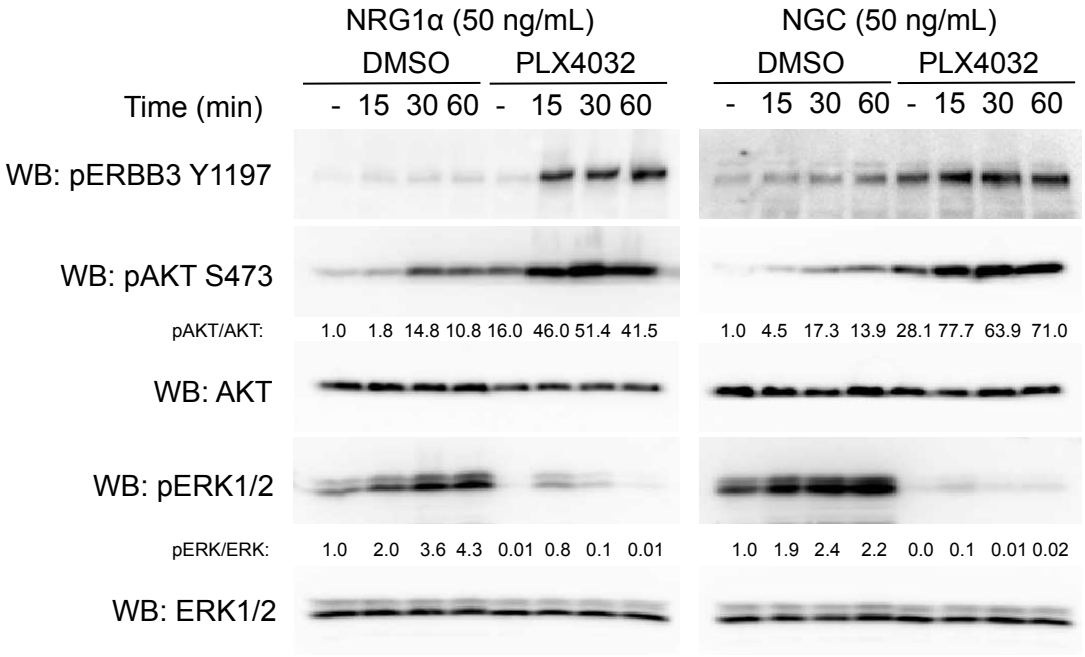
B

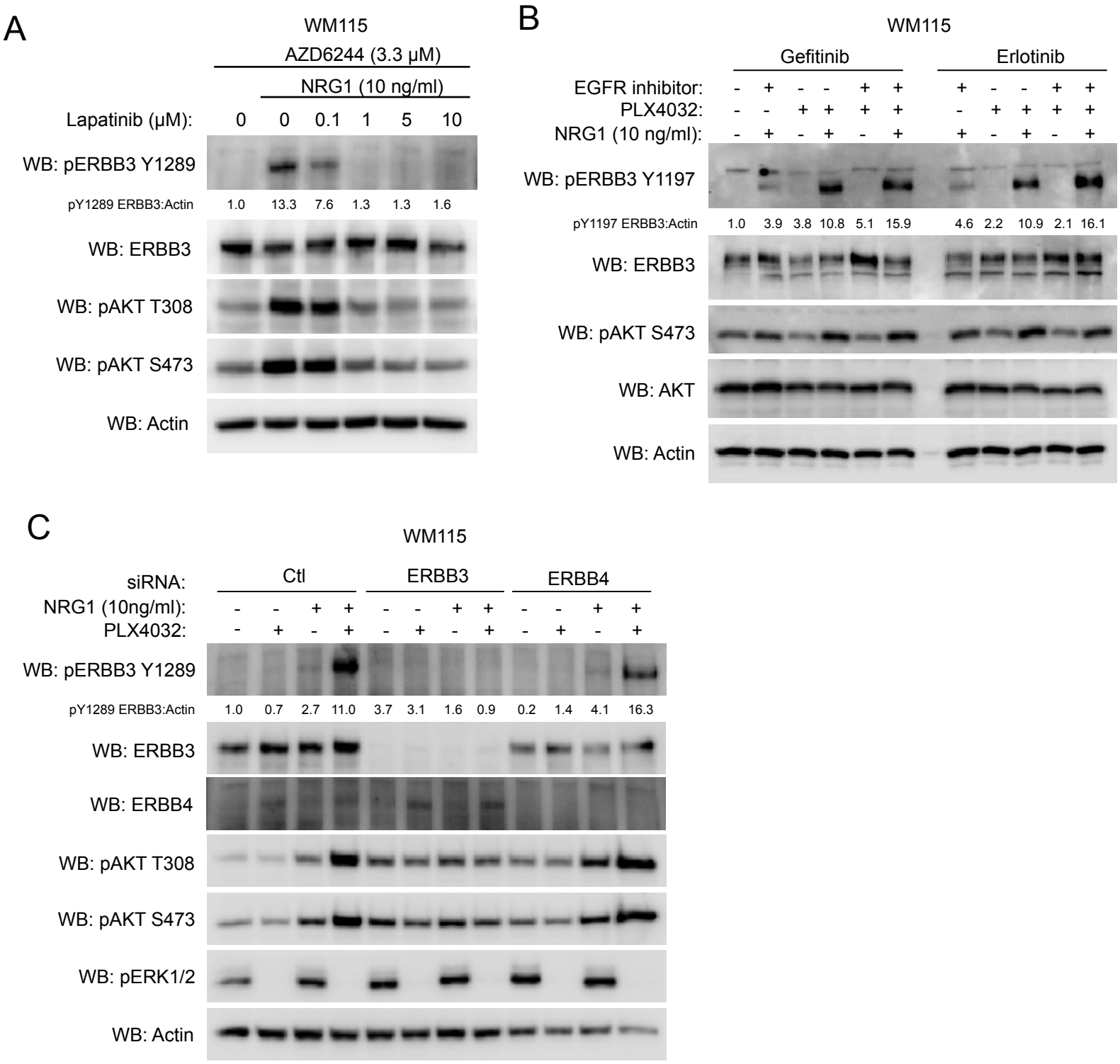
1205Lu



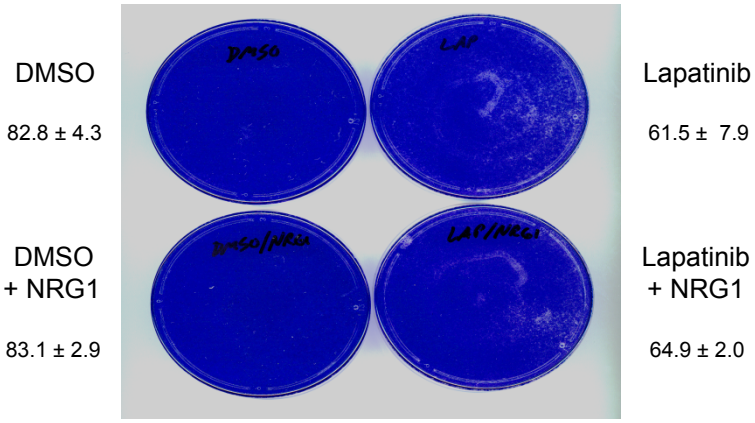
Abel et al, Supplemental Figure 3



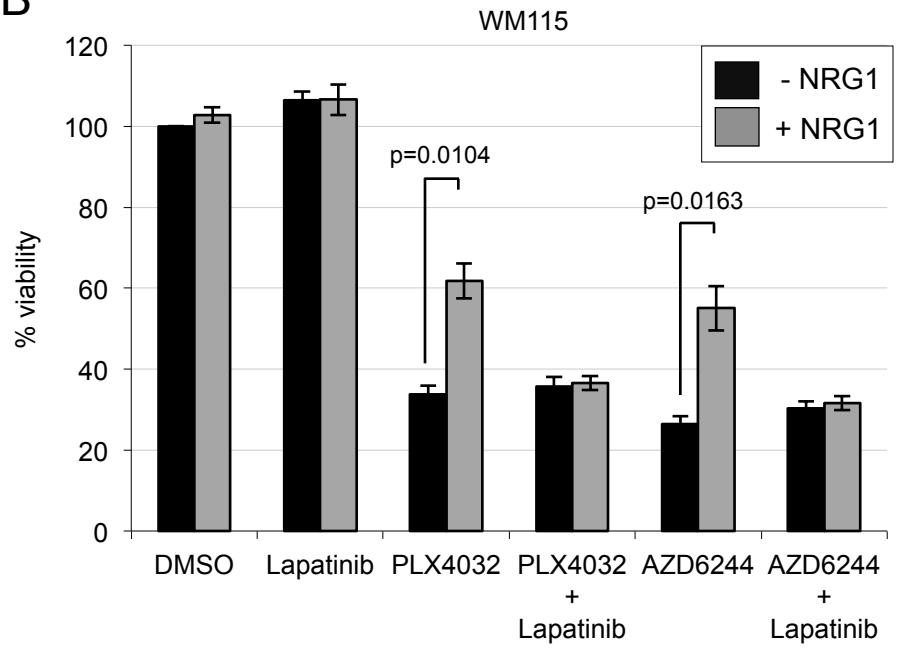

NRG1α (50 ng/mL)
NGC (50 ng/mL)
DMSO
PLX4032
DMSO
PLX4032
Time (min)
- 15 30 60 - 15 30 60
- 15 30 60 - 15 30 60
WB: pERBB3 Y1197
WB: pAKT S473
pAKT/AKT:
1.0 1.8 14.8 10.8 16.0 46.0 51.4 41.5
1.0 4.5 17.3 13.9 28.1 77.7 63.9 71.0
WB: AKT
WB: pERK1/2
pERK/ERK:
1.0 2.0 3.6 4.3 0.01 0.8 0.1 0.01
1.0 1.9 2.4 2.2 0.0 0.1 0.01 0.02
WB: ERK1/2


A
WM115
AZD6244 (3.3 μM)
NRG1 (10 ng/ml)
Lapatinib (μM): 0 0 0.1 1 5 10
WB: pERBB3 Y1289
pY1289 ERBB3:Actin 1.0 13.3 7.6 1.3 1.3 1.6
WB: ERBB3
WB: pAKT T308
WB: pAKT S473
WB: Actin
B
WM115
Gefitinib
Erlotinib
EGFR inhibitor: - + - - + + + - - + +
PLX4032: - - + + + + - + + + +
NRG1 (10 ng/ml): - + - + - + + - + - +
WB: pERBB3 Y1197
pY1197 ERBB3:Actin 1.0 3.9 3.8 10.8 5.1 15.9 4.6 2.2 10.9 2.1 16.1
WB: ERBB3
WB: pAKT S473
WB: AKT
WB: Actin
C
WM115
siRNA: Ctl ERBB3 ERBB4
NRG1 (10ng/ml): - - + + - - + + - - + +
PLX4032: - + - + - + - + - + - +
WB: pERBB3 Y1289
pY1289 ERBB3:Actin 1.0 0.7 2.7 11.0 3.7 3.1 1.6 0.9 0.2 1.4 4.1 16.3
WB: ERBB3
WB: ERBB4
WB: pAKT T308
WB: pAKT S473
WB: pERK1/2
WB: Actin

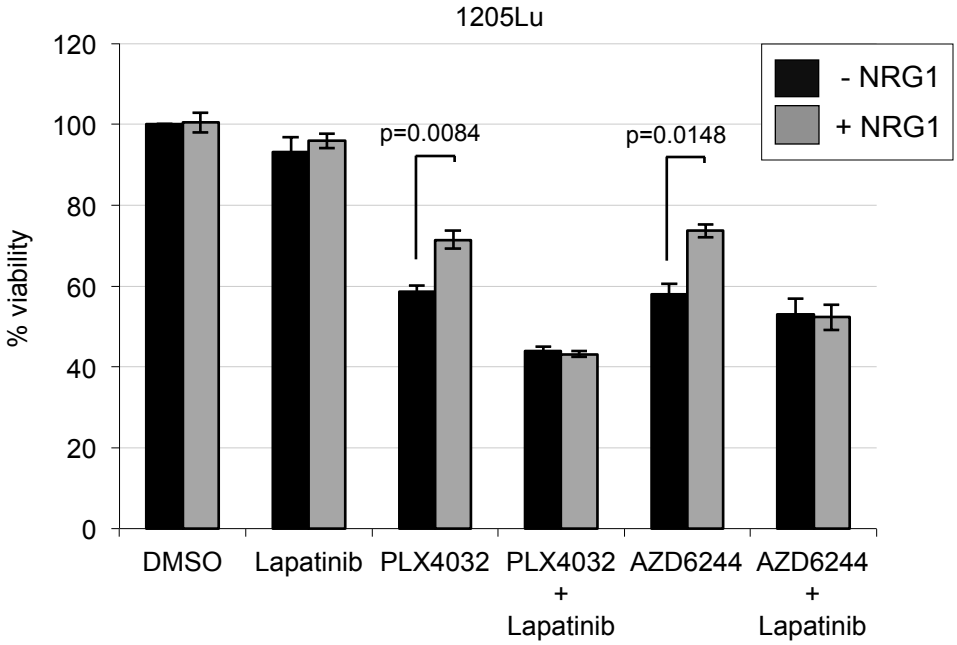
A


DMSO
82.8 ± 4.3
Lapatinib
61.5 ± 7.9
DMSO
+ NRG1
83.1 ± 2.9
Lapatinib
+ NRG1
64.9 ± 2.0

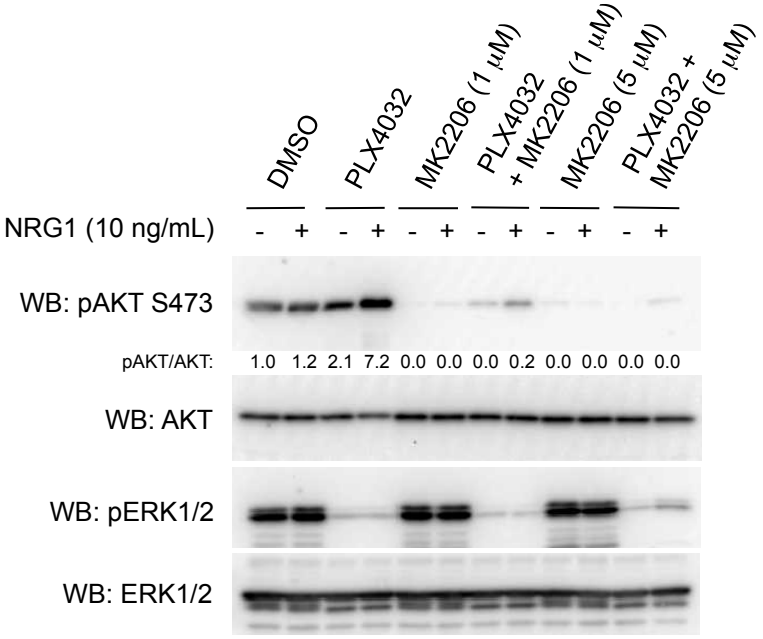
B


WM115
- NRG1
+ NRG1
% viability
120
100
80
60
40
20
0
p=0.0104
p=0.0163
DMSO
Lapatinib
PLX4032
PLX4032 + Lapatinib
AZD6244
AZD6244 + Lapatinib

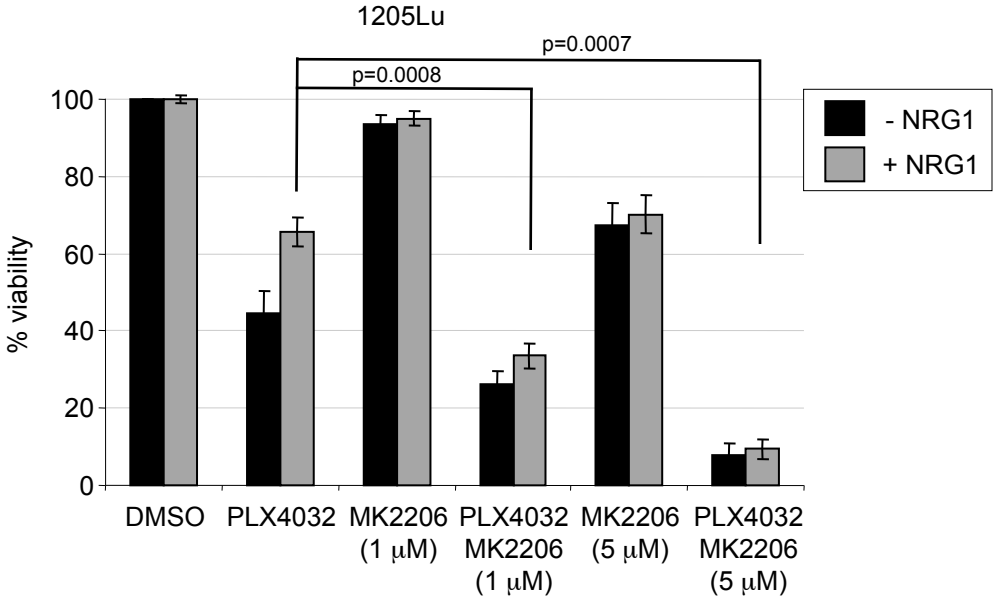
C


1205Lu
- NRG1
+ NRG1
% viability
120
100
80
60
40
20
0
p=0.0084
p=0.0148
DMSO
Lapatinib
PLX4032
PLX4032 + Lapatinib
AZD6244
AZD6244 + Lapatinib

A


DMSO
PLX4032
MK2206 (1 μM)
PLX4032 + MK2206 (1 μM)
MK2206 (5 μM)
PLX4032 + MK2206 (5 μM)
NRG1 (10 ng/mL)
- + - + - + - + - + - +
WB: pAKT S473
pAKT/AKT: 1.0 1.2 2.1 7.2 0.0 0.0 0.0 0.2 0.0 0.0 0.0 0.0
WB: AKT
WB: pERK1/2
WB: ERK1/2

B


1205Lu
p=0.0007
p=0.0008
- NRG1
+ NRG1
% viability
0
20
40
60
80
100
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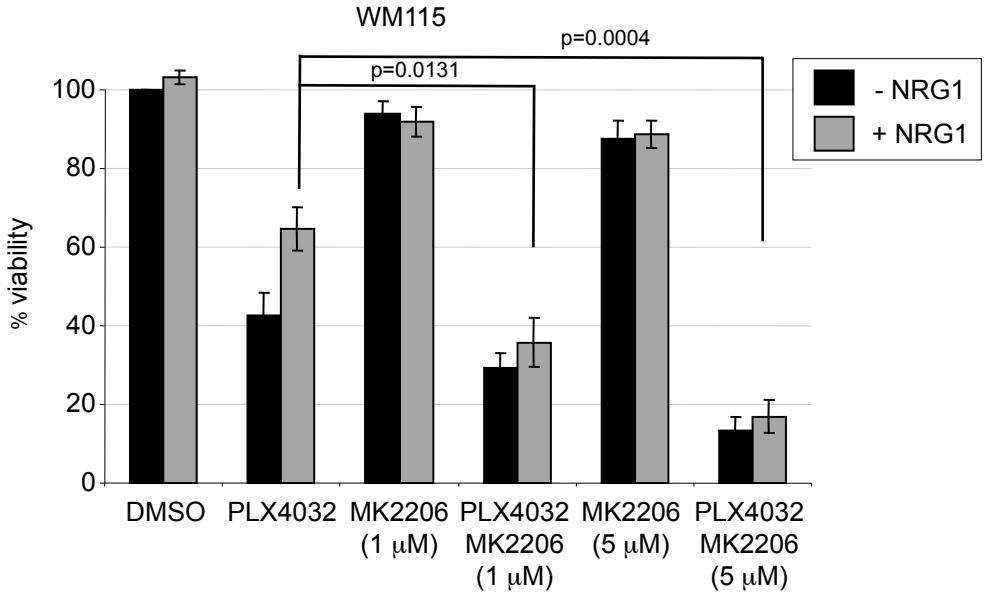
MK2206 (1 μM)
PLX4032 MK2206 (1 μM)
MK2206 (5 μM)
PLX4032 MK2206 (5 μM)

C


WM115
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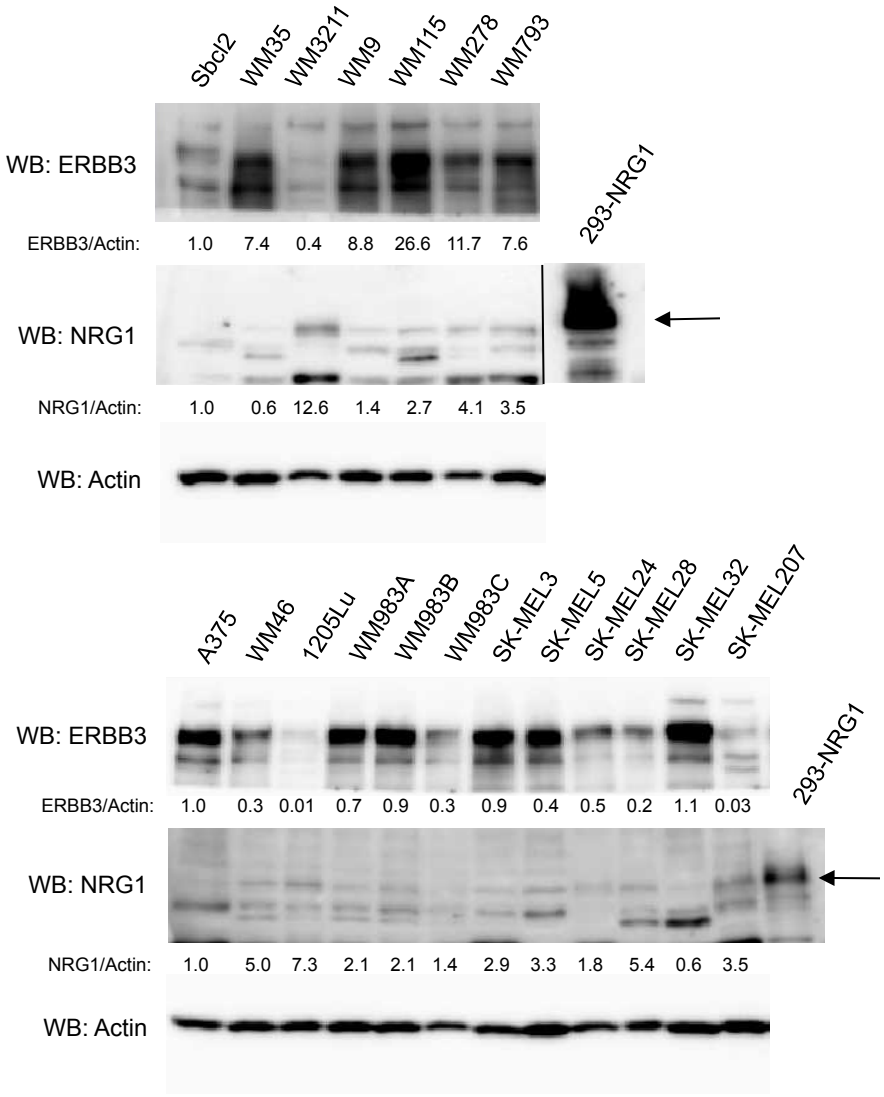
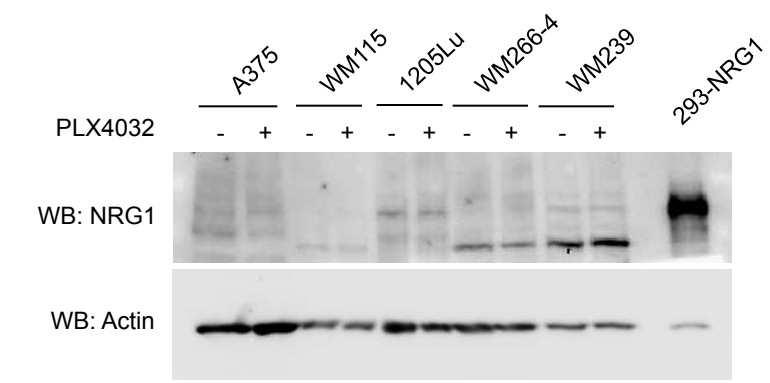
p=0.0131
- NRG1
+ NRG1
% viability
0
20
40
60
80
100
DMSO
PLX4032
MK2206 (1 μM)
PLX4032 MK2206 (1 μM)
MK2206 (5 μM)
PLX4032 MK2206 (5 μM)

A


Sbcl2
WM35
WM3211
WM9
WM115
WM278
WM793
WB: ERBB3
293-NRG1
ERBB3/Actin: 1.0 7.4 0.4 8.8 26.6 11.7 7.6
WB: NRG1
NRG1/Actin: 1.0 0.6 12.6 1.4 2.7 4.1 3.5
WB: Actin
A375
WM46
1205Lu
WM983A
WM983B
WM983C
SK-MEL3
SK-MEL5
SK-MEL24
SK-MEL28
SK-MEL32
SK-MEL207
WB: ERBB3
293-NRG1
ERBB3/Actin: 1.0 0.3 0.01 0.7 0.9 0.3 0.9 0.4 0.5 0.2 1.1 0.03
WB: NRG1
NRG1/Actin: 1.0 5.0 7.3 2.1 2.1 1.4 2.9 3.3 1.8 5.4 0.6 3.5
WB: Actin

B


A375
WM115
1205Lu
WM266-4
WM239
293-NRG1
PLX4032
- + - + - + - + - +
WB: NRG1
WB: Actin