Supplemental Data

The K-Ras effector p38γ MAPK confers intrinsic resistance to tyrosine kinase inhibitors by stimulating *EGFR* transcription and EGFR de-phosphorylation

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This PDF file contains:

Supplemental Experimental Procedures
Six Suppl. Figures
Suppl. Figure S1. Roles of K-Ras mutation and EGFR/Y1173 phosphorylation in resistance to TKIs.
Suppl. Figure S2. Roles of c-Jun and EGFR in resistance to TKIs downstream of p38γ
Suppl. Figure S3. Nuclear co-localization of p38γ and c-Jun in K-Ras mutant cells and sensitization of K-Ras mutant cells to TKIs by c-Jun knockdown
Suppl. Figure S4. p38γ and PTPH1 knockdown sensitizes K-Ras mutant cells to TKI-induced growth inhibition
Suppl. Figure S5. Knockdown of PTPH1 fails to consistently affect the sensitivity of K-Ras WT cells to TKIs.
Suppl. Figure S6. PFD treatment increases the sensitivity of K-Ras mutant cancer cells to TKIs and disrupts the EGFR complexes with PTPH1 and p38γ proteins in K-Ras mutant tumors.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Antibodies and other reagents

The antibody against PTPH1 (mouse) was kindly provided by Dr. N. K. Tonks (1). Other antibodies used in this study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These include Anti-GAPDH (sc-47724), anti-β-actin (sc-47778), anti-EGFR (mouse, sc-53274), anti- EGFR (rabbit, sc-03) anti-EGFR (goat, sc-03G), anti-p-EGFR/Y1173 (goat, sc-12351), anti-PTPH1 (goat, sc9789), anti-α-Actinin (sc-17829), anti-c-Jun (sc-44) and anti-Tubulin (sc-6199). Anti-p-EGFR/Y1173 (rabbit, 4407L) was obtained from Cell Signaling and anti-p38γ (AF1347) was from R&D Systems. The dual EGFR/Her2 inhibitor lapatinib (Lap) and the EGFR inhibitor gefitinib (Gef) were obtained Selleckem. Pirfenidone (PFD) for cell culture and animal studies was purchased from Sigma and Pirespa (Shionogi & Co.), respectively.

Cell fractionation, immunoprecipitation, immunoblot analysis, and immune-staining

For cell fractionation analysis, our previously published protocol was used (2,3). For immunoprecipitation (IP), equal protein amounts were used for analysis, with aliquots of whole cell lysates (WCL) used as input controls. Briefly, cells were washed with cold PBS and lysed in modified RIPA buffer (50mM Tris-HCL, pH 7.5, 1mM phenylmethylsulfonyl fluoride, 1mM dithiothreitol, 10mM sodium fluoride, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin, and 1% NP-40) as described (2,3). Cleared lysates were then incubated with the indicated antibodies or IgG overnight at 4^0C. Precipitates were then washed and pellets were re-suspended in 2X loading buffer. For direct Western blot (WB), cells were directly lysed in 1X loading buffer. After heating, samples were separated on SDS-PAGE and the rest of the procedure was the same as previously described (4). For immune-staining analysis, cells were plated on coverslips and fixed in 3.7% formaldehyde. Cells were then permeabilized in a buffer containing 0.5% Triton X-100 and 0.5% NP40, and then incubated with a blocking buffer (PBS containing 3% bovine serum) prior to immunostaining.

Primers used for these studies

<u>shRNA Target Sequences:</u>

shLuc: GTGCGTTGCTAGTACCAAC;

shp38γ#1: CTCATGAAACATGAGAAGCTA;

shp38γ#2: GAAGGAGATCATGAAGGTGAC;

shc-Jun#1: TTAAGCTGTGCCACCTGTTCC;

shc-Jun#2: GGCACAGCTTAAACAGAAAGTC

shEGFR#1: GAGAACTCTGAGTGCATACAG

shEGFR#2: GCTCTCTTGAGGATCTTGAAG

Primers used in qRT-PCR:

EGFR rat forward: 5' CCCACAGCAAGGCTTCTTCA 3';

EGFR rat reverse: 5' CACGGCAGCTCCCATTTCTA 3';

EGFR human forward: 5' CCACCAAATTAGCCTGGACA 3';

EGFR human reverse: 5' CGCGACCCTTAGGTATTCTG 3';

GAPDH forward: 5' GGTGGTCTCCTCTGACTTCAACA 3';

GAPDH reverse: 5' GTTGCTGTAGCCAAATTCGTTGT 3'

Primers used in the ChIP assay (the EGFR promoter region):

between -889/-785 containing 2 AP-1 sites,

EGFR promoter -889/-785 region forward: 5' AGAGGGTCCCGTAGTGCTG 3';

EGFR promoter -889/-785 region reverse: 5' AGACTGGCCCCTGGCATTTCTCCT 3'

References

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Suppl. Figures

Suppl. Figure S1. Roles of K-Ras mutation and EGFR/Y1173 phosphorylation in resistance to TKIs. ***A.*** Representative images of colony formation for Figs. 1A/B in K-Ras mutant and K-Ras-disrupted sublines (5-9). ***B.*** Expression of mutant K-Ras (G12V) in Hke3 cells confers TKI resistance in Hke3 cells (left, WB and right, relative colony formation over DMSO in K-Ras transduced cells, mean ± SD, n = 3). ***C,*** representative colony images for Fig. 1E.

Suppl. Figure S2. Roles of c-Jun and EGFR in resistance to TKIs downstream of p38γ. ***A-C.*** c-Jun and EGFR were stably depleted from cells via lentiviral shRNA infection and engineered cells were analyzed for protein expression by WB (**A, B**), and for colony formation (**C**, mean ± SD, n = 3) (10).

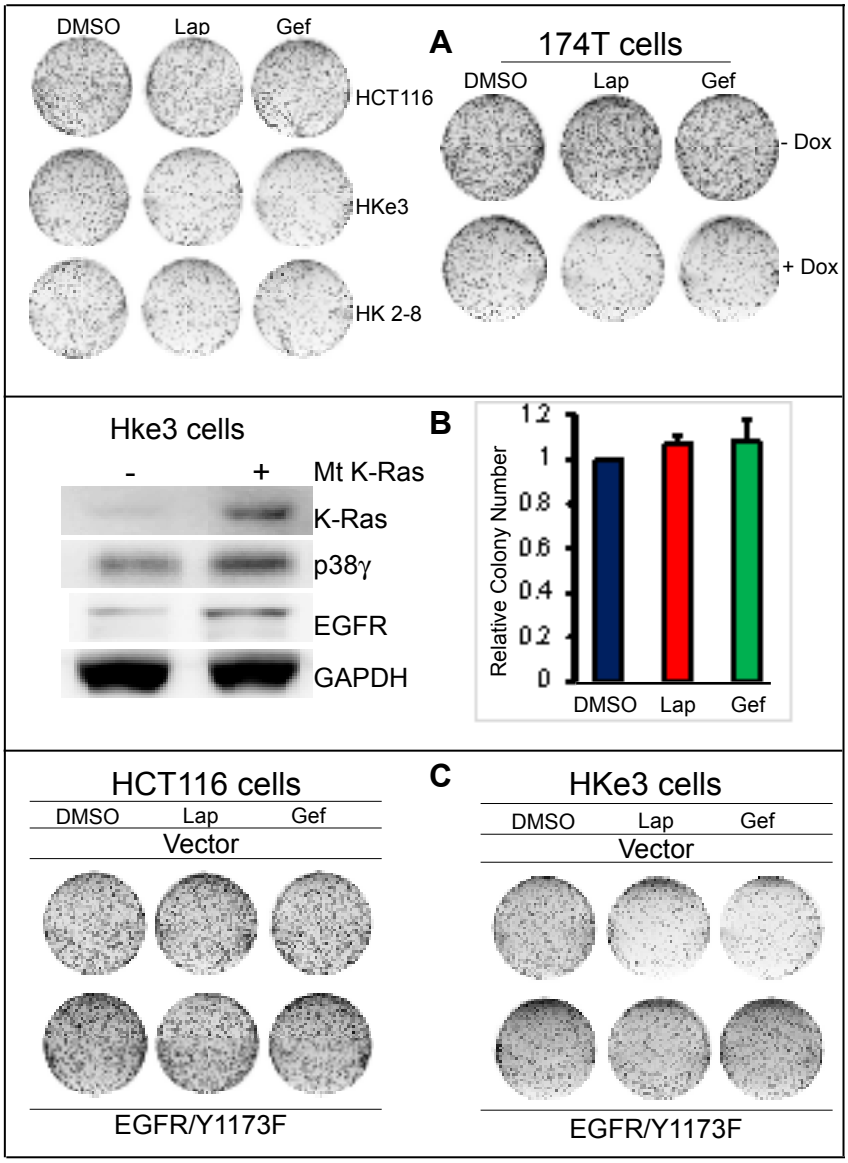
Suppl. Figure S3. Nuclear co-localization of p38γ and c-Jun in K-Ras mutant cells and sensitization of K-Ras mutant cells to TKIs by c-Jun knockdown. ***A.*** Immuno-fluorescence co-staining was performed as described (4) (scale bar = 100μm). ***B.*** Cells were depleted of c-Jun protein by lentiviral mediated shc-Jun (#1shRNA) delivery and the resultant cells were analyzed for growth inhibition by TKIs (the same conditions as Figure 1A). Results of 3 separate experiments are shown at left (mean ± SD) with representative images of colony formation at bottom right. WB results (top, right panel) show c-Jun knockdown by shRNA.

Suppl. Figure S4. p38γ and PTPH1 knockdown sensitizes K-Ras mutant cells to TKI-induced growth inhibition. ***A, B.*** Cells were stably depleted of p38γ or PTPH1 by lentiviral shRNA infection. The resultant cells were assessed for protein expression (left top) and colony formation. Results of summarized colony formation are given in the bar graph (Mean ± SD, n = 3) and representative colony images are presented at right.

Suppl. Figure S5. Knockdown of PTPH1 fails to consistently affect the sensitivity of K-Ras WT cells to TKIs and p38γ and PTPH1 collaborate to regulate EGFR expression and de-phosphorylation. ***A, B.*** PTPH1 stable knockdown cells (from Fig. 5A) were analyzed for growth-inhibition by TKIs (the same conditions as in Fig. 1A). Mean ± SD of 3 experiments are presented in **A** and representative images of colony formation are shown in **B**.

Suppl. Figure S6. PFD treatment increases the sensitivity of K-Ras mutant cancer cells to TKIs and disrupts the EGFR complexes with PTPH1 and p38γ proteins in K-Ras mutant tumors. ***A.*** Cells were treated for about 2 weeks and colonies formed were stained and photographed (for Fig. 6A). ***B.*** Protein lysates were prepared from tumors growing in nude mice treated with PFD ± Lap (Fig. 6C), which were then analyzed by WB. Results (left) are from 3 tumors from 3 separate mice treated with PFD or control DMSO for decreased p38γ activity (reduced p-PTPH1 levels) (7,8). Equal protein amounts from the indicated tumor lysates were immune-precipitated with specific anti-EGFR antibody or control IgG and precipitates were analyzed for PTPH1 and p38γ by WB. The numbers indicate EGFR-bound PTPH1 or p38γ measured from EGFR precipitates.

Figure S1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Gef
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B
-
+
Mt K-Ras
K-Ras
p38γ
EGFR
GAPDH
Relative Colony Number
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0.8
0.6
0.4
0.2
0
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Lap
Gef
HCT116 cells
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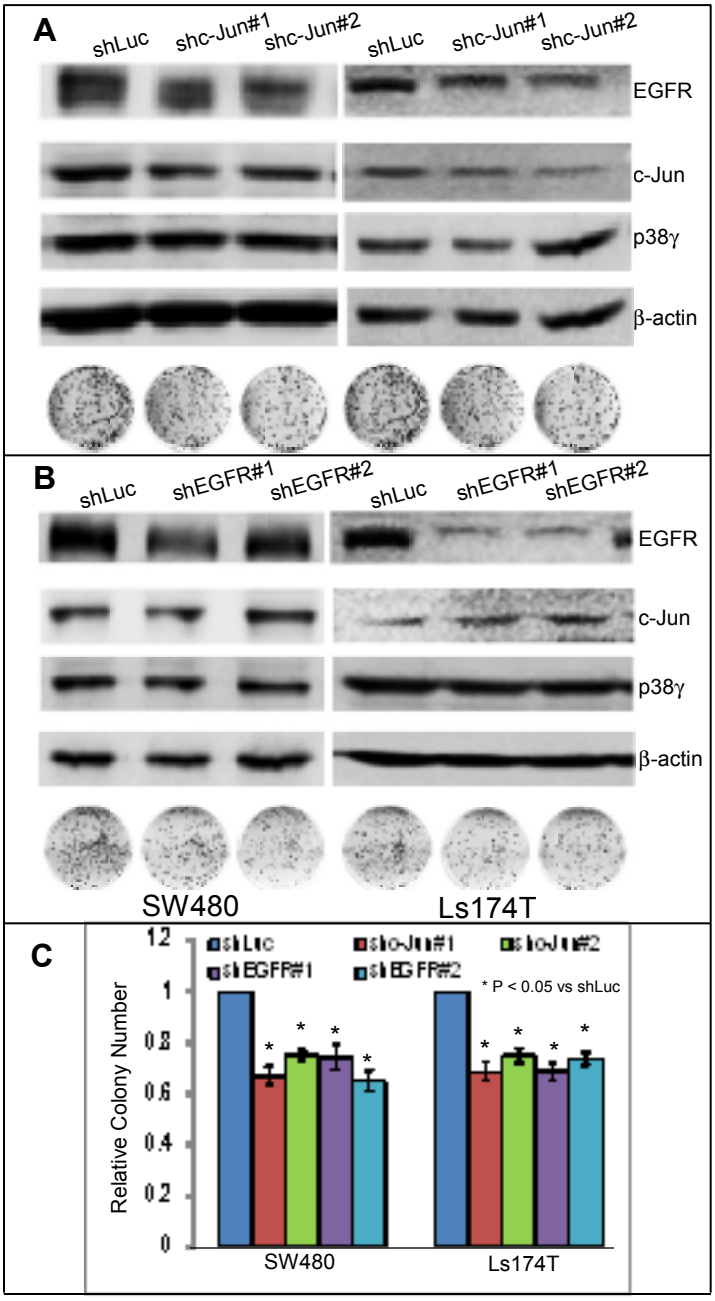
Figure S2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B
shLuc
shEGFR#1
shEGFR#2
shLuc
shEGFR#1
shEGFR#2
EGFR
c-Jun
p38γ
β-actin
SW480
Ls174T
C
shLuc
shc-Jun#1
shc-Jun#2
shEGFP#1
shEGFP#2
* P < 0.05 vs shLuc
Relative Colony Number
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0.4
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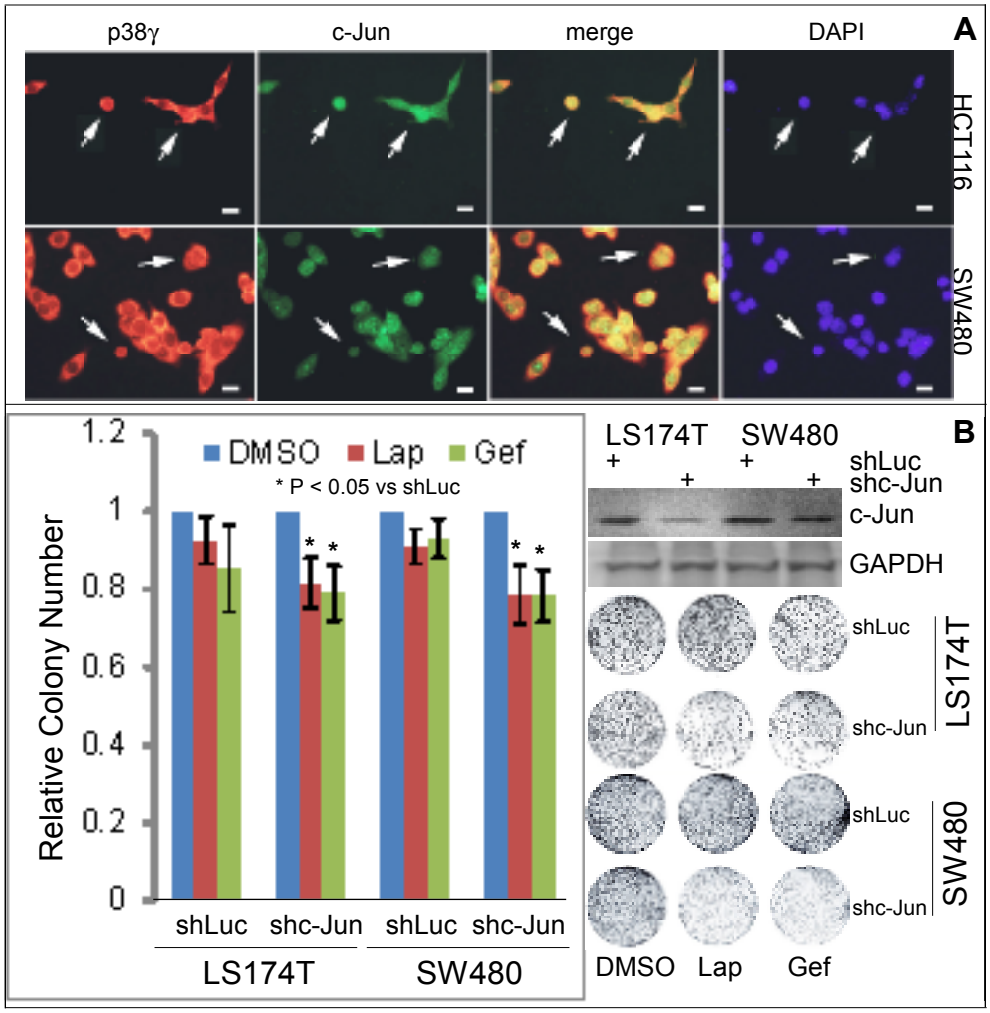
0
SW480
Ls174T

Figure S3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c-Jun
merge
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HCT116
SW480
B
Relative Colony Number
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Lap
Gef
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shc-Jun
shLuc
shc-Jun
LS174T
SW480
LS174T
SW480
+
+
+
+
shLuc
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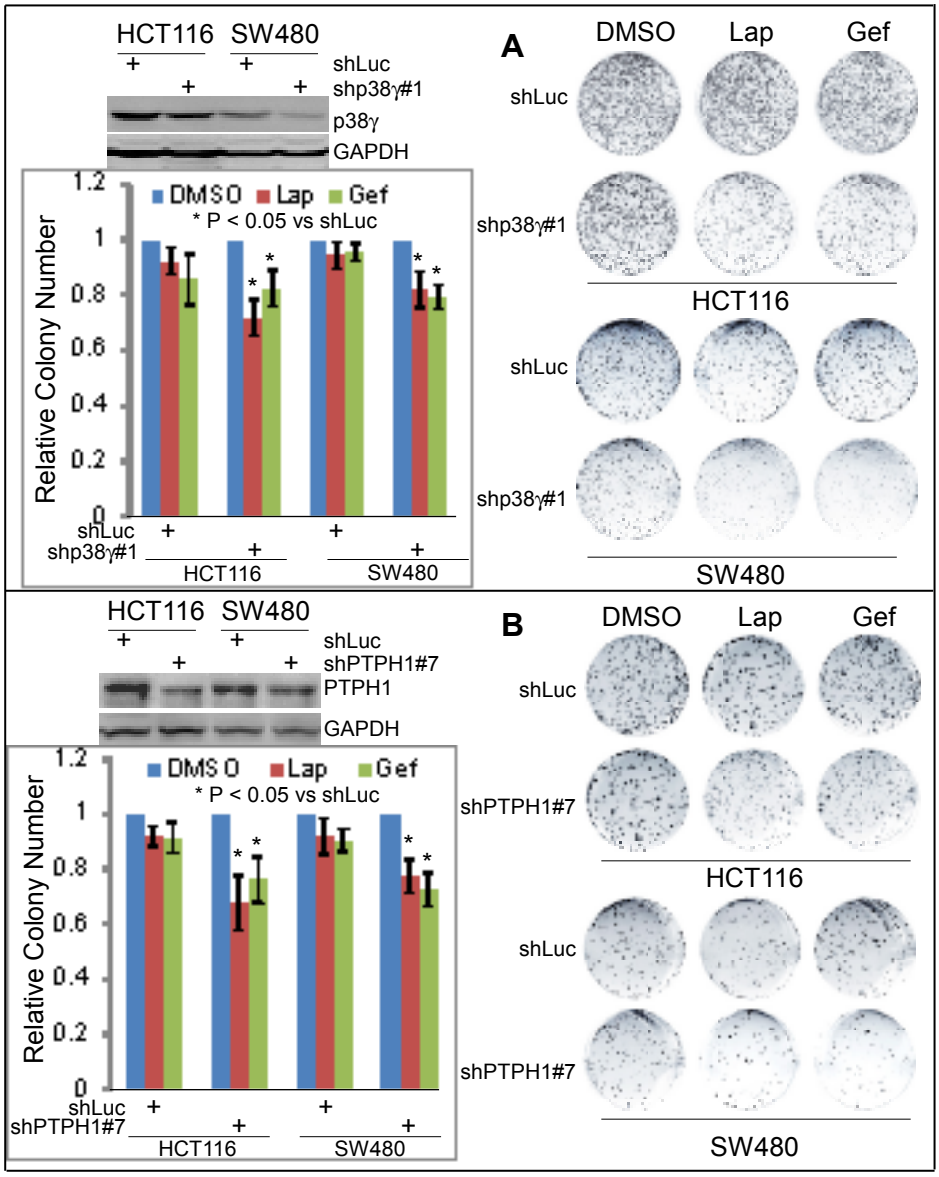
Lap
Gef

Figure S4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p38γ
GAPDH
DMSO
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Gef
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Relative Colony Number
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1
0.8
0.6
0.4
0.2
0
shLuc
shp38γ#1
HCT116
SW480
A
DMSO
Lap
Gef
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HCT116
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SW480
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shPTPH1#7
PTPH1
GAPDH
DMSO
Lap
Gef
* P < 0.05 vs shLuc
Relative Colony Number
1.2
1
0.8
0.6
0.4
0.2
0
shLuc
shPTPH1#7
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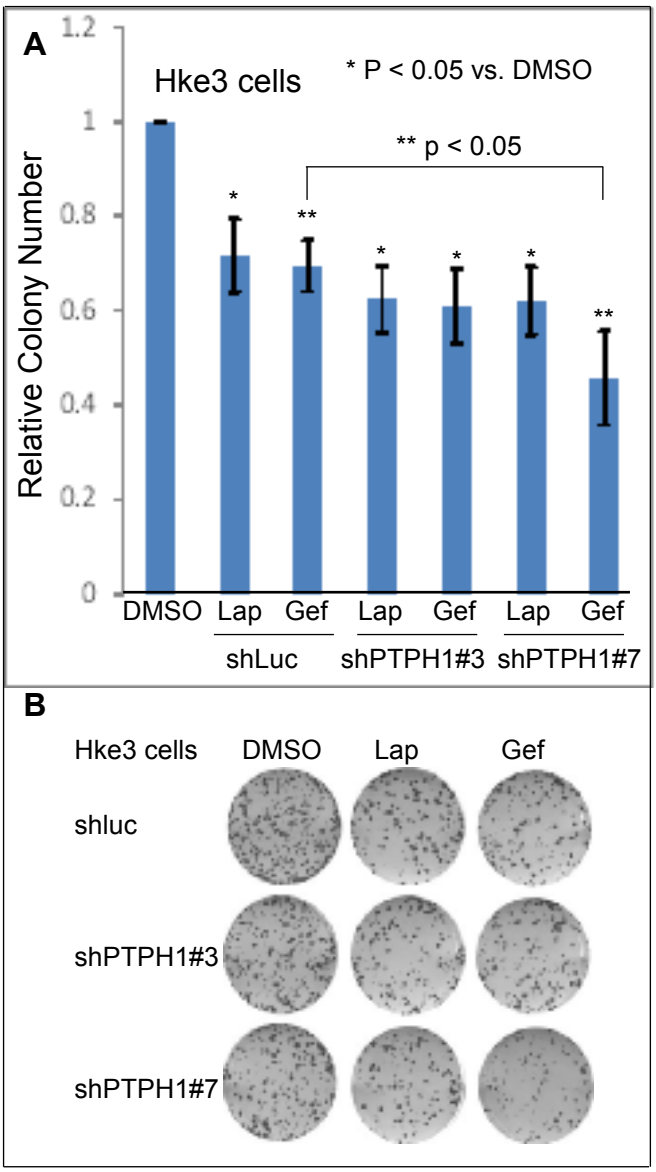
SW480

Figure S5


A
Hke3 cells
* P < 0.05 vs. DMSO
** p < 0.05
Relative Colony Number
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1
0.8
0.6
0.4
0.2
0
DMSO
Lap
Gef
Lap
Gef
Lap
Gef
shLuc
shPTPH1#3
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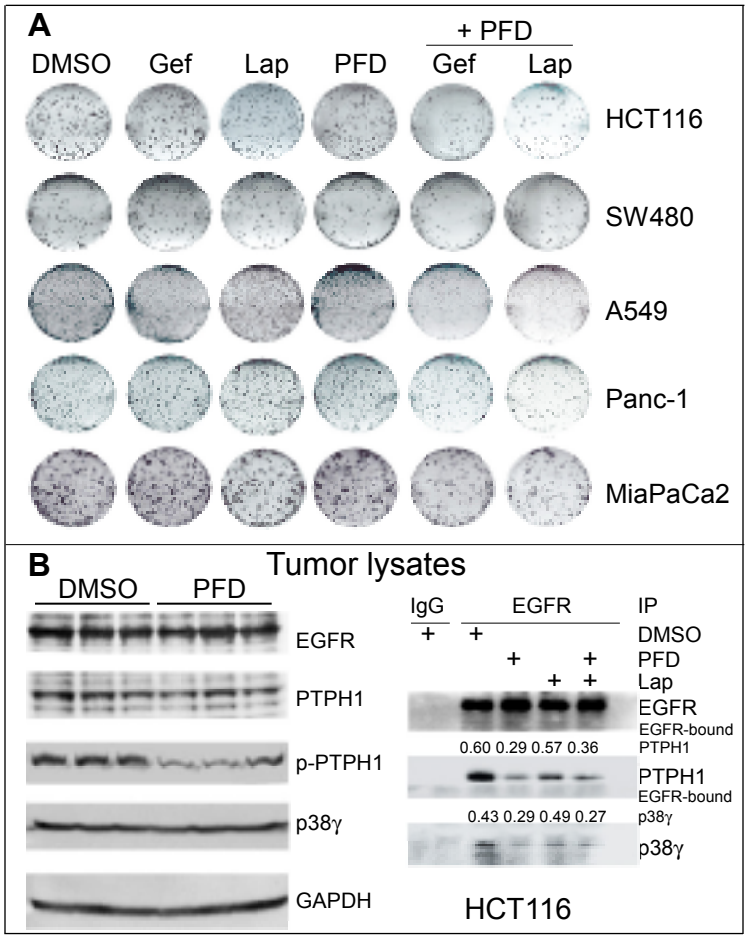
B
Hke3 cells
DMSO
Lap
Gef
shluc
shPTPH1#3
shPTPH1#7

Figure S6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+ PFD
DMSO
Gef
Lap
PFD
Gef
Lap
HCT116
SW480
A549
Panc-1
MiaPaCa2
B
Tumor lysates
DMSO
PFD
EGFR
PTPH1
p-PTPH1
p38γ
GAPDH
IgG
EGFR
IP
DMSO
PFD
Lap
EGFR
EGFR-bound PTPH1
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PTPH1
EGFR-bound p38γ
0.43 0.29 0.49 0.27
p38γ
HCT116