Supplemental data

p38γ MAPK signals through phosphorylating its phosphatase PTPH1 in regulating Ras oncogenesis and stress response

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This PDF file contains the following:

1) Supplemental experimental procedures;

2) Supplemental Figures (S1 to S5) and Figure legends;

3) Supplemental references

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Constructs, cell lines, antibodies, and other reagents-His-tagged p38α and p38γ, Flag-tagged p38γ, HA-tagged MKK6/2E, and HA-tagged PTPH1 expressing constructs were provided by Drs. Jia-Huai Han and Nicholas Tonks, respectively. Flag-tagged p38γΔ4, GST-tagged PTPH1 and retrovirus LZRS-K-Ras have been described previously (1,2). IEC-6 cells and human colon cancer cell lines T84, CaCo2, HCT116, HT29, Skco-1, SW480, ls-174T were purchased from ATCC. p38γ+/+ and -/- mouse embryonic fibroblasts MEFs (passage 3 to 5) were provided by Dr. Ana Cuenda (3), which were immortalized by infection with pBabe-H-Ras, and the KO cells were further re-expressed with p38γ or with the control vector (4). The antibodies used in this study include p-p38, p-ERK, p-JNK, anti-p38δ that were purchased from Cell Signaling and the specific antibody against phospho-PTPH1/S459 (p-PTPH1) was generated by PhosphoSolutions Inc. Other antibodies from Santa Cruz are anti-Flag (sc-807), anti-PTPH1 (sc-9789), anti-GST (sc-138), anti-GAPDH (sc-47724), anti-HA (sc-7392), anti-α-Actinin (sc-17829), anti-p38α (sc-535), anti-MKK6 (sc-1991), anti-phospho-ATF2 (sc-8398), and anti-ATF2 (sc-6233). Anti-PTPH1 mouse monoclonal antibody was a gift from Dr. Nicholas Tonks, while anti-p38β polycolonal antibody was a gift from Dr. Jiahuai Han. Anti-p38γ goat and rabbit antibodies were bought from R&D Systems. Protein synthesis inhibitor cycloheximide (CHX) and pirfenidone (PFD) were purchased from Sigma, cell culture materials from Gibco, SB203508 (SB) from Calbiochem, and all other chemicals were from Sigma.

Plasmid construction-PTPH1 truncated mutant HA-PTPH1Δ402, HA-PTPH1Δ490 were created by PCR using pcDNA3-PTPH1 as template. The resultant PCR products were digested with *BamH*I and *Xho*I and inserted into pcDNA3.

Primers for HA-tagged PTPH1Δ402 and PTPH1Δ490 are:

Forward: cgtacGAATTCaccatggcaacctacatcacggaaacggaagat (PTPH1Δ402);

cgtacGAATTCaccatggaggacgccagccagtactactgt (PTPH1Δ490).

Reverse: cgtacCTCGAGatcgctggcatagtcaggcacgtcataaggataactaggatccagcatttggactaaac.

PTPH1 point mutation mutants HA-PTPH1/S459A, HA-PTPH1/S469A, HA-PTPH1/S459A/S469A, HA-PTPH1/SH3, HA-PTPH1/S459D were created by site-directed mutagenesis using pc-DNA3-PTPH1 as template.GST-PTPH1/S459A was created by site-directed mutagenesis using pGEX-6P-2-PTPH1 as template.

The primers used are:

Forward: gtcatccagttctgtggctccatcttcaaatg, reverse: catttgaagatggagccacagaactggatgac (HA-PTPH1/S459A),

forward: ccaggctcctgcgcacctgacggcg , reverse: cgccgtcaggtgcgcaggagcctgg (HA-PTPH1/S469A),

forward:ccagttctgtggctccatcttcaaatgctccaggctcctgcgcacctgacggcgttg,

reverse: caacgccgtcaggtgcgcaggagcctggagcatttgaagatggagccacagaactgg (HA-PTPH1/S459A/S469A),

forward: ctgccttctcgtgcccctcccattgctcccaactggcgagctcctcggctccggc,

reverse: gccggagccgaggagctcgccagttgggagcaatgggaggggcacgagaaggcag (HA-PTPH1/SH3),

forward: aagtcatccagttctgtggatccatcttcaaatgctcca, reverse: tggagcatttgaagatggatccacagaactggatgactt (HA-PTPH1/S459D).

Flag-tagged p38γ/EATA was created by PCR using pcDNA3-p38γ as template. The resultant fragment was digested with *Hind*III and *Xho*I and inserted into pcDNA3.

Primers used are:

Forward: cgtacAAGCTTatggattacaaggatgac, reverse: cgtacCTCGAGtcacgcaggcgcctccttggagaccctggc.

In vitro kinase assay and protein stability experiment-Kinase assay was performed as previously described (5). Briefly, kinase and its substrate were added together in the reaction buffer (20 mM Hepes pH 7.6, 20 mM $MgCl_2$, 15 μM ATP, 20 mM β-glycerolphosphate, 20 mM ρ-nitrophenylphophate, 0.5 mM Na_3VO_4, 2 mM DTT). Then, the mixture was incubated at 30°C for 30 minutes and phosphorylated proteins were separated with SDS-PAGE and detected with phospho-specific antibodies as indicated. For autoradiography, γ-^{32}P-ATP was added at the final concentration of 10 μM γ-^{32}P-ATP to the reaction buffer and the mixtures were separated in SDS-PAGE and detected with a

phospho-imager (5). For protein stability experiments, cells (293T) were transfected with plasmid DNAs as indicated for 48 hrs, which were then treated with CHX for indicated time before Western analysis. For HCT116 cells, stable p38γ depleted and control cells (2) were incubated with CHX and analyzed by Western blot.

Immunoprecipetation and immunoblotting-For immunoprecipitation, cells were harvested and lysed in RIPA buffer containing several phosphatase and protease inhibitors (2,6). Then, the lysates were pre-cleared with protein A or G agarose beads, followed by incubating with the precipitating antibody for overnight. The immune complexes were then washed with RIPA buffer for four times and the pellets were re-suspended in 1 X loading buffer, followed by boiling at 100^{o}C for 5 min. Protein samples from total cell lysates (input) or from immunoprecipitates (IP) were resolved by SDS-PAGE, which were then transferred to nitrocellulose membrane, blocked in 5% nonfat milk, and blotted with the appropriate antibody. The rest procedures were the same as previously described (2,6).

His-tagged protein purification-His-p38α and His-p38γ were purified from IPTG-induced *BL21(DE3)pLysS* according to the Promega manual. Briefly, 250 ml of exponentially growing bacteria was induced with 1mM IPTG for 4 hours at 37°C. After centrifugation, cells were re-suspended in 15 ml of lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM immidazole ph 8.0) and sonicated. Ni-NTA beads were added into the supernatants, which were then incubated on a rotator at 4°C for 1 hour. Thereafter, the mixtures were loaded onto a polypropylene column, followed by washing twice with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM immidazole pH 8.0) and then washing twice with washing buffer (0.5% Triton-X100, 50 mM NaH_2PO_4, 300 mM NaCl, 50 mM immidazole, 5 mM β-mecaptoethnol pH 8.0), which were eluted with the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM immidazole pH 8.0). The elutes were preserved in the buffer containing 20 mM Hepes, 10% glycerol and 150 mM NaCl.

GST-tagged protein purification-GST-PTPH1 and GST-PTPH1/S459A were purified from IPTG-induced BL21(DE3)pLysS based on the protocol described previously. Briefly, exponentially growing bacteria (250 ml) was induced with 0.1 mM IPTG for 2 hours at 30°C. After centrifugation, cells were re-suspended into 15 ml of ice-cold buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 100 μg/ml of lysozyme and incubated on ice for 15min. DTT was then added to final concentration of 5 mM. Thereafter, bacteria was lysed by the addition of N-laurylsarcosine to 1.5% final concentration from a 10% stock. Cells were sonicated on ice for 1 min and the lysate was clarified by centrifugation for 5 min at 4°C to which Triton X-100 was added to the final concentration of 2%. Glutathione agarose beads were then added and the mixture incubated at 4°C on a rotator for 1 hour. Beads were then washed 6-8 times with ice cold PBS, and GST-PTPH1 and GST-PTPH1/S459A were eluted with elution buffer (50 mM Tris-HCl, pH 8.0, and 15 mM reduced glutathione).

Phosphomapping by Mass Spectrometry-The SDS-PAGE gel containing the proteins of interest were identified by a gamma counter, which was then cut and used for in gel digestion, TiO_2 enrichment and LC-MS/MS analysis. The followings are brief description.

In gel Protein Digestion: The gel plugs were washed with 250 μl 50% acetonitrile/50% water for 5 minutes followed by 250 μl of 50 mM ammonium bicarbonate/50% acetonitrile/50% water for 30 minutes. One final wash was done using 10 mM ammonium bicarbonate/50% acetonitrile/50% water for 30 minutes. After washing, the gel plugs were dried in a Speedvac and rehydrated with 0.1μg of trypsin (Promega) per (approximately) $15mm^3$ of gel in 15 μl 10mM ammonium bicarbonate. Samples were digested at 37 °C for 16 hours.

Titanium dioxide enrichment: The digest is acidified with 0.5% TFA, 50% acetonitrile. Top Tips (Glygen Corp.) were prepared by washing three times with 40 μl of each100% acetonitrile, followed by 0.2 M sodium phosphate pH 7.0, and 0.5% TFA, 50% acetonitrile. Washes were spun through into an eppendorf tube at 2,000 rpm for 1 minute. The acidified digest supernatant is loaded into the TopTip, spun at 1,000 rpm for 1 minute, and then 3,000 rpm for 2 minutes. Gel pieces were rinsed with 40 μl

0.5% TFA, 50% acetonitrile, with the supernatant transferred to the Top Tip and the spin repeated. The Top Tip was then washed with 40 µl 0.5% TFA, 50% acetonitrile and the spin repeated. The flow through from these washes were saved and analyzed by LC-MS/MS as below. Phosphopeptides were eluted from the TopTip by three times in 30 µl 28% ammonium hydroxide. Both the flow through and eluted fractions were evaporated to dryness in a speedvac. Samples were dissolved in 3 µl 70% formic acid, vortexed, diluted with 7 µl 0.1% TFA, spun and transferred to LC-MS/MS vials of which 5 µl was injected for mass spectral analysis.

LC-MS/MS on the LTQ Orbitrap MS: Tandem MS experiments were performed on an LTQ Orbitrap equipped with a Waters nanoAcquity UPLC system with a Waters Waters Symmetry® C18 180µm x 20mm trap column and a 1.7 µm, 75 µm x 250 mm nanoAcquity™ UPLC™ column (35ºC) for peptide separation. Trapping was done at 15µl/min, 99% Buffer A (100% water, 0.1% formic acid) for 1 min. Peptide separation was performed at 300 nl/min with Buffer A: 100% water, 0.1% formic acid and Buffer B: 100% CH_3CN, 0.075% formic acid. A linear gradient (51 minutes) was run with 5% buffer B at initial conditions, 50% B at 50 minutes, and 85% B at 51 minutes. Data were acquired in the Orbitrap using 1 microscan, and a maximum inject time of 900 followed by four data dependant MS/MS acquisitions in the ion trap. Multistage activation was used for neutral losses of 98.0, 49.0, 32.7 and 24.5 amu. The data were searched using Mascot Distiller and the Mascot search algorithm.

Data analysis: All MS/MS spectra were searched in-house using the Mascot algorithm (Hirosawa et al, 1993, version 2.2.0) for un-interpreted MS/MS spectra after using the Mascot Distiller program to generate Mascot compatible files. The Mascot Distiller program combines sequential MS/MS scans from profile data that have the same precursor ion. A charge state of +2 and +3 were preferentially located with a signal to noise ratio of 1.2 or greater and a peak list was generated for database searching. Using the Mascot database search algorithm, the Keck Facility considers a protein identified when Mascot lists it as significant and more than two unique peptides match the same protein.

The data were searched against NCBInr, which was chosen over genome specific databases since a match to the correct species has more significance in the larger databases and, for some incomplete

genomes, a match may be found based on homology to another species. The Mascot significance score match was based on a MOWSE score and relies on multiple matches to more than one peptide from the same protein. Parameters used for searching were partial methionine oxidation and carboxamidomethylated cysteine, a peptide tolerance of ± 20 ppm, MS/MS fragment tolerance of ±0.6 Da, and peptide charges of +2 or +3. Normal and decoy database searches were run.

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Supplemental Figure legends:

Figure S1. **p38γ phosphorylates PTPH1 at S459 *in vitro* and *in vivo***. *A, B,* HA-tagged (*A*) or GST-tagged (*B*) PTPH1 and its mutants were incubated with the same amount of bacterially expressed His-p38γ and *in vitro* kinase activity was determined as described (5). Phosphoryted proteins were detected by autoradiography (*A*) or phospho-specific antibody (*B*). *C*, the *in vivo* PTPH1/S459 phosphorylation by Flag-p38γ was performed by the co-expression and Western blot (WB) analysis. *D*, Myc-tagged EGFR was incubated *in vitro* with indicated concentrations of GST-PTPH1 and resultant p-EGFR/Y1173 was detected with a specific antibody (top). Effects of PTPH1 and its S459A mutant on *in vivo* p-EGFR/Y1173 expression were performed by their co-expression in 293T cells, followed by direct WB analysis (bottom). *E,* the sequence alignment analysis shows that S459 is conversed in different species. *F,* p38γ similarly binds and phosphorylates PTPH1 and its catalytic deficient PTPH1/DA mutant (7), as demonstrated by their co-transfection in 293T cells and PTPH1 IP/WB analysis.

Figure S2. **p38γ increases the Ras transformation through stimulating PTPH1/S459 phosphorylation**. *A, B, C,* IEC-6/K-Ras cells were stably expressed with p38γ or p38γ/AGF through G418 selection (*A*) or infected with retrovirus pSUPER to stably deplete endogenous p38γ (*B, C*) (1) and the soft-agar growth was performed (*A, B*) as described previously (2). The colony numbers from 20 fields per 60-mm dish were counted based on the photograph taken about two weeks later. Results shown are mean from three separate experiments with each in triplicate and were expressed as relative to vector controls (mean $\pm$ SD, n=3, * $P < 0.05$ vs. Vector or siLuc; ** $P < 0.05$ vs. p38γ, student's t test). *D*, representative pictures of stained colonies in IEC-6/K-Ras cells stably expressed with PTPH1 or PTPH1/S459A for Fig.2C were shown as compared with the Vector-transfected cells.

Figure S3. **PTPH1 suppresses SOB-induced cell-death and growth-inhibition through S459 and the maximal p38γ activation occurs before the peak of p-PTPH1 expression in response to both SOB and TPA.** *A,* indicated cells were analyzed for cell-death as described in Fig.3D ($\pm$SD, n=3, * P <

0.05 vs. Vector; ** P < 0.05 vs. PTPH1). *B*, indicated cells were treated with ANI or SOB for 24 and 48 h and viable cells were counted and divided by those of their respective untreated controls (Fig.S3A) for the growth inhibition (±SD, n=3, * P > 0.05 vs. PTPH1, ** P < 0.05 vs. PTPH1, and P < 0.05 for all vs. Vector). *C*, protein expression and phosphorylation were analyzed by WB after incubation with and without ANI/SOB as described in Fig.3A. *D, E,* cells were treated with SOB (0.5M) or TPA (200 μg/ml) for 1 h and total PTPH1 or p-p38 proteins were isolated and precipitates were examined for p-p38γ, p-p38α, and p-PTPH1 expression using their specific antibodies. In IEC-6 cells, p38γ and p38α contents from total p-p38 precipitates were shown as relative to 0 h incubation after SOB at different times, whereas the p-PTPH1/S459 level from total PTPH1 precipitates was presented as relative to 0 h (please see the number below IP, and the bands measured by the ImageQuant 5.0 software (*D*). For IEC-6/K-Ras cells (*E*), phosphorylated PTPH1 was expressed as relative to 0 h after normalization to total PTPH1 precipitated, whereas the p-p38γ level is expressed the same as in Fig.S3D.

Figure S4. **p38γ suppresses stress-induced growth-inhibition and/or cell-death.** *A, B,* MEFs were treated with ANI (100 μg/ml) and SOB (0.5M) or a control solvent for 24 h and cell proliferation was performed according to the CyQuant NF cell proliferation assay kit manual (Cat: C35006) as previously described (8). Results shown are mean absorbance from 6 wells of one experiment (± SD) and similar results were obtained from an independent experiment (Figs.4B/D) (* P<0.05 vs. Vector; ** P<0.05 vs. WT or Vector cells). *C*, cells were treated and assessed for cell-death as described in Fig.4E (± SD, n=3). *D,* indicated cells were treated with ANI or SOB for 24 and 48 h, and the growth inhibition was performed and calculated as described in Fig.S3B (±SD, n=3, * P < 0.05 for p38γ+/+ vs. p38γ-/- and for KO p38γ vs. KO Vector; ** P > 0.05 for the same comparisons; recalculated from Fig.4E and Fig.S4C).

Figure S5 **MKK6 or its p38γ fusion protein expression induces endogenous PTPH1/S459 phosphorylation, p38γ stabilizes PTPH1 through PDZ-binding, and S459 is important for PTPH1**

stability. *A*, cells (IEC-6 or MCF-7) were expressed with MKK6 by adenoviral infection (2) or with MKK6-p38γ (9) by the Tet-on system (6) and the resultant p-PTPH1 expression was examined by direct WB. *B, C,* cells were transiently expressed with indicated constructs for 48 h, which were then incubated with CHX (100 μg/ml) for indicated time and examined for protein expression by WB. Results shown are representative from two separate experiments. The number on top of panel *B* and bottom of panel *C* was relative to 0 h with CHX, which was obtained by dividing endogenous PTPH1 (*B*) or transfected HA-PTPH1 (*C*) with that of the corresponding GAPDH band (measured by the ImageQuant 5.0 software). *D*, HCT116 cells with stably p38γ knockdown (shp38γ) or the vector control (shLuc) (2) were analyzed for protein expression by WB after CHX and the number shown at the bottom is relative PTPH1 content (as compared with CHX treatment at 0 h) after normalization to the GAPDH.

Figure S1

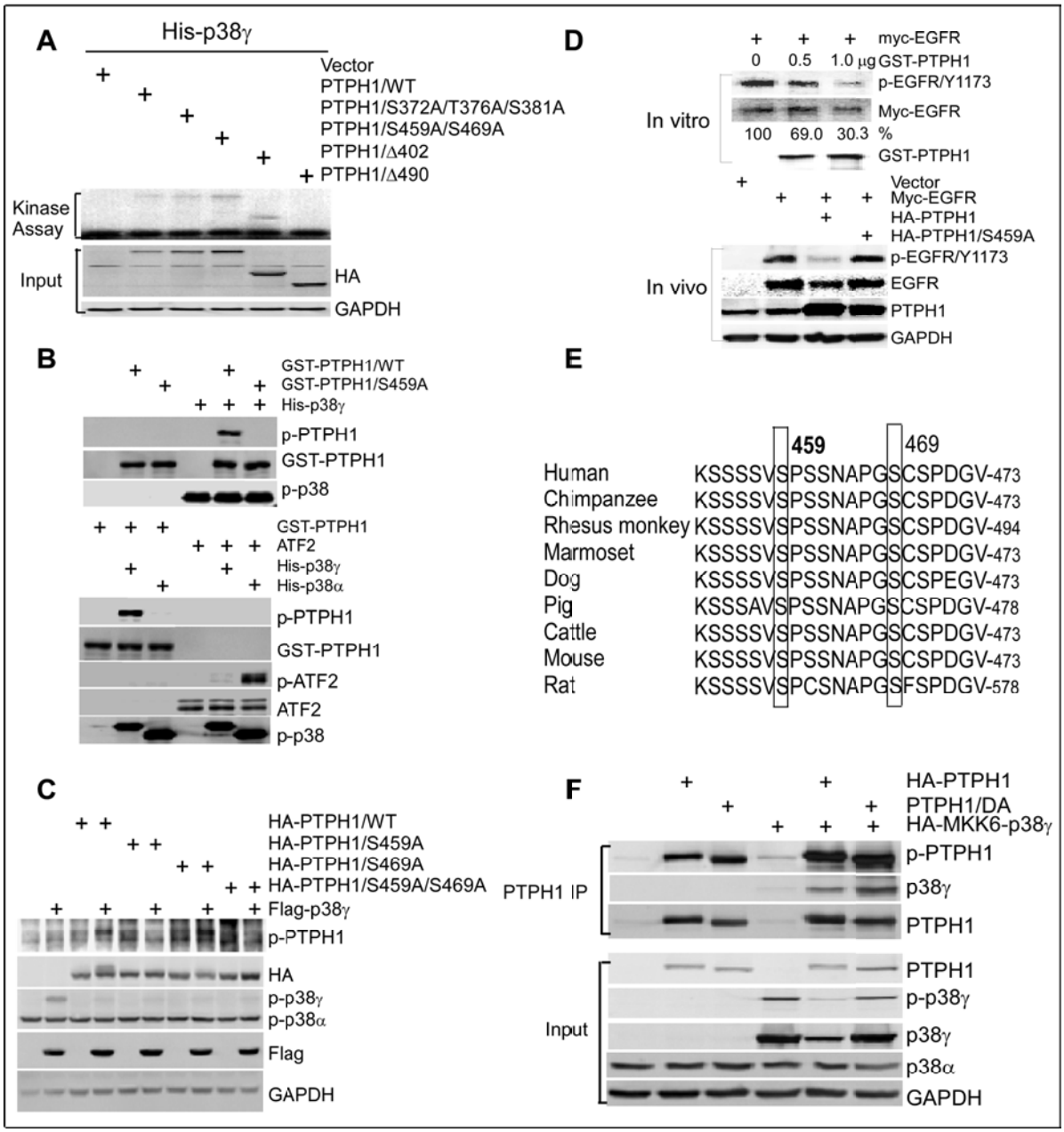
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Figure S2

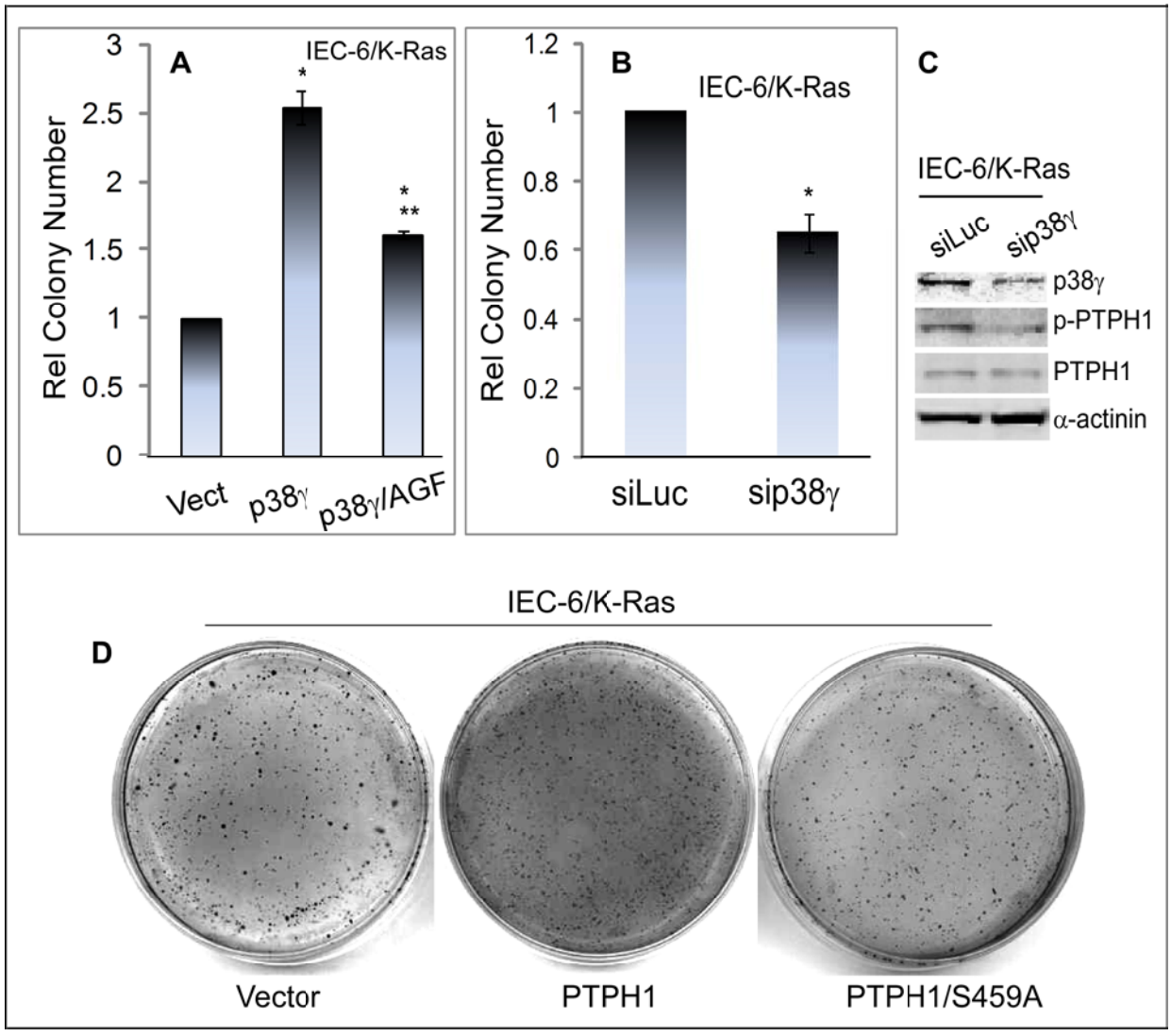
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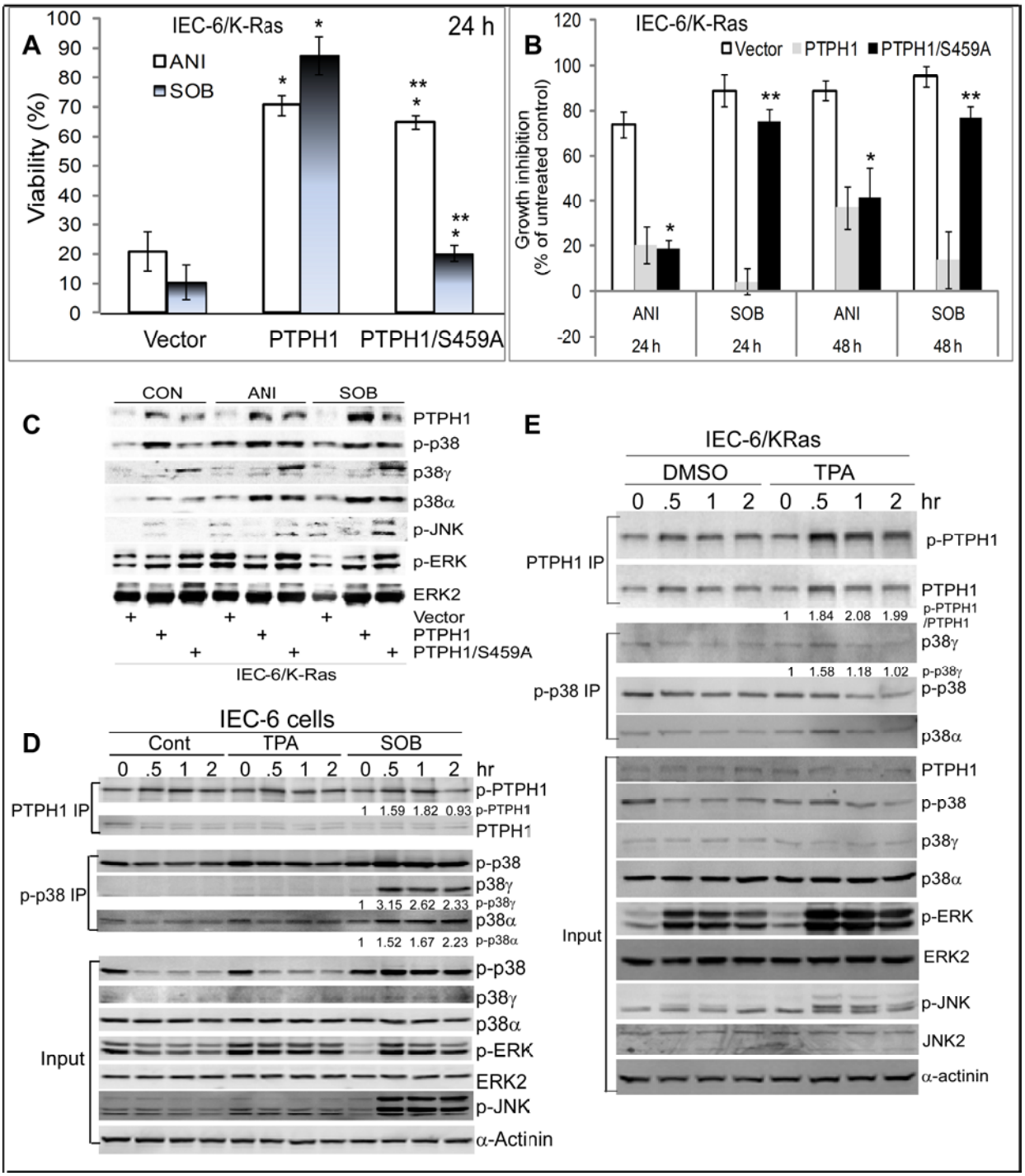
Figure S3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24 h
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SOB
Viability (%)
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PTPH1/S459A
Growth inhibition (% of untreated control)
ANI 24 h
SOB 24 h
ANI 48 h
SOB 48 h
C
CON
ANI
SOB
PTPH1
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p-ERK
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D
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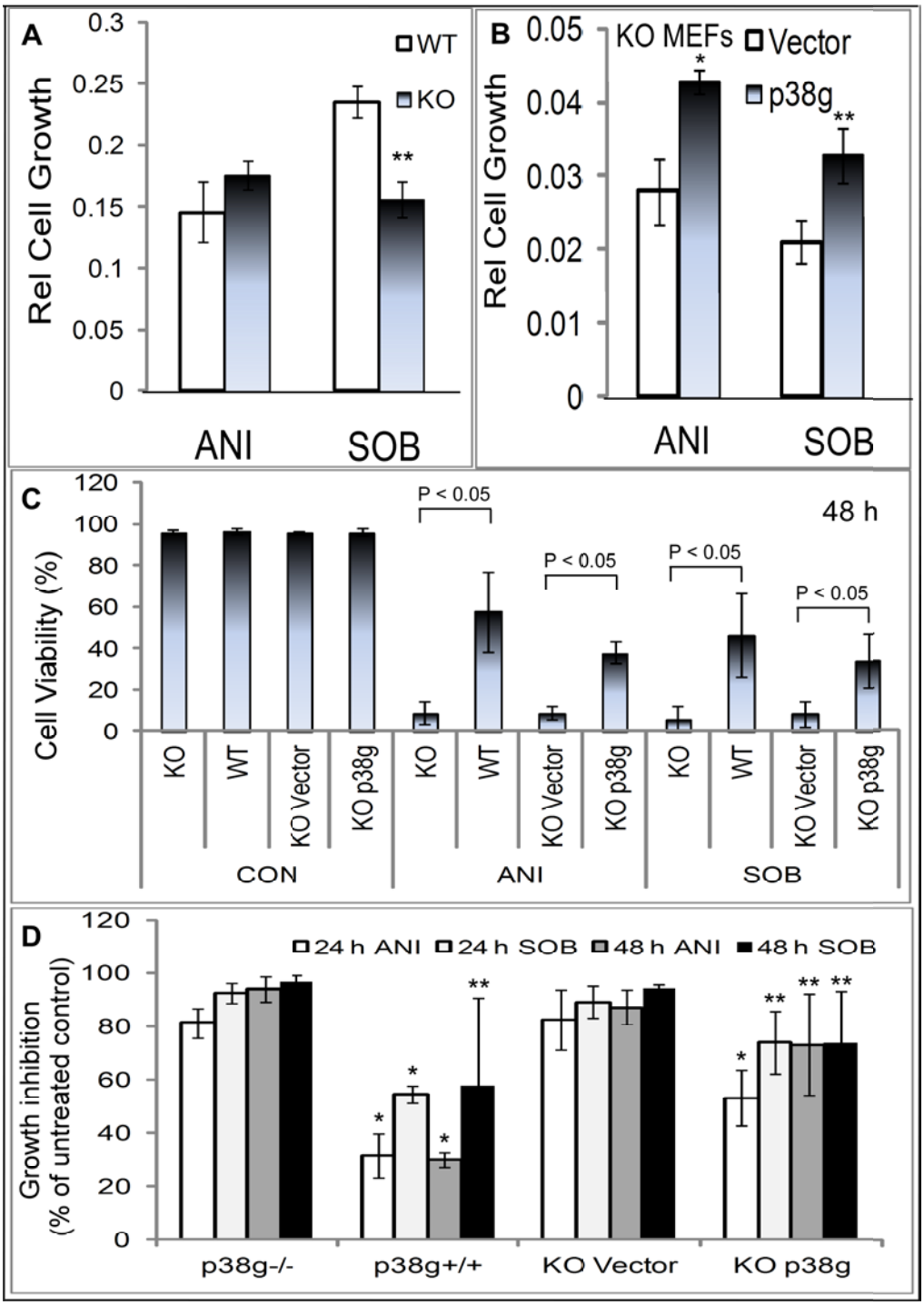
Figure S4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0.04
0.03
0.02
0.01
0
KO MEFs
Vector
p38g
*
**
ANI
SOB
C
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100
80
60
40
20
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P < 0.05
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48 h
KO
WT
KO Vector
KO p38g
CON
ANI
SOB
D
Growth inhibition
(% of untreated control)
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100
80
60
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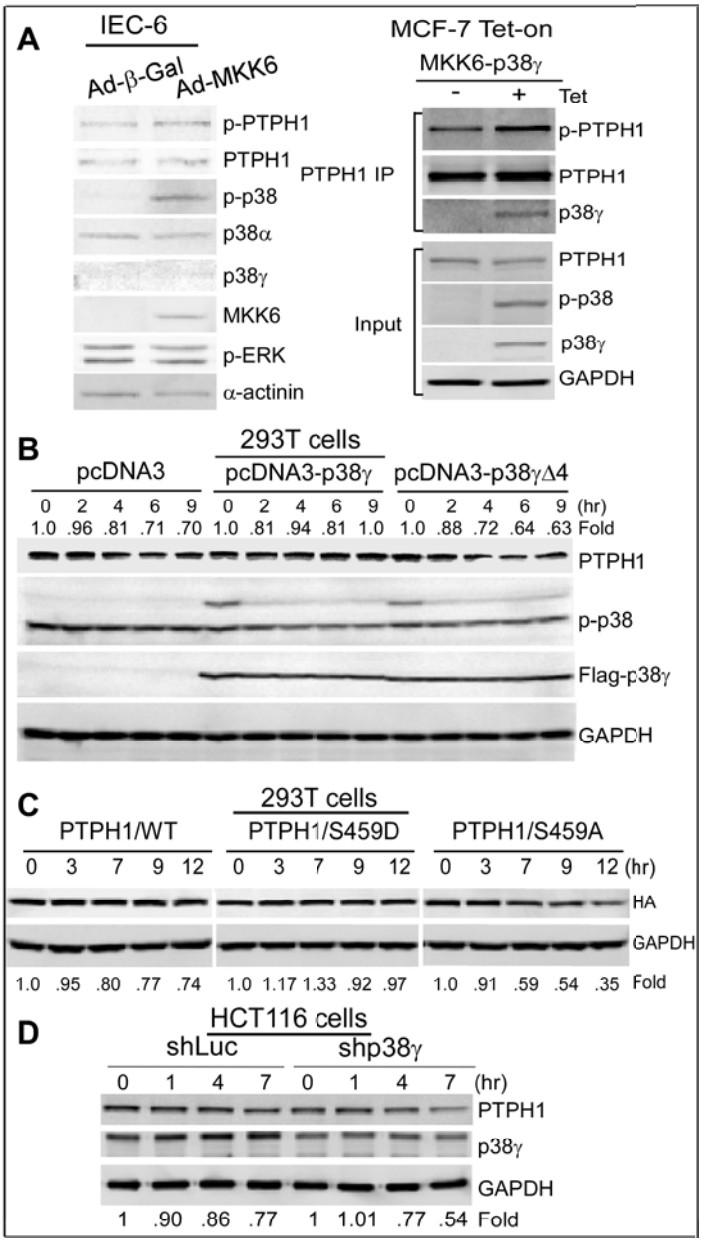
Figure S5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GAPDH
1.0 .95 .80 .77 .74 1.0 1.17 1.33 .92 .97 1.0 .91 .59 .54 .35 Fold
D
HCT116 cells
shLuc
shp38γ
0 1 4 7 0 1 4 7 (hr)
PTPH1
p38γ
GAPDH
1 .90 .86 .77 1 1.01 .77 .54 Fold