Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supplementary Information

Isoform-Specific Optical Activation of Kinase Function Reveals p38-ERK Signaling Crosstalk

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Protocols

Plasmid Construction: General Protocol

Site-directed mutagenesis was performed in a 50 µL reaction volume containing template DNA (1-100 ng), 10 µL of 5x Phusion HF buffer (Thermo Scientific, F530L), 1 µL of dNTP mix (1 mM, each, Thermo Scientific, R0191), 1 µL of both forward and reverse primers (10 µM, each, Sigma Aldrich), and 0.5 µL of Phusion polymerase (2 U/µL, Thermo Scientific, F530L). The reactions included an initial denaturing step (98 °C, 3 minutes), followed by 36 cycles of denaturing (98 °C, 30 s), annealing (melting temperature and melting temperature – 5 °C, 30 s), and elongation steps (72 °C, 1 kbp/30 sec). The thermocycler program was completed with a final elongation (72 °C, 10 minutes) and cooling (12 °C, 5 minutes) steps, and the samples were stored at 4 °C until further use. Template DNA was digested by combining 44 µL of the PCR reaction mixture with 5 µL of 10x CutSmart buffer and 0.5 µL of DpnI (20 U/µL, NEB, R0176S) for 2 hours at 37 °C prior to transformation into Top10 chemically competent cells. Transformants were plated on 10 mL of LB agar supplemented with 10 µL of a 100 mg/mL ampicillin stock and incubated overnight at 37 °C. Three colonies were inoculated into 5 mL of LB broth supplemented with 5 µL of a 100 mg/mL ampicillin stock and grown overnight with shaking (37 °C, 250 rpm), followed by DNA miniprep (Thermo Scientific, K0503). Plasmid sequence was confirmed by Genewiz Sanger sequencing using EF1α forward, EGFP-N, and EGFP-C reverse universal primers (P1−P3, **Supplementary Table S1**).

All Gibson assembly cloning reagents and procedures were performed according to the published Gibson assembly method with the following details.[1] The 5x isothermal reaction buffer was prepared as follows and stored at –20 °C: 25% PEG-8000 (Alfa Aesar), 500 mM Tris-HCl (amresco) pH 7.5, 50 mM $MgCl_2$ (Alfa Aesar), 50 mM DTT (Fisher), 1 mM each of the 4 dNTPs (Fisher), and 5 mM NAD (NEB). The master mix was then prepared as follows and stored at –20 °C in 15 µL aliquots before use: 86 µL of autoclaved MilliQ water, 36 µL of 5x isothermal reaction buffer, 2 µL of Phusion DNA polymerase (2 U/µL Fisher), 0.064 µL of T5 exonuclease (10 U/µL, NEB), and 0.016 µL of Taq ligase (40 U/µL, abm).

For each Gibson assembly reaction, 15 µL of master mix was mixed with backbone and insert fragments with a 1:3 molar ratio (equimolar for all inserts if multiple) and autoclaved MilliQ water to make up to 20 µL. The reaction was carried out at 50 °C for 1 hr before heat-shock transformation (42 °C, 60 s) of 5 µL of Gibson assembly product into 50 µL of chemically competent TOP10 *E. coli* cells. The mixture was then placed on ice for 3 min before addition of 500 µL of SOC media and inoculation at 37 °C in shaker (250 rpm) for 1 hr. Approximately 100 µL of cell culture was then spread evenly on an agar plate containing the desired antibiotic(s). The agar plate was incubated at 37 °C for 16 hrs before colonies were picked for plasmid miniprep and confirmation by Sanger sequencing (Genewiz).

Plasmid Construction: cap38 KTAG Isoform Genes

The site-directed mutagenesis protocol described in the Plasmid Construction: General Protocol section was used to introduce the D176A (P4 and P5, annealing T_m = 62.7 °C, elongation time = 3.5 minutes) and K53TAG (P6 and P7, annealing T_m = 63.4 °C, elongation time = 3.5 minutes) mutations into pGEX-4T-1 3xFLAG-p38α (Addgene, 47575) to generate pGEX-4T-1 3xFLAG-cap38α K53TAG. The D176A (P8 and P9, annealing T_m = 67.3 °C, elongation time = 3.5 minutes) and K53TAG (P10 and P11, annealing T_m = 61.2 °C, elongation time = 3.5 minutes) mutations were introduced into pcDNA-FLAG-p38β2 (Addgene, 20355) to generate pcDNA-FLAG-cap38β K53TAG. The D179A (P12 and P13, annealing T_m = 62.9 °C, elongation time = 3.5 minutes) and K56TAG (P14 and P15, annealing T_m = 59.6 °C, elongation time = 3.5 minutes) mutations were introduced into pcDNA-FLAG-p38γ (Addgene, 20353) to generate pcDNA-FLAG-cap38γ K56TAG. The F324S (P16 and P17, annealing T_m = 65.9 °C, elongation time = 2 minutes) and K54TAG (P18 and P19, annealing T_m = 64.7 °C, elongation time = 2 minutes) mutations were introduced into pCellFree_G03-MAPK13 (Addgene, 67077) to generate pCellFree_cap38δ K54TAG.

Plasmid Construction: pE363_cap38-tagRFP KTAG_4xPylT

The cap38α K53TAG gene was amplified as described in the Plasmid Construction: General Protocol section from pGEX-4T-1 3xFLAG-cap38α K53TAG using primers P20 and P21 (elongation time = 1 minute). The tagRFP-HA gene was amplified from ptagRFP-T-EEA1 (Addgene, 42635) using primers P22 and P23 (elongation time = 1 minute). The backbone was generated by digesting 2000 ng of pE363_4xPylT[2] in a 50 µL reaction with 5 µL of 10x CutSmart buffer and 1 µL each of XbaI (20 U/µL, New England Biolabs, R0145S) and KpnI (20 U/µL, New England Biolabs, R3142S) restriction enzymes (37 °C, 4 h). Following treatment with 1 µL of Antarctic Phosphatase (5 U/µL, New England Biolabs, M0289S; 37 °C, 2 h) and 5 µL of the 10x Antarctic Phosphatase Reaction buffer (New England Biolabs, M0289S), the product was isolated through gel purification of the band corresponding to the correct backbone length (~7600 bp) using the GeneJET Gel Extraction Kit (Thermo Scientific, K0692) following the manufacturer's instructions. DNA fragments were assembled using the Gibson assembly protocol outlined in the Plasmid Construction: General Protocol section to generate pE363_cap38α-tagRFP K53TAG_4xPylT.

The cap38β K53TAG gene was amplified from pcDNA-FLAG-cap38β K53TAG using primers P24 and P25 (elongation time = 1 minute). The tagRFP-HA gene and backbone were generated as described above, and DNA fragments were assembled using the Gibson assembly protocol outlined in the Plasmid Construction: General Protocol section to generate pE363_cap38β-tagRFP K53TAG_4xPylT.

The cap38γ K56TAG gene was amplified from pcDNA-FLAG-cap38γ K56TAG using primers P26 and P27 (elongation time = 1 minute). The tagRFP-HA gene and backbone were generated as described above, and DNA fragments were assembled using the Gibson assembly protocol outlined in the Plasmid Construction: General Protocol section to generate pE363_cap38γ-tagRFP K56TAG_4xPylT.

The cap38δ K54TAG gene was amplified from pCellFree_cap38δ K54TAG using primers P28 and P29 (elongation time = 1 minute). The tagRFP-HA gene and backbone were generated as described above, and DNA fragments were assembled using the Gibson assembly protocol outlined in the Plasmid Construction: General Protocol section to generate pE363_cap38δ-tagRFP K54TAG_4xPylT.

Plasmid Construction: pcDNA_EF1α-cap38-tagRFP KTAG_4xPylT

Using the Gibson assembly protocol outlined in the Plasmid Construction: General Protocol section, the EF1α_cap38α-tagRFP K53TAG_puroR gene was amplified from pE363_cap38α-tagRFP K53TAG_4xPylT using primers P30 and P31 (elongation time = 2 minutes) and cloned into the pcDNA3.1[3] backbone (amplified with P32 and P33, elongation time = 1.5 minutes) for assembly of pcDNA_EF1α-cap38α-tagRFP K53TAG_4xPylT. This was repeated using the same primers to amplify: the EF1α_cap38β-tagRFP K53TAG_puroR gene from pE363_cap38β-tagRFP K53TAG_4xPylT for assembly of pcDNA_EF1α-cap38β-tagRFP K53TAG_4xPylT; the EF1α_cap38γ-tagRFP K56TAG_puroR gene from pE363_cap38γ-tagRFP K56TAG_4xPylT for assembly of pcDNA_EF1α-cap38γ-tagRFP K56TAG_4xPylT; and the EF1α_cap38δ-tagRFP K54TAG_puroR gene from pE363_cap38δ-tagRFP K54TAG_4xPylT for assembly of pcDNA_EF1α-cap38δ-tagRFP K54TAG_4xPylT.

Plasmid Construction: pE363_cap38-tagRFP KTAG_4xPylT_hPGK-ERK KTR Clover

The ERKKTRClover-WPRE gene was amplified from pLentiCMV Puro DEST ERKKTRClover (Addgene, 59150) using primers P34 and P35 (elongation time = 1 minute). The hPGK gene was amplified from pLentiPGK Puro DEST p38KTRClover (Addgene, 59152) using primers P36 and P37 (elongation time = 1 minute).

The cap38α-tagRFP K53TAG_4xPylT backbone was generated by digesting 2000 ng of pE363_cap38α-tagRFP K53TAG_4xPylT in a 50 µL reaction with 5 µL of 10x CutSmart buffer and 1 µL of EcoRV-HF (20 U/µL, New England Biolabs, R3195S) restriction enzyme (37 °C, 4 h).

The cap38βγ-tagRFP K56TAG_4xPylT backbone was generated by digesting 2000 ng of pE363_cap38γ-tagRFP K56TAG_4xPylT in a 50 µL reaction with 5 µL of 10x CutSmart buffer and 1 µL of EcoRV-HF (20 U/µL, New England Biolabs, R3195S) restriction enzyme (37 °C, 4 h).

The cap38δ-tagRFP K54TAG_4xPylT backbone was generated by digesting 2000 ng of pE363_cap38δ-tagRFP K54TAG_4xPylT in a 50 µL reaction with 5 µL of 10x CutSmart buffer and 1 µL of EcoRV-HF (20 U/µL, New England Biolabs, R3195S) restriction enzyme (37 °C, 4 h).

Following treatment of each solution with 1µL of Antarctic Phosphatase (5 U/µL, New England Biolabs, M0289S; 37 °C, 2 h) and 5 µL of 10x Antarctic Phosphatase Reaction buffer (New England Biolabs, M0289S), the corresponding product was isolated through gel purification of the band corresponding to the correct backbone length (~9500 bp) using the GeneJET Gel Extraction Kit (Thermo Scientific, K0692) following the manufacturer's instructions. DNA fragments were assembled using the Gibson assembly protocol outlined in the Plasmid Construction: General

Protocol section to generate pE363_cap38α-tagRFP K53TAG_4xPylT_hPGK-ERK KTR Clover, pE363_cap38γ-tagRFP K56TAG_4xPylT_hPGK-ERK KTR Clover, and pE363_cap38δ-tagRFP K54TAG_4xPylT_hPGK-ERK KTR Clover.

Plasmid Construction: pE363_cap38β-tagRFP K53TAG_4xPylT_hPGK-ERK KTR Clover

Because of an internal cut site recognized by the restriction enzyme, EcoRV-HF, within the cap38β gene, this construct was cloned using restriction ligation cloning in place of Gibson assembly. The pE363_4xPylT_hPGK-ERK KTR Clover backbone was generated by digesting 2000 ng of pE363_cap38δ-tagRFP K54TAG_4xPylT_hPGK-ERK KTR Clover in a 50 µL reaction with 5 µL of 10x CutSmart buffer and 1 µL each of NheI-HF (20 U/µL, New England Biolabs, R3131S) and SalI-HF (20 U/µL, New England Biolabs, R3138S) restriction enzymes (37 °C, 4 h). Simultaneously, the cap38β-tagRFP K54TAG insert was generated using the same restriction digest protocol to digest 2000 ng of pE363_cap38 β -tagRFP K56TAG_4xPylT. Following treatment of each solution with 1µL of Antarctic Phosphatase (5 U/µL, New England Biolabs, M0289S; 37 °C, 2 h) and 5 µL of 10x Antarctic Phosphatase Reaction buffer (New England Biolabs, M0289S), the corresponding products were isolated through gel purification of the band corresponding to the correct backbone length (~9200 bp) and insert length (~2500 bp) using the GeneJET Gel Extraction Kit (Thermo Scientific, K0692) following the manufacturer's instructions. The resulting DNA was then combined in a PCR tube, such that a 1:3 molar ratio of backbone:insert yielded 8.5 total microliters. Following treatment with 1 µL of T4 DNA Ligase Buffer (New England Biolabs M0203S) and 0.5 µL of T4 DNA ligase (New England Biolabs M0203S, room temperature, 1 h), the ligation mixture (5 µL) was transformed into chemically competent TOP10 cells and DNA isolated and sequenced as described above.

Decaging Assay

Solutions of Fmoc-HCK or Fmoc-lysine (50 µM) were made in water with 2% DMSO. The Fmoc-HCK sample was irradiated with 365 nm light (VWR UV Dual Transilluminator, 25 mW/cm^2) for various durations (0, 1, 2, 5, or 10 min). Following irradiation, each sample (10 µL) was analyzed by HPLC (**Fig. S1**) on a Zorbax StableBond C18 column (4.6 mm x 150 mm, 80 Å, 5 µm) using a gradient of 5 – 95% acetonitrile (0.1% TFA) in water (0.1% TFA), a flow rate of 1.1 mL/min, and an detection wavelength of 280 nm.

Cell Culture

All cell culture experiments were performed in a sterile laminar flow hood. NIH3T3 cells were maintained in antibiotic-free Dulbecco's Modified Eagle Medium (DMEM, Gibco, SH30003.03) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, F0926) at 37 °C with 5% CO_2. NIH3T3^{HCK} cells were maintained in DMEM supplemented with 1 mg/mL G-418 (VWR, 97063-060) and 10% (v/v) fetal bovine serum (complete medium, Sigma-Aldrich, F0926) at 37 °C with 5% CO_2. Cells were used between passage number 3 and 26 and tested for mycoplasma contamination every six months (Genlantis, MY01100).

Stable Cell Line Generation

Generic NIH3T3 cells were transfected with the pE323_chimeric HCKRS_4xPylT plasmid and the pcDNA_PiggyBac transposase plasmid in 6-well format for 36 hours prior to resuspension and 1:6 dilution to fresh media (DMEM with 10% fetal bovine serum). After 1:6 dilution to fresh media, cells were incubated at 37 °C, 5% CO2 for 48 hours before antibiotic selection. NIH3T3 cells successfully integrated with the desired cassette was selected using G-418 (Geneticin, 108321-42), with final concentrations of 0, 0.25, 0.5, 1, 2, and 5 mg/mL. After 2 weeks with selection media change every 2 days, cells continued to grow in media containing 1 mg/mL G-418 while all cells died when supplemented with 2 mg/mL G-418. For generation of monoclonal stable cell lines, the cells selected by 1 mg/mL G-418 were resuspended, diluted to 1 cell/100 µL of media (with 1 mg/mL G-418), and plated in 96-well plates with 100 µL of cell suspension per well. The cells were then incubated until ~90% confluent and those wells with sufficient cell growth were duplicated, with one plate preserved for passaging and the other plate transfected with pE363_mCherry-TAG-EGFP_4xPylT to screen for incorporation efficiency of HCK. A pool of 12 wells of cells with high EGFP/mCherry ratio and no obvious change in cell morphology were selected to be probed for the expression levels of FLAG-tagged chimeric HCKRS by western blots (data not shown). While these monoclonal cell lines were being propagated for further validation, five of them failed to proliferate efficiently under the constant selection pressure of 1 mg/mL G-418. Thus, the rest seven candidate cell lines were probed for PylT expression levels by qPCR of reverse transcribed cDNA (complementary DNA), using published primers[4] and the endogenous tRNA coding for methionine ($tRNA_{met}$) as control (data not shown). All seven candidate cell lines showed varied levels of PylT while the generic NIH3T3 cell line did not show expression of PylT. Of these candidate stable cell lines, the one with the highest incorporation efficiency of HCK (EGFP/mCherry ratio), highest expression level of FLAG-chimeric HCKRS (anti-FLAG/anti-GAPDH ratio), and the highest PylT expression level (PylT/$tRNA_{met}$ ratio) was then expanded, cryopreserved, and named as $NIH3T3^{HCK}$.

Cell Viability Assay

$3T3^{HCK}$ cells were plated at 2,000 cells per well in a clear 384-well plate (Greiner, 781182) with varying concentrations of doxorubicin (positive control) or hydroxycoumaryl alcohol (2% DMSO) in 40 µL of media (DMEM with 10% fetal bovine serum) in triplicate. After a 72-hour incubation, 16 µL of activated XTT reagent (8 µL of 1.7 mg/mL menadione diluted into 1 mL XTT reagent solution) to each well. Absorbance was measured at 450 nm and 630 nm (Tecan M1000 plate reader) immediately following addition and again after 2 hours. The background absorbance was subtracted from each well, then absorbance was normalized to a DMSO-containing control (**Fig. S2**).

Western Blotting

$NIH3T3^{HCK}$ cells were seeded into a 24-well plate (50,000 cells/well, Greiner, 665180) in 500 µL of complete medium. When cells reached ~90% confluency, the media in each well was replaced with 450 µL of fresh antibiotic-free DMEM. The media was supplemented with 0.25 mM hydroxycoumarin lysine (HCK)[5] for the appropriate wells in the following ratio: 5 µL of stock HCK solution (25 mM HCK in DMSO) into 445 µL of antibiotic-free DMEM, from a master mix of 12 mL. The cells in each well were transfected by combining 500 ng of pcDNA-EF1α-cap38α-tagRFP K53TAG, pcDNA-EF1α-cap38β-tagRFP K53TAG, pcDNA-EF1α-cap38γ-tagRFP K56TAG, or pcDNA-EF1α-cap38δ-tagRFP K54TAG with 500 ng of pE323_chimeric HCKRS_4xPylT and 2 µL

of P3000 reagent (Thermo Scientific, L3000001) and brought to 25 µL with OptiMEM (Thermo Scientific, 22600050) in a 1.7 mL microcentrifuge tube (Laboratory Products Sales, L211511). In a separate 1.7 mL tube, 2 µL of Lipofectamine 3000 (Thermo Scientific, L3000001) was combined with 23 µL of OptiMEM. The DNA mixture was added dropwise to the Lipofectamine 3000 solution, mixed by pipetting, and incubated for 20 minutes at room temperature. The mixture was added dropwise to the media of each replicate well (supplemented with HCK when necessary as described above), and the cells were incubated for 36 hours at 37°C.

For analysis of photocaged protein expression (**Fig. 2B**), each the transfection for each construct was duplicated and applied to cells in antibiotic-free DMEM in the absence or presence of HCK (prepared as described above). Following a 36-hour incubation at 37°C, the cells were washed with 250 µL of ice-cold PBS to remove residual medium, placed on ice, and lysed with 48 µL of ice-cold GE Healthcare mammalian protein extraction buffer (GE Healthcare, 28-9412-79) supplemented with 6 µL of 100x protease inhibitor cocktail (Thermo Scientific, 78429) and 6 µL of 100x EDTA (Thermo Scientific, 78429), with 250 RPM shaking for 20 minutes. Lysates were transferred to 1.7 mL microcentrifuge tubes (Laboratory Products Sales, L211511). Cell debris was pelleted by centrifugation at 21000 g for 20 minutes at 4 °C and supernatant (50 µL) was combined with 10 µL of 6x Laemmli SDS Sample buffer (375 mM Tris-HCl, pH 6.5, 9% (w/v) SDS, 50% (v/v) glycerol, 0.03% Brilliant Blue G; freshly mixed 9:1 with β-mercaptoethanol) and heated at 95 °C for 10 minutes. Samples were separated by 10% (v/v) SDS-PAGE gel electrophoresis (60 V for 30 minutes, 150 V for 1 hour) in an ice water bath, followed by protein transfer to a 0.45 µm PDVF membrane (Millipore, IPVH00010) at 90 V for 90 minutes using ice-cold Transfer Buffer (25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, pH = 8.3). The membrane was blocked for 1 hour at room temperature with 4 mL of blocking buffer (5% BSA in TBS with 0.1% (v/v) Tween 20, TBST) while rocking. Blots were probed with rabbit pAb anti-HA (1:1000 dilution, CST 3724S) and rabbit pAb anti-GAPDH (1:1000 dilution, ProteinTech 50-172-6351) primary antibodies in 4 mL of TBST overnight with rocking at 4 °C. After washing five times with 5 mL of ice-cold TBST, membranes were incubated for 1 hour at room temperature with goat anti-rabbit IgG HRP-linked secondary antibody (1:10000 dilution, Cell Signaling 7074S) in 4 mL of TBST. After washing five times with 5 mL of ice-cold TBST, blots were developed with SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, 34580) by mixing 2 mL of the luminol/enhancer solution with 2 mL of the peroxide solution and incubating the membrane in the resulting solution for 3 minutes at room temperature with rocking. The blots were imaged on a BioRad ChemiDoc system with automated exposure times.

For analysis of light-induced p38 activation (**Fig. 2C**), each construct was transfected in quadruplicate for timecourse analysis of pATF-2 generation. Following a 12-hour incubation with DMEM, 10% FBS, the cells were washed once by aspirating cell culture media and incubating the cells in 500 µL of PBS at 37 °C and 5% CO_2 for ten minutes. To serum starve, fresh FluoroBrite DMEM (500 µL, Gibco, A1896701) was applied. The media was supplemented with 0.25 mM HCK for the appropriate wells by diluting 5 µL of stock HCK solution into 495 µL of FluoroBrite DMEM. After the cells were incubated for 24 hours at 37 °C with 5% CO_2, they were washed once by incubating in 500 µL of PBS at 37 °C for ten minutes, and 500 µL of fresh, HCK-free FluoroBrite DMEM was applied. Cells were either kept in the dark or treated with 365 nm light (VWR UV Dual Transilluminator, 25 mW/cm^2) for 3 minutes either 60, 30, or 5 minutes prior to lysis. The plate was partially covered with foil to shield nonirradiated samples from light. Samples treated with light were irradiated from the bottom of the plate. The cells were washed with 500 µL of ice-cold PBS to remove residual medium, placed on ice, and lysed with 60 µL of ice-cold GE Healthcare mammalian protein extraction supplemented with protease inhibitor cocktail, 5 mM EDTA, and phosphatase cocktail (2 mM sodium fluoride, 1.125 mM sodium molybedate, 2 mM sodium orthovanadate, 4 mM sodium tartrate, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate)

with 250 RPM shaking for 20 minutes. Lysates were transferred to 1.7 mL microcentrifuge tubes. Cell debris was pelleted by centrifugation at 21000 g for 20 minutes at 4 °C and supernatant (50 µL) was combined with 10 µL of 6x Laemmli SDS Sample buffer and heated at 70 °C for 10 minutes. Samples were separated, transferred, and blocked as described above. Blots were probed with rabbit mAb anti-phospho-ATF-2 (1:1000 dilution, CST 27934T) and rabbit pAb anti-GAPDH (1:1000 dilution, ProteinTech 50-172-6351) primary antibodies in 4 mL of TBST overnight with rocking at 4 °C. The secondary antibody application, membrane development, and blot imaging was completed as described above. After imaging, the anti-phospho-ATF-2 blot was stripped by rocking the blot at room temperature with 4 mL of stripping buffer (200 mM glycine, 3.5 mM SDS, 1% (v/v) Tween 20, pH 2.2). The membrane was washed five times with 5 mL of ice-cold TBST and blocked as described previously. Blots were probed with rabbit mAb anti-ATF-2 (1:1000 dilution, CST 82870S) primary antibody in 4 mL of TBST overnight with rocking at 4 °C. The secondary antibody application, membrane development, and blot imaging was completed as described above.

Kinase Translocation Reporter Assay

For p38α-dependent suppression of ERK signaling (**Fig. 4** and **Fig. S5**) NIH3T3^{HCK} cells were seeded into an 8-well chambered glass slide (Cellvis, C8-1.5H-N, 30,000 cells/well) in 250 µL of complete medium. When cells reached ~90% confluency, the media in each well was replaced with 230 µL of antibiotic-free DMEM. The media was supplemented with 0.25 mM HCK for the appropriate wells in the following ratio: 2.5 µL of stock HCK solution (25 mM in DMSO) into 227.5 µL of antibiotic-free DMEM, from a master mix of 12 mL. Each well was transfected by combining 200 ng of pE363_cap38α-tagRFP K53TAG_4xPylT_hPGK-ERK KTR Clover and 0.8 µL of P3000 (Thermo Scientific, L3000001) and brought to 10 µL with OptiMEM (Thermo Scientific, 22600050) in a 1.7 mL microcentrifuge tube (Laboratory Products Sales, L211511). In a separate 1.7 mL tube, 0.8 µL of Lipofectamine 3000 (Thermo Scientific, L3000001) was combined with 9.2 µL of OptiMEM. The DNA mixture was added dropwise to the Lipofectamine 3000 solution, mixed by pipetting, and incubated for 20 minutes at room temperature. The mixture was added dropwise to the media of each replicate well, and the cells were incubated at 37°C. This transfection was repeated in duplicate for expression in the absence or presence of HCK. Following a 36-hour incubation, the cells were washed by incubating in 200 µL of phosphate buffered saline (PBS) at 37 °C for ten minutes. To serum starve, fresh FluoroBrite DMEM (250 µL) was applied. The media was supplemented with 0.25 mM HCK for the appropriate wells by diluting 2.5 µL of stock HCK solution into 247.5 µL of FluoroBrite DMEM. After the cells were incubated for 4 hours at 37 °C with 5% CO_2, they were washed once by incubating with 250 µL of PBS at 37 °C for ten minutes, and 225 µL of fresh, HCK-free FluoroBrite DMEM was applied. Following a 20-minute incubation period, cells were treated with light from the bottom of the well using the Zeiss Axio Observer Z1 microscope DAPI filter (49, Ex. G 365; Em. BP 445/50) for 2 seconds (Φ = 4.6 x 10^{13} s^{-1}). Within 25 minutes of irradiation, cells were stimulated by diluting 25 µL of a 1 µg/mL stock of FGF (Gibco, PHG0264) directly into media containing cells for a final concentration of 100 ng/mL FGF. Cells were imaged at intervals using a Tokai Hit (Inu) on a Zeiss Axio Observer Z1 with the LD Plan-Apochromat 20X objective and both the FITC (Chroma, Ex. BP 470/40, Em. BP 525/50) and dsRed (43 HE, Ex. BP 550/25, Em. BP 605/70) filter sets. Images were exported in TIFF format using ZEN 2 Blue Edition (Zeiss) imaging software. Image processing for ERK KTR Clover localization and C/N quantification was completed using ImageJ (National Institutes of Health) software package. C/N ratios were determined by measuring the mean fluorescence intensity of a representative area (between 200 and 1000 µm^2) within the cellular regions of interest and averaging individual cellular C/N values. All images generated for KTR analysis were exported by Zen 2 Blue Edition with the same low and high thresholds, with the high threshold being higher than the intensity of the brightest pixel in the region of interest.

For p38-dependent activation of p38 or ERK signaling (**Fig. 3** and **Fig. S4**), NIH3T3 cells were seeded into an 8-well chambered glass slide in 250 µL of antibiotic-free DMEM. When cells reached ~90% confluency, the media in each well was replaced with 230 µL of antibiotic-free DMEM, supplemented with 0.25 mM HCK by diluting 2.5 µL of stock HCK solution into 227.5 µL of antibiotic-free DMEM. Each well was transfected by combining 80 ng of pE363_cap38-tagRFP KTAG_4xPylT (α, K53TAG; β, K53TAG; γ, K56TAG; δ, K54TAG), with 80 ng of pE323_ chimeric HCKRS_4xPylT, 20 ng of either pLenti-CMV Puro DEST ERK KTR Clover or pLenti-PHK Puro DEST p38 KTR Clover, and 0.4 µL of P3000 reagent (Thermo Scientific, L3000001) and brought to 10 µL with OptiMEM (Thermo Scientific, 22600050) in a 1.7 mL microcentrifuge tube (Laboratory Products Sales, L211511). In a separate 1.7 mL tube, 0.4 µL of Lipofectamine 3000 (Thermo Scientific, L3000001) was combined with 9.6 µL of OptiMEM. The DNA mixture was added dropwise to the Lipofectamine 3000 solution, mixed by pipetting, and incubated for 20 minutes at room temperature. The mixture was added dropwise to the media of each replicate well, and the cells were incubated at 37°C. Following a 36-hour incubation, the cells were washed and serum starved as described above. Upon the start of the timecourse imaging experiment and following an initial 10-minute incubation period, the cells were irradiated and imaged as described above. Images were exported in TIFF format by SlideBook 6 imaging software. Imaged processing was completed as described above.

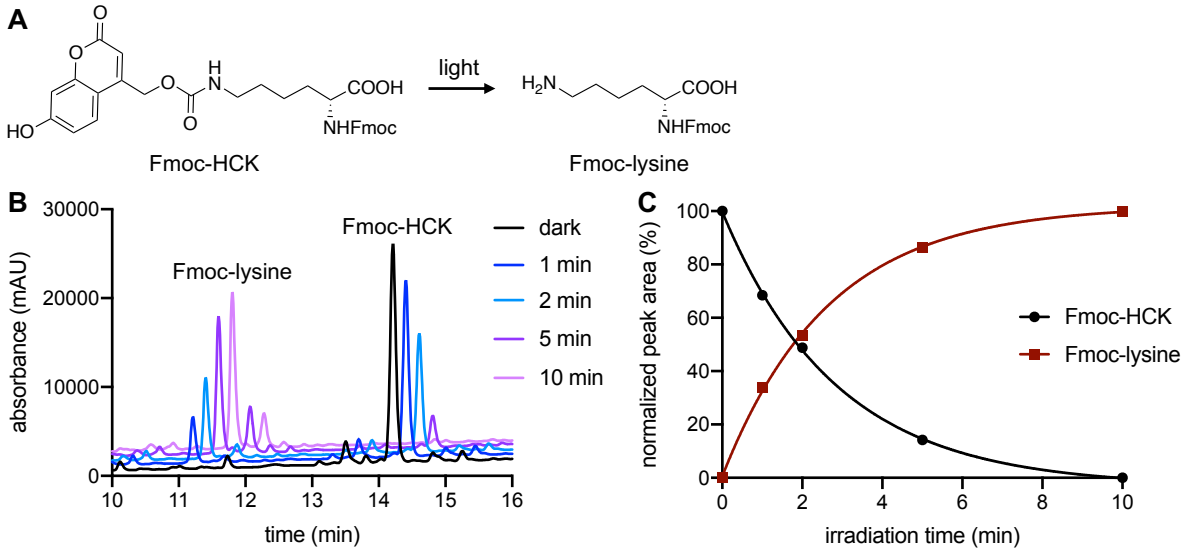
For analysis of cap38γ/δ-dependent activation of ERK signaling in the presence of ERK/MAPK pathway inhibitors (**Fig. 6**), NIH3T3 cells were seeded into an 8-well chambered glass slide in 250 µL of antibiotic-free DMEM. When cells reached ~90% confluency, the media in each well was replaced with 230 µL of antibiotic-free DMEM, supplemented with 0.25 mM HCK by diluting 2.5 µL of stock HCK solution into 227.5 µL of antibiotic-free DMEM. Each well was transfected by combining 80 ng of pE363_cap38-tagRFP KTAG_4xPylT (γ, K56TAG; δ, K54TAG), with 80 ng of pE323_ chimeric HCKRS_4xPylT, 20 ng of pLenti-CMV Puro DEST ERK KTR Clover, and 0.4 µL of P3000 reagent (Thermo Scientific, L3000001) and brought to 10 µL with OptiMEM (Thermo Scientific, 22600050) in a 1.7 mL microcentrifuge tube (Laboratory Products Sales, L211511). In a separate 1.7 mL tube, 0.4 µL of Lipofectamine 3000 (Thermo Scientific, L3000001) was combined with 9.6 µL of OptiMEM. The DNA mixture was added dropwise to the Lipofectamine 3000 solution, mixed by pipetting, and incubated for 20 minutes at room temperature. The mixture was added dropwise to the media of each replicate well, and the cells were incubated at 37°C. Following a 36-hour incubation, the cells were washed and serum starved as described above. Prior to the start of the timecourse imaging experiment, cells were incubated with DMSO or small molecule inhibitors for Raf (LY3009120, 1 µM), MEK (U0126, 10 µM), or ERK (SCH772984, 1 µM) for 30 minutes at 37 °C. Upon the start of the timecourse imaging experiment and following an initial 10-minute incubation period, the cells were irradiated and imaged as described above. Images were exported in TIFF format by SlideBook 6 imaging software. Imaged processing was completed as described above.

Immunofluorescence Imaging

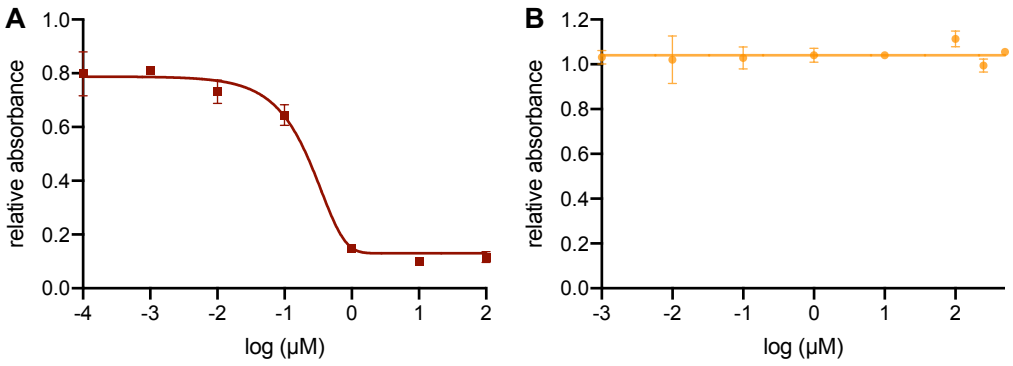
For immunofluorescence detection of ERK activation upon p38γ/δ decaging (**Fig. 5** and **Fig. S6**), NIH3T3 cells were seeded into a polyD-lysine treated black, clear-bottom, 96-well plate (15,000 cells/well) in 100 µL of DMEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (Corning, 30002CI). When cells reached 80-90% confluency, the media in each well was replaced with 90 µL of antibiotic-free DMEM. The media was supplemented with 0.5 mM HCK for the appropriate wells by diluting 1 µL of stock solution (50 mM HCK in DMSO) into 89 µL of antibiotic-free DMEM. Each well was transfected by combining 100 ng of pE363_cap38-tagRFP KTAG_4xPylT (γ, K56TAG; δ K54TAG) or pmCherry-HA with 100 ng of

pE323 PylRS and 0.4 µL of P3000 reagent (Thermo Scientific, L3000001) and brought to 10 µL with OptiMEM (Thermo Scientific, 22600050) in a 1.7 mL microcentrifuge tube (Laboratory Products Sales, L211511). In a separate 1.7 mL tube, 0.4 µL of Lipofectamine 3000 (Thermo Scientific, L3000001) was combined with 9.6 µL of OptiMEM. The DNA mixture was added dropwise to the Lipofectamine 3000 solution, mixed by pipetting, and incubated for 20 minutes at room temperature. The mixture was added dropwise to the media of each replicate well, and the cells were incubated at 37°C. Following a 36-hour incubation, the cells were washed three times by incubating in 100 µL of PBS at 37 °C for ten minutes. To serum starve, fresh Live Cell Imaging Solution (100 µL, Invitrogen, A14291DJ) was applied and cells were incubated for 5 hours at 37 °C with 5% CO_2. The cells were treated with or without light from the top of the well using a 405 nm LED for 1 minute. Following a 60-minute incubation period, the cells were washed three times with 100 µL of ice-cold PBS to remove residual medium and fixed with an ice-cold 1% paraformaldehyde (PFA) solution in PBS for 15 minutes on ice. After washing three times with 100 µL of PBS, the cells were permeabilized with 50 µL of 100% methanol for 10 minutes at −20 °C. Following another three washes with 100 µL of PBS, the cells were blocked for 1 hour at room temperature with 100 µL of blocking buffer (5% goat serum and 0.3% Triton X-100 in PBS). After another three washes with 100 µL of PBS, cells were simultaneously probed with rabbit pAb anti-HA (1:1000 dilution, CST 3724S) and mouse mAb anti-phospho-ERK1/2 (1:200 dilution, CST 5726S) primary antibodies in 100 µL of antibody dilution buffer (1% BSA and 0.3% Triton X-100 in PBS) overnight at 4 °C. After washing three times with 100 µL of PBS, membranes were incubated for 1 hour at room temperature simultaneously with goat anti-rabbit IgG Alexa fluor 488 (1:1000 dilution, Invitrogen A11008) and goat anti-mouse IgG Alexa fluor 647 (1:1000 dilution, Invitrogen A21235) secondary antibodies in 100 µL of antibody dilution buffer while protected by light. Following three washes with 100 µL of PBS, cells were incubated with 50 µL of Hoechst 3342 (Thermo Fisher Scientific) at room temperature for 5 minutes. The cells were washed four final times with 100 µL of PBS before being imaged using the CellInsight CX5 High Content Screening Platform with the 10X Olympus objective (0.30NA) and both the green (Ex. 485, Em. 521) and deep red (Ex. 650, Em. 694) channels. The associated HCS Studio software was used to complete the imaging experiment. The mCherry-HA control data was plotted by cell size and mean fluorescence intensity of Alexa 488 (detecting mCherry expression through recognition of the HA epitope) and arbitrary limits were set to select single cells (**Fig. S6A**) transiently expressing the protein of interest (**Fig. S6B**). These limits were applied to the p38δ and p38γ data sets and raw fluorescence values were sorted by mean fluorescence intensity of Alexa 488 (detecting p38 expression through recognition of the HA epitope). The mean fluorescence intensity of Alexa 647 (detecting phospho-ERK1/2) within the population of cells displaying high Alexa 488 intensity was plotted and compared between −UV and +UV replicates.

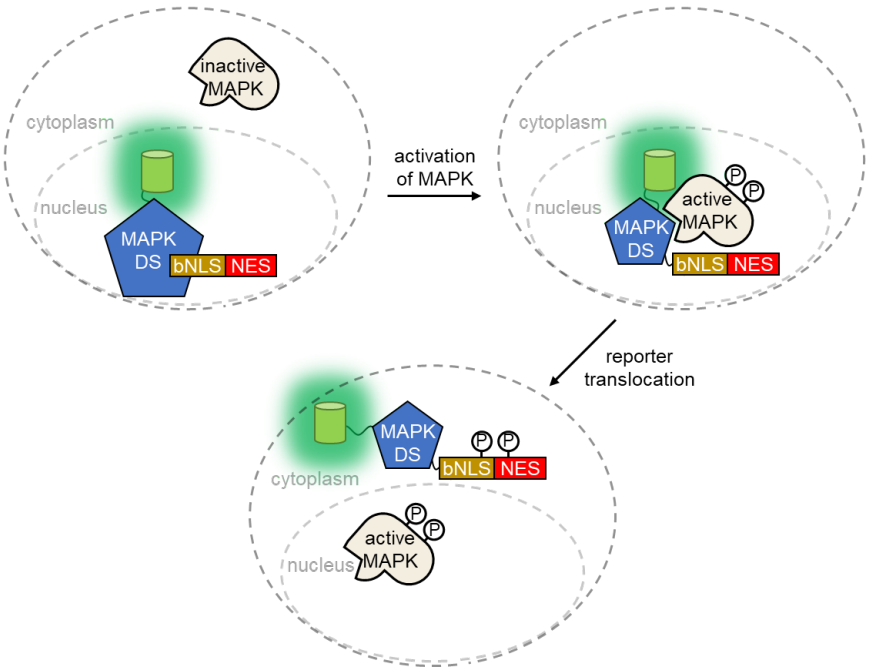
Supplementary Figures and Tables



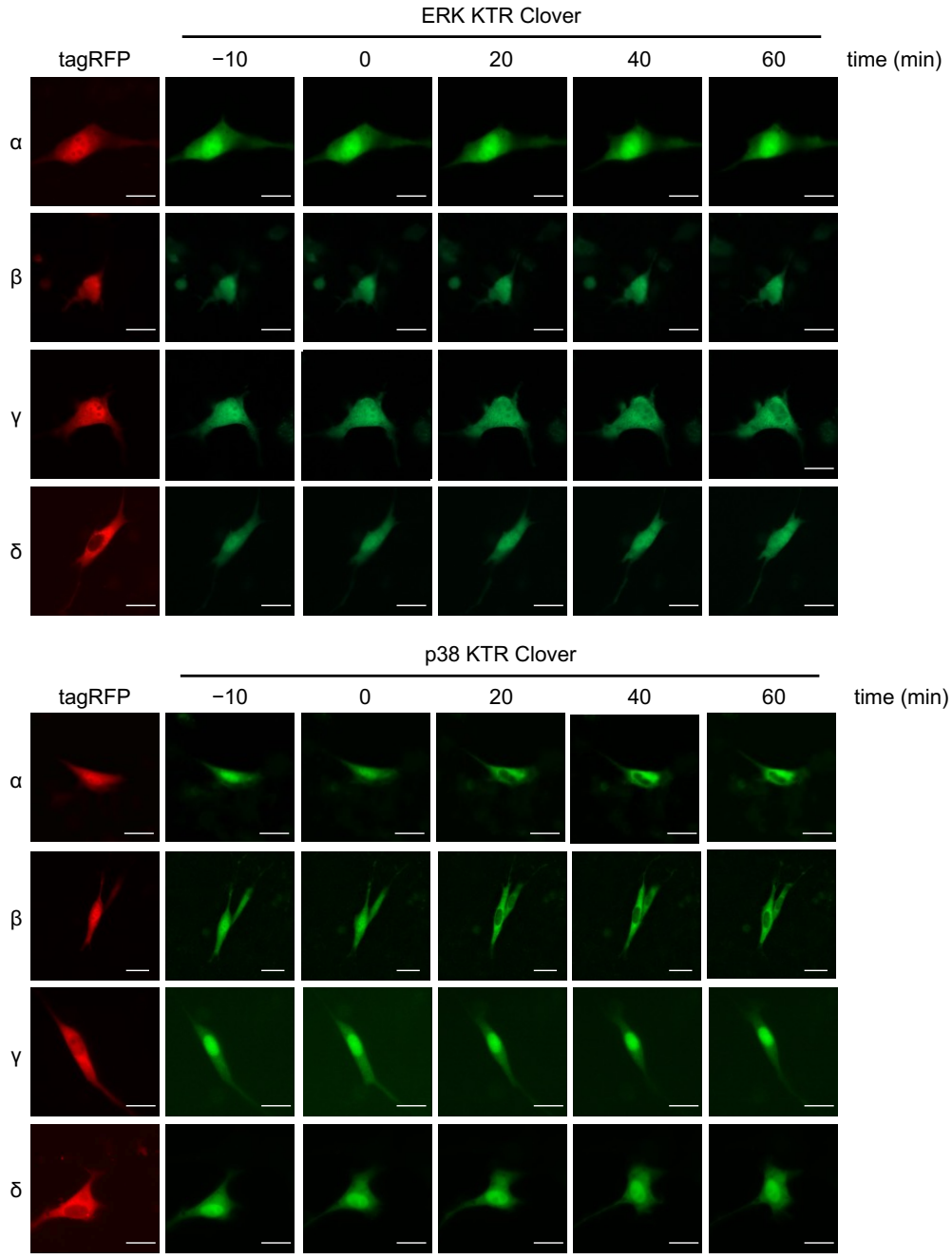
Supplementary Figure S1. Decaging study of Fmoc-protected HCK. In order to visualize light-activation of HCK using HPLC, an Fmoc-protected synthetic intermediate was utilized. (A) Schematic representation of Fmoc-HCK decaging to release Fmoc-lysine upon irradiation with a 365 nm UV transilluminator. (B) HPLC chromatograms showing decaging of Fmoc-HCK to Fmoc-lysine with increasing irradiation times. (C) Quantification of relative peak area with increasing lengths of irradiation.



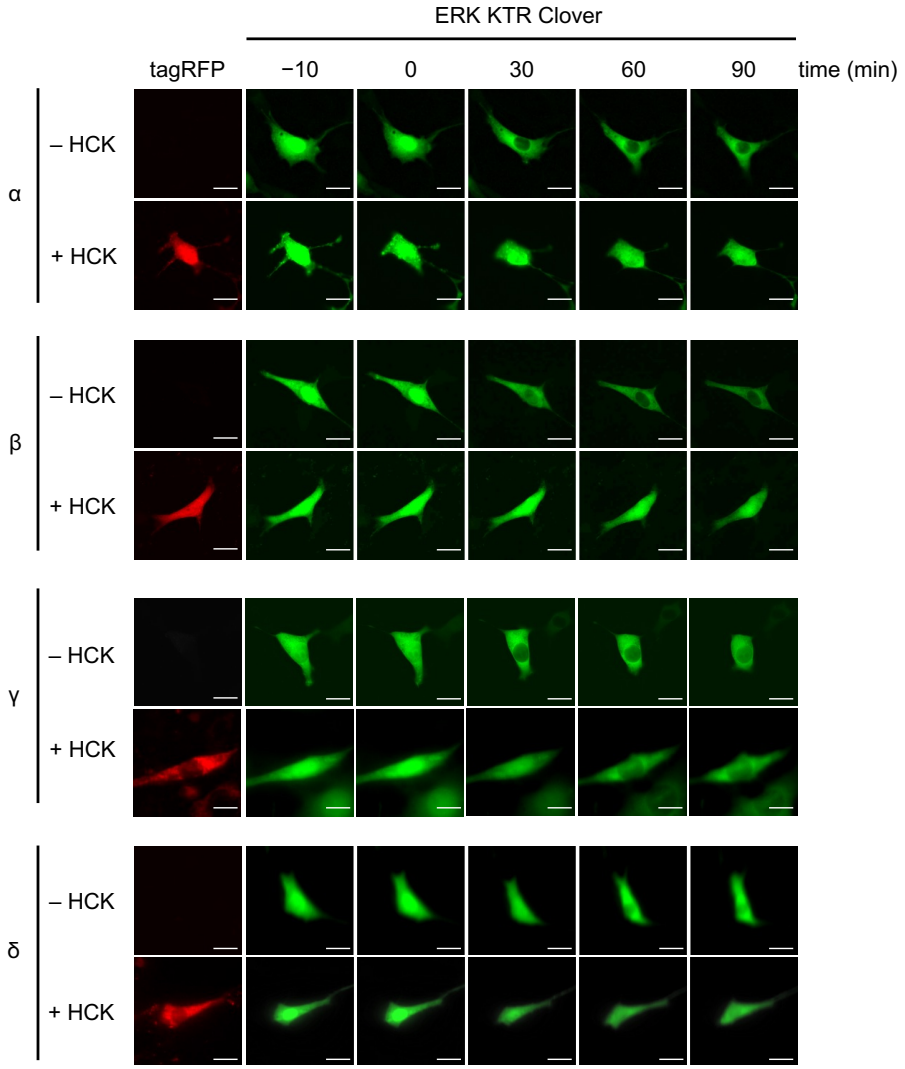
Supplementary Figure S2. Cell viability of 3T3^{HCK} cells in response to photolysis product. Cells were treated with various concentrations of either (**A**) doxorubicin, as a positive control for toxicity, or (**B**) hydroxycoumaryl alcohol, the photolysis product of HCK. Hydroxycoumaryl alcohol was found to elicit no toxicity up to 500 μM. Error bars represent standard deviation from three biological replicates.



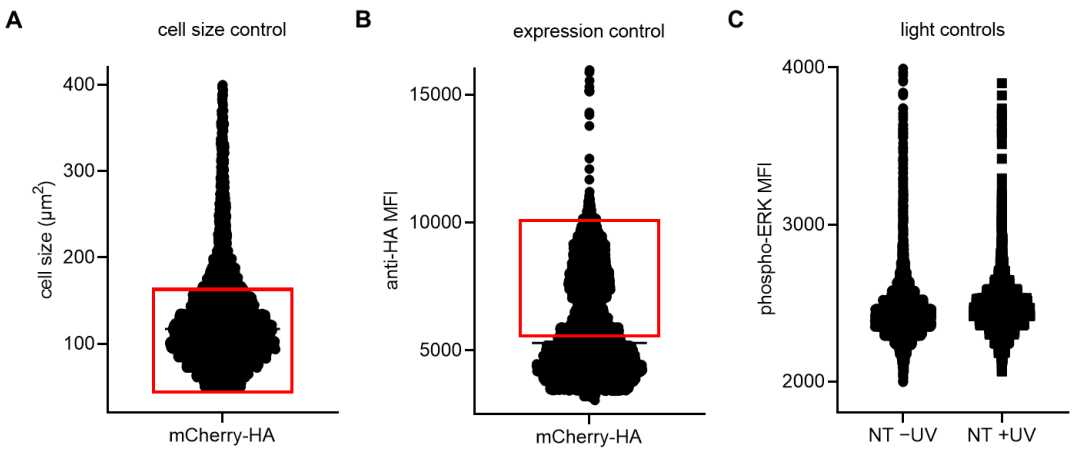
Supplementary Figure S3. Mechanism of the MAPK activity reporter, KTR Clover. Schematic illustration of the mechanism of MAPK KTR Clover. In the presence of the inactive MAPK enzyme, the reporter is nuclear localized. Upon activation and nuclear translocation of the MAPK activator, a nuclear export signal (NES) is triggered by modification of an adjacent phosphorylation site and the activity of a suboptimal bipartite nuclear translocation signal (bNLS) is negatively regulated by phosphorylation at either side of its linker region. The reporter is subsequently translocated to the cytoplasm. Activity of the kinase can thus be related to a change in the ratio of cytoplasmic and nuclear fluorescence. MAPK DS = MAPK docking site.



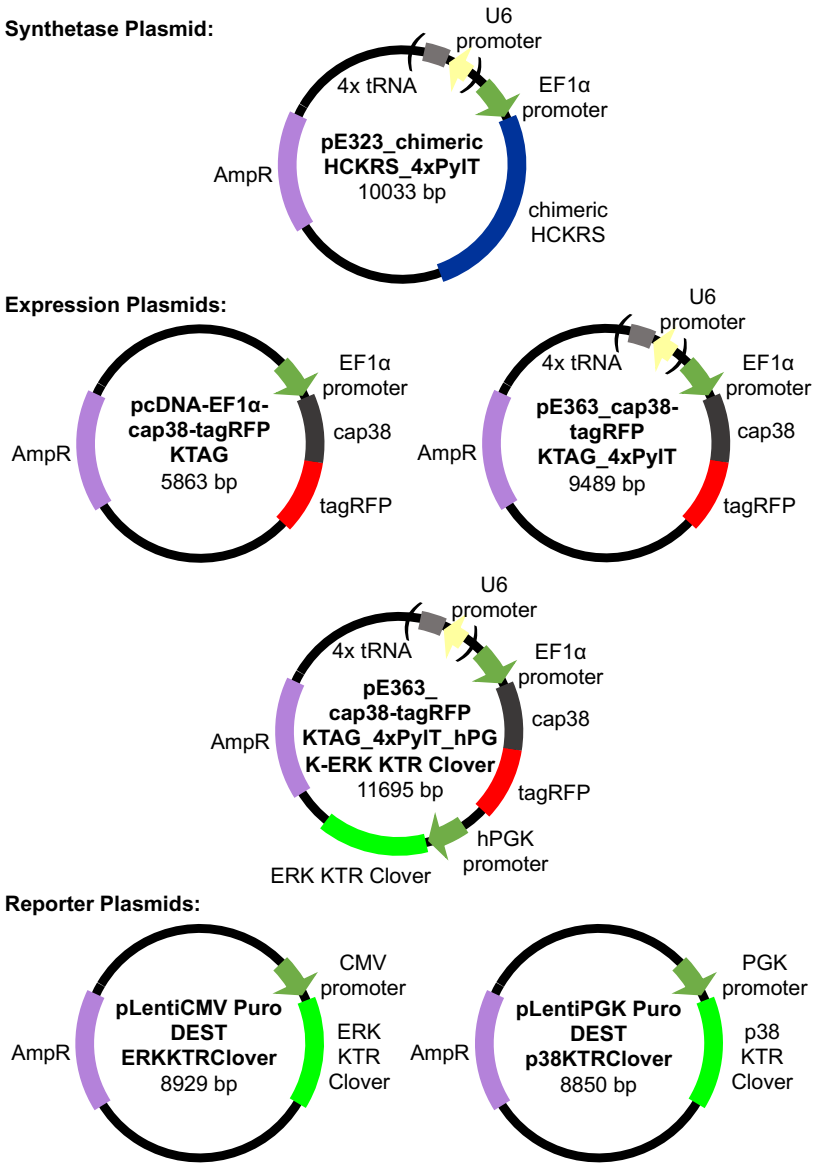
Supplementary Figure S4. Full ERK/p38 KTR timecourse micrographs. Full timecourse fluorescence microscopy images of the ERK KTR Clover or p38 KTR Clover reporters in the presence of caged p38α, p38β, p38γ, or p38δ (abbreviated in **Figure 3**). Scale bars = 20 μm.



Supplementary Figure S5. Full ERK/p38 crosstalk micrographs. Full timecourse fluorescence microscopy images of the ERK KTR Clover reporter in the presence of caged p38α, p38β, p38γ, or p38δ (data plotted in **Figure 4**). Scale bars = 20 μm.



Supplementary Figure S6. Immunofluorescence imaging gating controls. (**A**) Cell size and (**B**) transfection efficiency (through anti-HA MFI) plotted for mCherry-HA transfected cells. Limits were determined (cell size, 50 – 150 μm^2; anti-HA, 5500 – 10000 MFI) and applied to the p38δ and p38γ data sets. (**C**) ERK stimulation plotted (through phospho-ERK MFI) for nontransfected (NT) cells incubated for 60 minutes following treatment with or without light (405 nm LED, 1 min).



Supplementary Figure S7. Plasmid maps. Plasmid maps for mammalian cell culture expression of synthetase, photocaged p38 isoforms, and reporter constructs.

Supplementary Table S1. Primers. List of primers used for plasmid construction (shown 5' to 3'). Base mutations are indicated by capitalization.

Primer	Sequence
P1	gtacgtcgtctttag
P2	cgtcgccgtccagctcgacca
P3	gttcaggggaggtgtg
P4	cggcacactGCCgatgagatgacaggctac
P5	ctgtcatctcatcGGCagtgtgccgagccag
P6	catcgtgtggcagttTAGaagctgtcgagaccgttt
P7	gatggactgaaacggtctcgacagcttCTAaactgccac
P8	gcgccaggcgGCCgaggagatgaccggc
P9	gtcatctcctcGGCcgcctggcgcgccag
P10	ggtggcggtgTAGaagctgtcgcgcccc
P11	gcgacagcttCTAcaccgccaccttctg
P12	caggcaggcgGCCagtgagatgacagga
P13	gtcatctcactGGCcgcctgcctggcaag
P14	ggtggccatcTAGaagttgtaccggccctt
P15	cggtacaacttCTAgatggccaccttgttgcc
P16	cccagcagccgAGCgatgattccttagaacacgaga
P17	ctaaggaatcatcGCTcggctgctgggcctccgtc
P18	gaaggtggccatcTAGaagctgagccgacccctttca
P19	gggtcggctcagcttCTAgatggccaccttctcccc
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P36	agtccagtgtggtggaattctgcagatatcaacaag
P37	tatatatattttcttgttatagatatcgcggggaggcggcccaaa

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