

Visualisation of Endogenous ERK1/2 in Cells with a Bioorthogonal Covalent Probe

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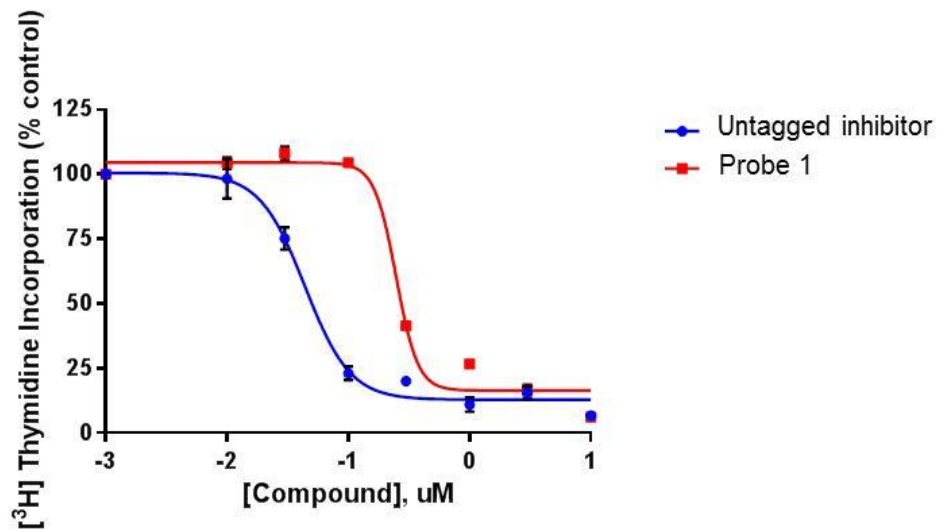
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Supplementary Figures 1-10;

Experimental procedures for LC-MS, cell culture, in-gel fluorescence, cell proliferation assay, immunoblotting, transfection, fluorescence imaging and immunofluorescence, and ERK1/2 inhibitor competition experiments.

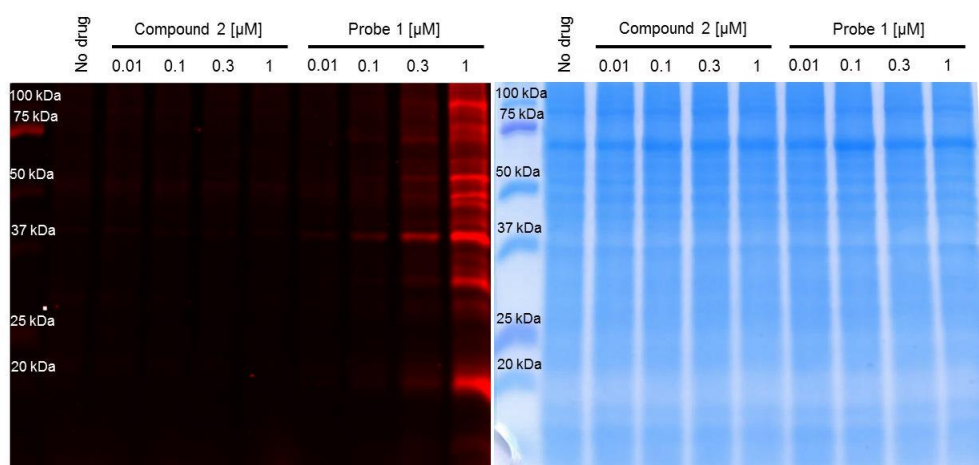
Supplementary Figure 1. Cell proliferation inhibitory activity of Probe 1.

The cell proliferation inhibitory activities of Probe 1 and its untagged analogue were determined using a ^3H thymidine incorporation assay. SW620 cells were treated with increasing concentrations of either Probe 1 or its untagged analogue. Following cell lysis, the radioactivity in the DNA was measured. Data shown are mean, normalised scintillation counts \pm SD, of three biological replicates.



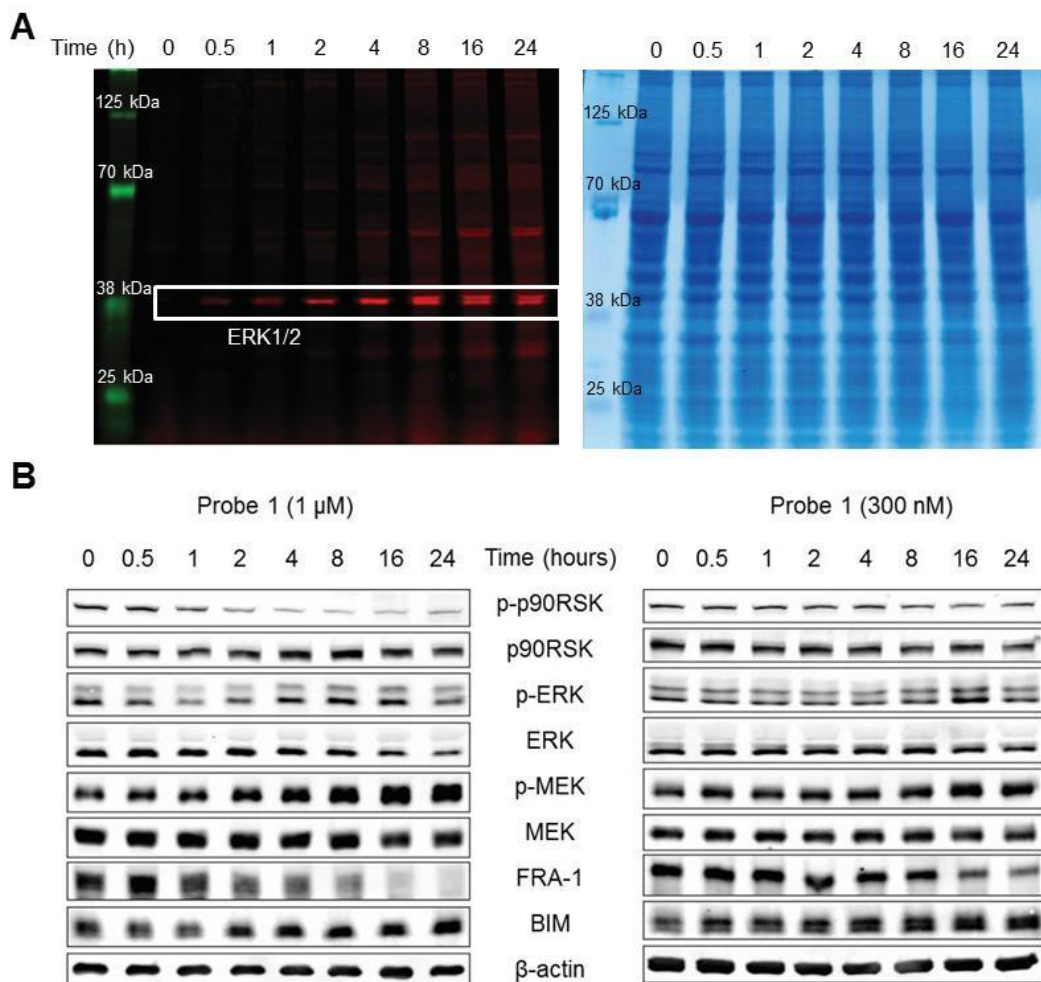
Supplementary Figure 2. Selectivity profile of Probe 1.

(Left panel) In-gel fluorescence showing the selectivity profile of Probe 1. SW620 cells were treated with Compound 2 or with Probe 1 at different concentrations for 3 hours. Following cell lysis, the lysates were incubated with Sulfo-Cy5-Tz (10 μ M, 6 h, 4 $^{\circ}$ C) (for click reaction) and the proteins were separated by SDS-PAGE. No fluorescence was observed when cells were not treated or were incubated with Compound 2 as the Tz-dye could not react with the untagged compound. The selectivity profile of Probe 1 was found acceptable up to 300 nM. (Right panel) Coomassie staining confirming equivalent protein loading for each track. Data shown is representative of at least 3 biological replicate experiments.



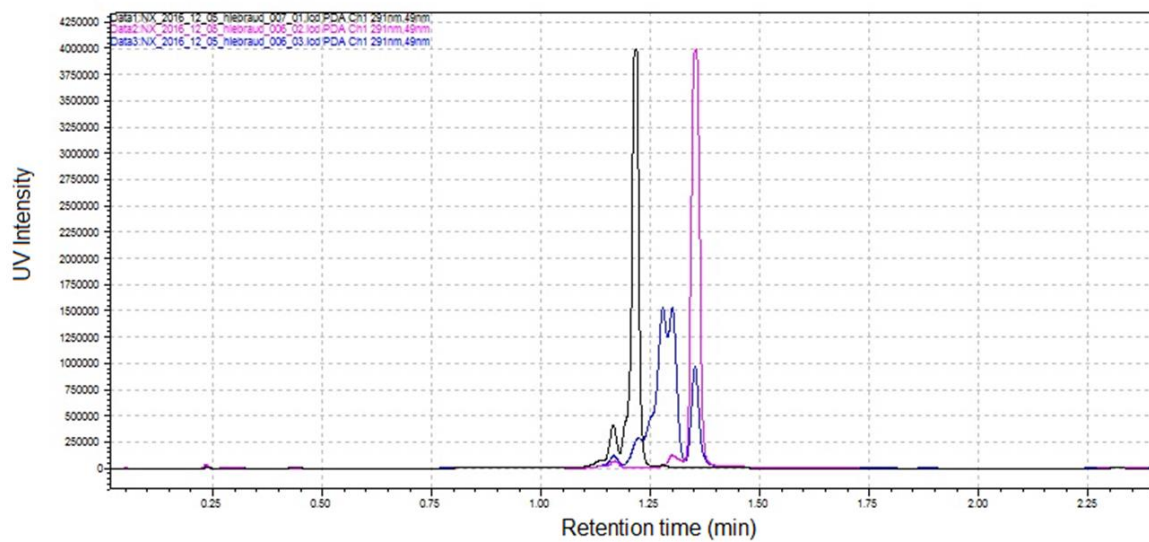
Supplementary Figure 3. Time course experiment to study the formation of the ERK-Probe 1 adduct.

A. (Left panel) In-gel fluorescence showing the time dependent formation of the ERK-Probe 1 adduct. SW620 cells were treated with Probe 1 (300 nM) and lysed. The lysates were incubated with Sulfo-Cy5-Tz (10 μ M, 6 h, 4 $^{\circ}$ C) (for click reaction) and the proteins were separated by SDS-PAGE. The formation appeared complete after 8 hours of incubation. (Right panel) Coomassie staining confirming equivalent protein loading for each track. B. Immunoblots for ERK1/2, its downstream substrates and activating kinases (MEK1/2) showing the pharmacological profile of Probe 1 in time dependent experiments. SW620 cells were treated with Probe 1 (1 μ M or 300 nM). Data shown is representative of 3 biological replicate experiments.



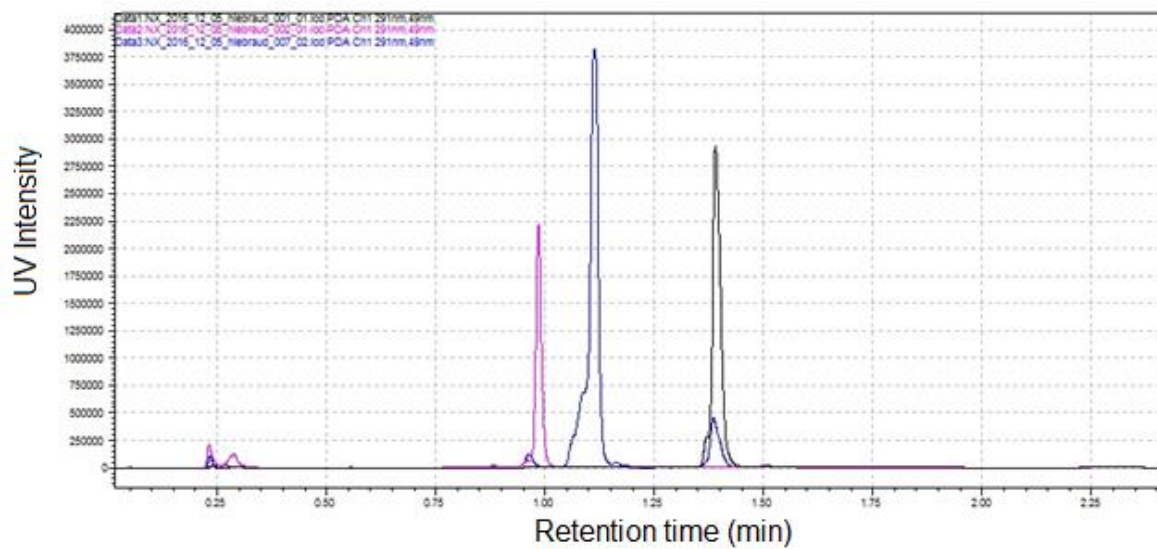
Supplementary Figure 4. LC-MS Profile of the click reaction between Probe 1 and BODIPY-Tz.

LC-MS profiles of Probe 1 (10 mM in MeOH, black), BODIPY-Tz (10 mM in MeOH, pink) and after the reaction between Probe 1 and BODIPY-Tz (1:1.5, 10 min, r.t., blue).



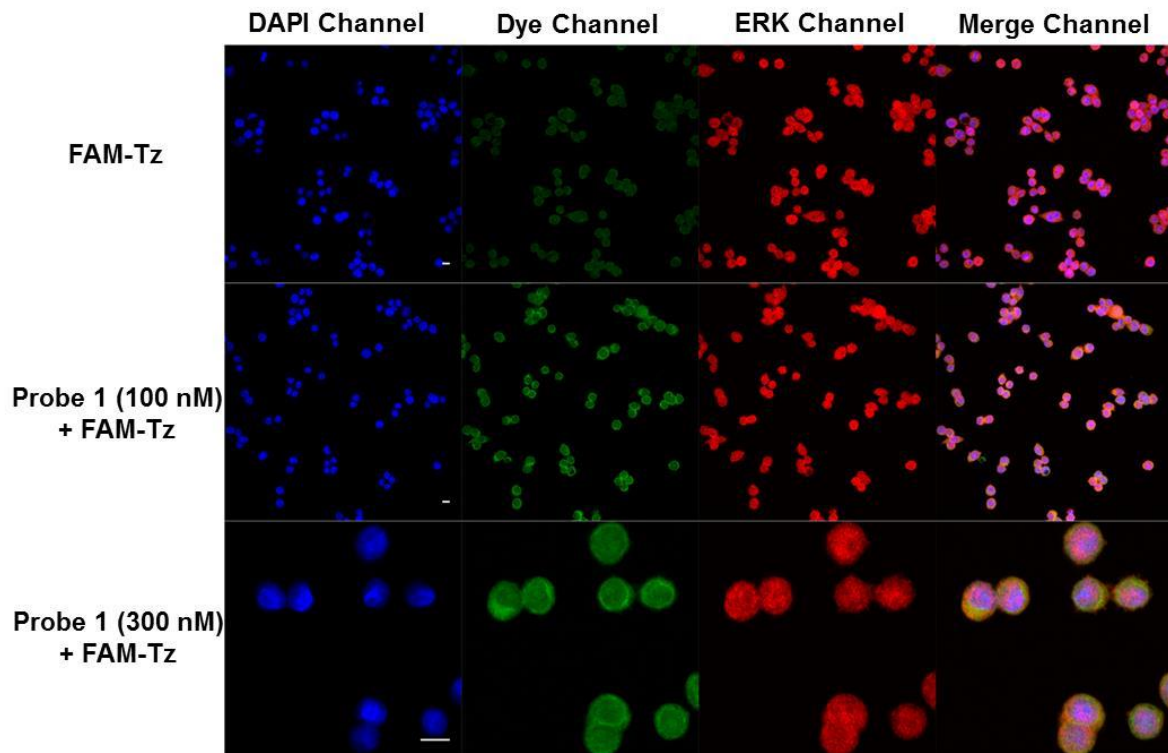
Supplementary Figure 5. LC-MS Profile of the click reaction between Probe 1 and FAM-Tz.

LC-MS profiles of Probe 1 (10 mM in MeOH, black), FAM-Tz (10 mM in MeOH, pink) and after the reaction between Probe 1 and FAM-Tz (1:1, 5 min, r.t., blue).



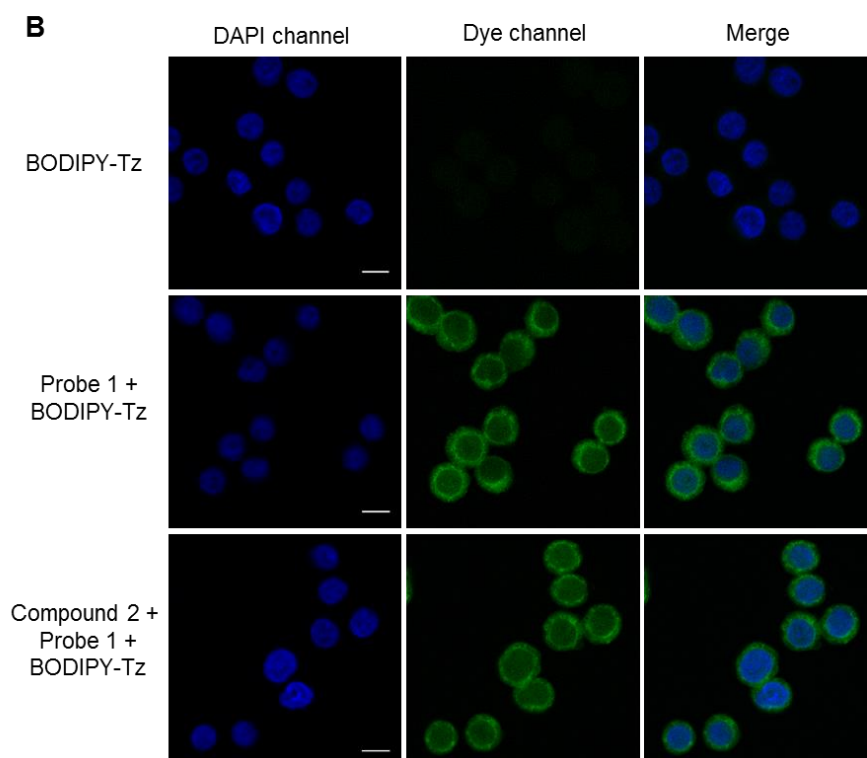
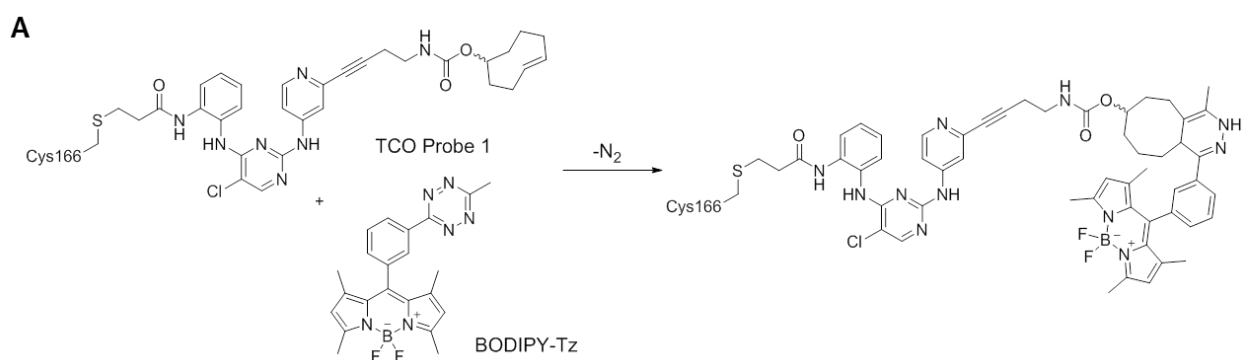
Supplementary Figure 6. Variation of Probe 1 concentration for ERK imaging.

Probe 1 was used at varying concentrations to ascertain at which concentration Probe 1 could be detected above background levels using FAM-Tz, alongside ERK1/2 immunofluorescence. Probe 1 used at a dose of 300 nM provided signal well above that of background signal whilst at 100 nM the signal was closer to that of background fluorescence. SW620 cells were treated with the indicated doses of Probe 1 for 3 hours then fixed and stained for ERK1/2 followed by FAM-Tz (1 μ M, 10 mins), as described in the methods section below. The nuclei are shown in blue (DAPI channel) and the fluorescence from the click reaction between Probe 1 and FAM-Tz is shown in green (FAM channel), ERK1/2 immunofluorescence is shown in red (ERK1/2 channel), and the three channels merged (Merge). Data shown is representative of 2 biological replicate experiments. The white bar represents 10 μ m.



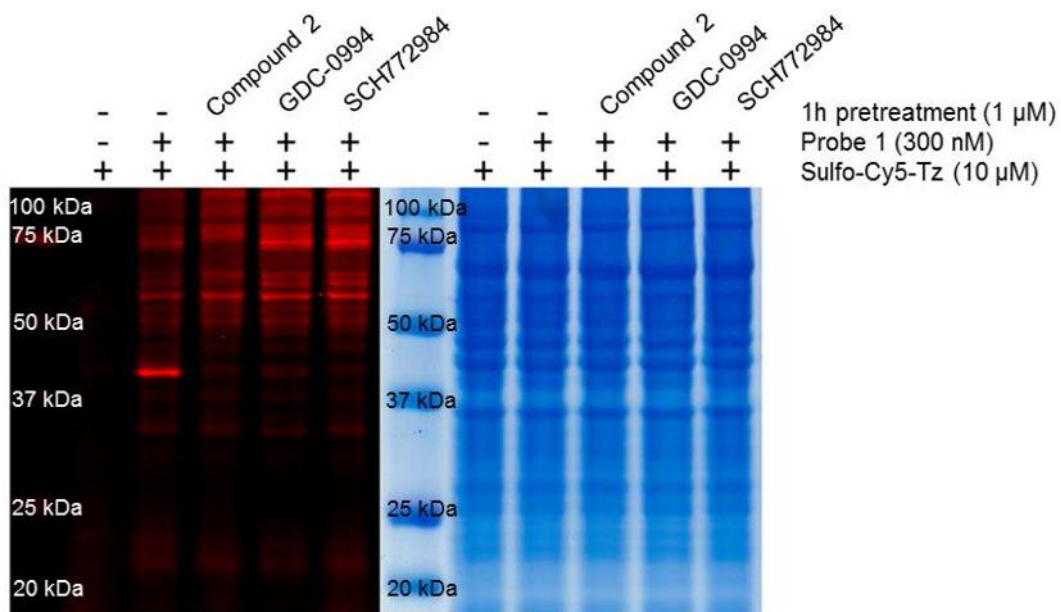
Supplementary Figure 7. Imaging illustrating the reaction between Probe 1 and BODIPY-Tz in SW620 cells.

A. ‘Click’ reaction between Probe 1 and BODIPY-Tz. B. Images showing fixed SW620 cells after treatment with BODIPY-Tz (1 μ M, 15 min) alone, with Probe 1 (300 nM, 3 hours) and BODIPY-Tz (1 μ M, 15 min), and when the cells were pre-treated with compound 2 (1 μ M, 4 hours) followed by Probe 1 (300 nM, 3 hours) and BODIPY-Tz (1 μ M, 15 min). The nuclei are shown in blue (DAPI channel) and the fluorescence from the click reaction is shown in green (dye channel) and a merged image of the two channels (merge). Data shown is representative of at least 2 biological replicate experiments. The white bar represents 10 μ m.



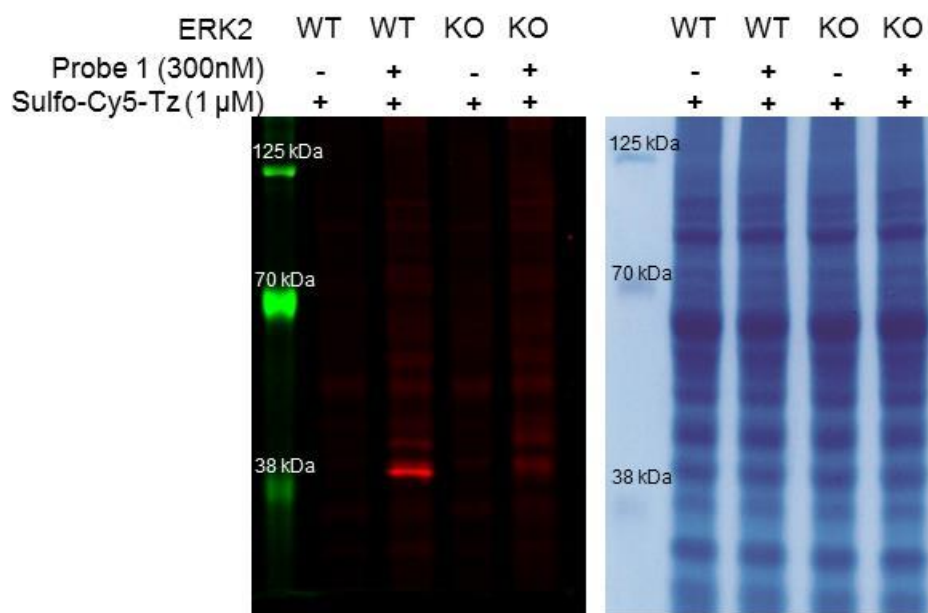
Supplementary Figure 8. In-gel fluorescence showing the binding of Probe 1 to ERK1/2 in WT and ERK2 KO MEF cells.

SW620 cells were pre-treated with ERK inhibitors; Compound 2, GDC-0994 or SCH772984 (1 μ M, 4 hours) followed by Probe 1 (300 nM, 3 hours). The cleared cell lysates were then treated with Sulfo-Cy5-Tz dye (10 μ M, 6 hours, 4 $^{\circ}$ C) (for click reaction) and the proteins were separated by SDS-PAGE. A decrease of fluorescence of the band at 42 kDa (ERK2) in cells pre-treated with ERK inhibitors was observed. (Right panel) Coomassie staining confirming equivalent protein loading for each track. Data shown is representative of 2 biological replicate experiments.



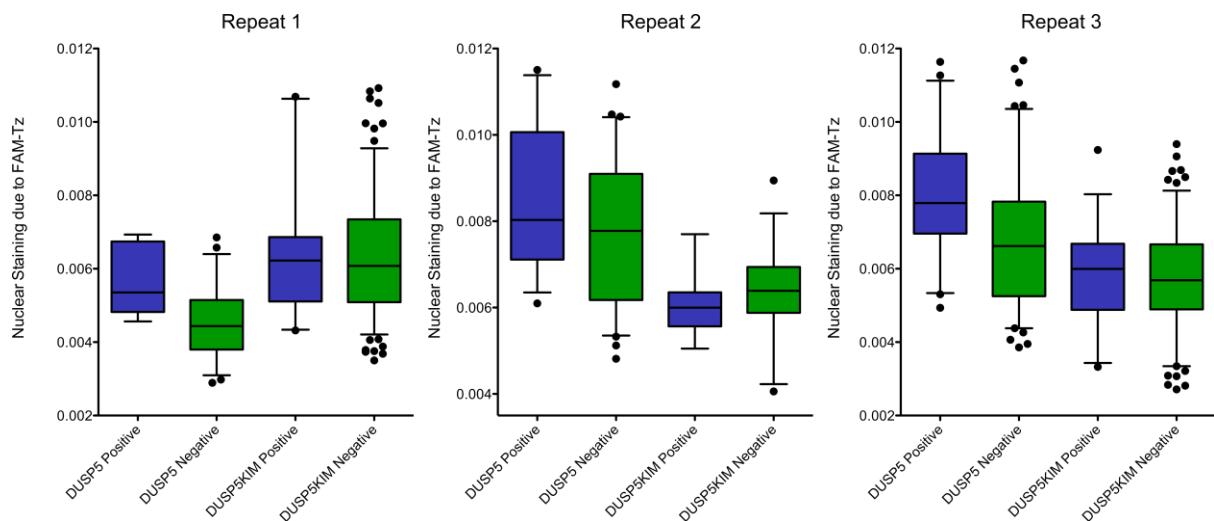
Supplementary Figure 9. In-gel fluorescence showing the binding of Probe 1 to ERK1/2 in WT and ERK2 KO MEF cells.

WT or ERK2 KO MEF cells were treated with Probe 1 at 300 nM for 3 hours. Following cell lysis, the lysates were incubated with Sulfo-Cy5-Tz (10 μ M, 6 h, 4 $^{\circ}$ C) (for click reaction) and the proteins were separated by SDS-PAGE. (Left panel) No fluorescence was observed when cells were not treated with Probe 1. After treatment with Probe 1, a decrease in fluorescence of the band at 42 kDa (ERK2) in ERK2 KO cells was observed. (Right panel) Coomassie staining confirming equivalent protein loading for each track. Data shown is representative of 2 biological replicate experiments.



Supplementary Figure 10. Statistical analysis of nuclear fluorescence due to FAM-Tz and Probe 1 upon the expression of DUSP5 and DUSP5KIM.

Samples were prepared as outlined in the experimental procedures. Nuclei, and the average intensity of their staining, were identified using CellProfiler software. Any cells where the nuclei had been incorrectly assigned or merged with other nuclei were removed. Within each experimental repeat, the number of DUSP5-MYC and DUSP5KIM-MYC expressing cells within the images taken were identified by the intensity of their nuclear staining as a result of MYC antibody. This created 4 bins across the two conditions: DUSP5 positive cells, DUSP5 negative cells, DUSP5KIM positive cells and DUSP5KIM negative cells. The average intensity of nuclear staining due to the FAM-Tz in each of these bins was then assessed and plotted against each other. In those cells expressing DUSP5 there is an increase in the nuclear staining due to FAM-Tz, indicating a re-localisation of Probe 1 into the nucleus ($p = 0.011$). In DUSP5KIM expressing cells, no increase in nuclear staining was observed ($p = 0.985$). Two extreme outliers were excluded from the analysis. Boxes show 25th and 75th percentile with the line representing the mean value. Whiskers show 10th and 90th percentile values. The analysis was performed using a 2-way ANOVA accounting for the experimental variability. Data is taken from 3 biological replicate experiments.



Experimental Procedures

LC-MS method

The compounds were solubilised in MeOH to generate a 10 mM solution. Pure and mixed samples (TCO ligand: Tz dye) were then analysed by LC-MS (liquid chromatography mass spectrometry) on a Shimadzu Nexera UPLC coupled with a Shimadzu LCMS-2020 single-quadrupole MS using a YMC-Triart C18 column (50 x 2.0 mm, 1.9 μ m) at 45 °C. Gradient elution was performed from 3% acetonitrile to 99% acetonitrile in 10 mM ammonium bicarbonate pH 9.4 over 0.7 min (for the reaction between Probe 1 and FAM-Tz) or from 3% acetonitrile to 99% acetonitrile with 0.1% formic acid over 0.7 min (for the reaction between Probe 1 and BODIPY-Tz).

Cell culture

SW620 cells were cultured in Liebovitz's L-15 medium, HEK293 cells and MEFS were grown in DMEM (Dulbecco's modified Eagle's medium) with SW620 media containing 0.0075% (v/v) NaHCO₃ and both supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), 2 mM glutamine and 10% (v/v) FBS.

In-gel fluorescence

Cells were grown to around 80 % confluency then treated with the appropriate inhibitors for the indicated times. Cells were then lysed in ice-cold TG lysis buffer and the lysates were cleared by centrifugation (12,000 x g, 10 min, 4 °C). The protein levels were determined by Bradford assay. Samples were then incubated with 1-10 μ M Sulfo-Cy5-Tz for 6 hours at 4 °C. Samples were then made to 1 X laemmli buffer and boiled for 10 min at 90 °C prior to SDS-PAGE separation. Once complete, gels were washed in H₂O (3 x 10 min) then were imaged using Odyssey Imaging System LICOR at 700 nm. Gels were stained using Simplyblue Safestain following the manufacturers procedures, then washed 2 x 15 min in

H₂O then scanned.

Cell proliferation assay

The ³H thymidine incorporation assay were performed as described previously.²

Immunoblotting

Cell lysates were prepared using ice-cold TG lysis buffer, assayed for protein content, and fractionated by SDS-PAGE as described previously.¹⁻²

Transfection

For transient expression, cells were seeded in 6-well plates. 2 µg of the vector was diluted in Opti-MEM and mixed with 4 µl of Lipofectamine 2000 in an equal volume of Opti-MEM. These two solutions were mixed and incubated for 15 min prior to cell treatment in pen-strep free media for 6 hours. Cells were then washed with PBS then incubated overnight in full media prior to treatment with Probe 1 and relevant Tz-dye.

Fluorescence imaging and immunofluorescence

Cells were seeded into 6-well plates containing a sterilised coverslip. Cells were fixed with 4% paraformaldehyde solution in PBS pH 7 for 10 min then permeabilised in ice cold MeOH for 10 min at 4 °C.

If using BODIPY-Tz, the dye was added to the medium (1 µM, 15 mins) on live cells, then cells were washed twice in serum free media and then briefly washed in PBS prior to fixation.

If using FAM-Tz, the dye was applied post fixation and permeabilisation, with the slides being incubated (1 µM in PBS, 10 min) at room temperature. Slides were then washed five times in PBS.

When performing immunofluorescence samples were treated the same way up to

permeabilisation with MeOH, slides were then blocked in a solution of 2% w/v BSA/PBS containing 0.02% v/v sodium azide at room temperature for 1 hour. Slides were then incubated in primary ERK1/2 antibody diluted in blocking solution for 16 hours at 4 °C. Slides were incubated with secondary antibody diluted in blocking solution for 1 h at 4 °C in the dark. If samples were to be double stained, the cells were then incubated in the second primary MYC antibody for 16 h and subsequent secondary antibody as described above. After these steps FAM-Tz was applied as described above.

Slides were mounted onto microscope slides using VectaShield mounting media plus DAPI and sealed. The slides were then either used immediately or stored at 4 °C. Images were recorded using Nikon A1R confocal microscope.

ERK1/2 inhibitor competition experiments

The experiments were carried out as described above, however, prior to the addition of Probe 1, cells were treated for 1 hour with either Compound 2 (1 µM), GDC-0994 (1 µM) or SCH772984 (1 µM). Following this pre-treatment, the experimental procedures described above were followed.

References

- (1) Ley, R., Balmanno, K., Hadfield, K., Weston, C., Cook, S. J. (2003) Activation of the ERK1/2 Signaling Pathway Promotes Phosphorylation and Proteasome-dependent Degradation of the BH3-only Protein, Bim. *J. Biol. Chem.* 278, 18811-18816.
- (2) Todd, D. E., Densham, R. M., Molton, S. A., Balmanno, K., Newson, C., Weston, C. R., Garner, A. P., Scott, L., Cook, S. J. (2004) ERK1//2 and p38 cooperate to induce a p21CIP1-dependent G1 cell cycle arrest. *Oncogene* 23, 3284-3295.