Expanding the “minimalist” small molecule tagging approach to different bioactive compounds

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Experimental

All chemicals were purchased from commercial vendors and used without further purification, unless otherwise noted. N, N-Dimethylformamide (DMF) and Dichloromethane (CH2Cl2, DCM) were distilled over CaH2. All non-aqueous reactions were carried out under nitrogen atmosphere in oven-dried glassware. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254, 250 μm thicknesses) and spots were visualized by ceric ammonium molybdate, ninhydrin, basic KMnO4, UV light or iodine. Flash column chromatography was carried out using Merck silica gel (0.040-0.063). UV-vis absorption and fluorescence spectra were measured by using a Shimadzu UV-vis spectrophotometer and a Perkin Elmer LS50 spectrofluorometer, respectively. All the measurements were performed at room temperature. 1H NMR and 13C NMR spectra were recorded on a Bruker model Advance 300 MHz or DPX-500 MHz NMR spectrometer. Chemical shifts are reported in parts per million relative to internal standard tetramethyldisilane (Si(CH3)4 = 0.00 ppm) or residual solvent peaks (CDCl3 = 7.26 ppm, MeOD = 3.31 ppm). 1H NMR coupling constants (J) are reported in Hertz (Hz) and multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet). MS spectra were recorded on a Finnigan LCQ mass spectrometer, a Shimadzu LC-IT-TOF spectrometer or a Shimadzu LC-ESI spectrometer. Analytical HPLC was carried out on Shimadzu LC-IT-TOF or LC-ESI systems equipped with an autosampler, using reverse-phase Phenomenex Luna 5 μm C18 100 Å 50 × 3.0 mm columns.

Antibodies against TNKS1, PPARδ, PHD1, TOP1, IDO and HDAC3 were purchased from Abcam. Plasmids carrying TNKS1/2 genes (catalytic domains with His tags) were provided by Johan Weigelt’s group.1 HRP-conjugated secondary antibodies: Goat anti-Rabbit IgG (#31210, Pierce). Nuclear staining reagent Hoechst 33342, trihydrochloride, trihydrate (10 mg/mL solution in water) were purchased from Invitrogen. Western blot was done by using the SuperSignal® West Dura Extended Duration Substrate Kit (200 mL Kit, Thermal Scientific).
Imaging was done by a Leica TCS SP5X Confocal microscope equipped with a 40X water immersion objective. Cell lines (HepG2, HeLa, MCF-7, NIH3T3, HT29) were provided by the National Cancer Institute Developmental Therapeutics Program (NCI-60), which were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen), 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Scientific) and maintained in a humidified 37 °C incubator with 5% CO2. Protein concentration was determined by Bradford protein assay.

Chemistry

(S)-4-ethyl-3,14-dioxo-3,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl 3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanoate (CA)

To a stirred solution of L1 (2.9 mg, 0.017 mmol) in DMF at 0 °C, EDC (4.5 mg, 0.029 mmol), DMAP (0.18 mg, 0.0014 mmol) were added. After 10 min Camptothecin (5 mg, 0.014 mmol) was added. The resulting solution was continued to stir overnight, after which the reaction was quenched by water and the organic layer was extracted by EA. The crude was purified by flash silica gel chromatography using 95:5 DCM/methanol as running solvent to give a colourless compound CA (3.9 mg, 55%). 1H NMR (300 MHz, CDCl3) δ 8.40 (s, 1H), 8.22 (d, J = 8.5 Hz, 1H), 7.94 (d, J = 8.1 Hz, 1H), 7.84 (t, J = 7.7 Hz, 1H), 7.67 (t, J = 7.5 Hz, 1H), 7.23 (s, 1H), 5.54 (dd, J = 85.8, 17.3 Hz, 2H), 5.29 (s, 2H), 2.52-2.07 (m, 4H), 2.07-1.91 (m, 3H), 1.81 (t, J = 7.7 Hz, 2H), 1.63 (t, 2H), 0.98 (t, J = 7.5 Hz, 3H); 13C NMR (126 MHz, CDCl3) δ 171.1, 167.3, 157.3, 152.3, 148.9, 146.3, 145.7, 131.2, 130.7, 129.6, 128.4, 128.2, 128.1, 120.2, 96.0, 82.5, 76.2, 69.4, 67.1, 49.94 (s), 32.1, 31.8, 28.1, 27.8, 27.4, 13.2, 7.6, 1.0. ESI-HRMS: [M+Na]+, calcd: 519.1639, found: 519.1636.

(4-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)ethoxy)-2-(4-(trifluoromethyl)phenyl)-7,8-dihydro-5H-thiopyrano[4,3-d]pyrimidine (XA)

To a stirred solution of XAV-939 (5.0 mg, 0.016 mmol) in DMF, K2CO3 (2.7 mg, 0.019 mmol) and L3 (4.8 mg, 0.019 mmol) were added. After stirring overnight and extraction, the crude mixture was purified by flash silica gel chromatography using 20:1 hexane/EA as running solvent so as to give a pale yellow compound XA (5.8 mg, 84%). 1H NMR (300 MHz, CDCl3) δ 8.48 (d, J = 8.5 Hz, 2H), 7.70 (d, J = 8.6 Hz, 2H), 4.42 (t, J = 6.2 Hz, 2H), 3.79 (s, 2H), 3.20 (t, J = 6.0 Hz, 2H), 2.98 (t, J = 6.0 Hz, 2H), 2.07 (td, J = 7.3, 2.3 Hz, 2H), 1.98 (dd, J = 7.7, 4.6
Hz, 3H), 1.75 (t, J = 7.4 Hz, 2H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 165.7, 164.6, 159.6, 140.8, 128.2, 125.3, 113.5, 82.5, 77.2, 69.4, 61.4, 33.5, 32.4, 26.5, 25.3, 23.1, 13.3. ESI-HRMS: m/z [M+H]*, calcd: 433.1304, found: 433.1310.

1-cyclohexyl-2-(5H-imidazo [5,1-a]isoindol-5-yl)ethyl 3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl) propanoate (NL)

To a stirred solution of \(L_1\) (3.5 mg, 0.021 mmol) in DCM at 0 °C, EDC (4.5 mg, 0.035 mmol), DMAP (0.18 mg, 0.0021 mmol) were added. After 10 min NLG919 (5 mg, 0.018 mmol) was added. The crude was purified by flash silica gel chromatography using 95:5 DCM/methanol as running solvent so as to give a colourless compound NL (3.7 mg, 49%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.71 (s, 1H), 7.53 (d, J = 8.0 Hz, 2H), 7.37 (t, J = 7.7 Hz, 1H), 7.31-7.22 (m, 1H), 7.19 (s, 1H), 5.16 (dd, J = 8.1, 3.7 Hz, 1H), 5.01-4.89 (m, 1H), 2.50-2.30 (m, 1H), 2.22-2.06 (m, 1H), 2.07-1.54 (m, 19H), 1.47 (s, 1H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 171.5, 144.2, 128.5, 126.4, 123.9, 120.1, 82.5, 77.1, 73.7, 69.2, 57.5, 42.0, 37.3, 32.2, 28.4, 28.1, 27.8, 27.7, 27.5, 26.1, 25.8, 13.2. ESI-HRMS: m/z [M+H]^+, calcd: 431.2442, found: 431.2456.

N-(2-((2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl) ethyl)amino)-2-oxoethyl)-1-hydroxy-4-methyl-6-phenoxy-2-naphthamide (FG)

To a stirred solution of FG-4592 (5.0 mg, 0.014 mmol) in DMF, EDC (3.3 mg, 0.021 mmol), DIEA (2.2 mg, 0.017 mmol) and \(L_2\) (1.7 mg, 0.013 mmol) were added. The crude reaction was purified by flash silica gel chromatography using 2:1 hexane/EA as running solvent to give a pale yellow compound FG (5.2 mg, 79%). \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 12.55 (s, 1H), 8.60 (s, 1H), 8.36 (d, J = 9.4 Hz, 1H), 7.51-7.37 (m, 4H), 7.22 (t, J = 7.4 Hz, 1H), 7.16-7.07 (m, 2H), 6.32 (s, 1H), 4.16 (d, J = 6.2 Hz, 2H), 3.17 (q, J = 6.6 Hz, 2H), 2.69 (s, 3H), 2.04-1.95 (m, 3H), 1.72 (t, J = 6.7 Hz, 2H), 1.68-1.61 (m, 3H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 170.7, 168.8, 158.6, 156.0, 153.8, 147.2, 132.3, 130.1, 125.7, 124.5, 124.2, 122.3, 119.7, 119.3, 111.8, 82.7, 77.2, 69.4, 43.2, 34.4, 32.5, 32.1, 26.7, 21.7, 13.2. ESI-HRMS: m/z [M+Na]^+, calcd: 494.1799, found: 494.1811.

N-(2-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl) ethyl)-2-(4-(((2-(3-fluoro-4-(trifluoromethyl) phenyl)-4-methylthiazol-5-yl)methyl)thio)-2-methylphenoxy) acetamide (GW)
To a stirred solution of GW0742 (5.0 mg, 0.01 mmol) in DMF, EDC (3.3 mg, 0.021 mmol), TEA (1.9 mg, 0.013 mmol) and L2 (1.7 mg, 0.013 mmol) were added and stirred overnight. After extraction, crude mixture was purified by flash silica gel chromatography using 3:1 hexane/EA as running solvent to give a pale yellow compound GW (5.3 mg, 85%). ^1H NMR (500 MHz, CDCl$_3$) δ 7.76-7.67 (m, 2H), 7.63 (t, $J = 7.6$ Hz, 1H), 7.20 (dd, $J = 11.5$, 3.2 Hz, 2H), 6.70 (d, $J = 8.3$ Hz, 1H), 6.62 (s, 1H), 4.47 (s, 2H), 4.13 (s, 2H), 3.22 (q, $J = 6.4$ Hz, 2H), 2.29 (s, 3H), 2.22 (s, 3H), 2.00 (td, $J = 7.2$, 2.6 Hz, 2H), 1.96 (t, $J = 2.6$ Hz, 1H), 1.77 (t, $J = 6.6$ Hz, 2H), 1.64 (t, $J = 7.3$ Hz, 2H); ^13C NMR (75 MHz, CDCl$_3$) δ 168.0, 155.5, 151.6, 135.9, 132.2, 131.5, 127.7, 126.0, 121.5, 114.3, 114.0, 112.0, 82.5, 69.4, 67.4, 33.9, 33.9, 32.4, 32.3, 32.1, 29.7, 26.7, 16.3, 14.9, 13.1. ESI-HRMS: m/z [M+H]+, calcd: 591.1506, found: 591.1507.

(R)-N-(1-(3-benzyl-7-chloro-4-oxo-3, 4-dihydro quinazolin-2-yl)-2-methylpropyl)-N-(3-(3-(3-(3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanamido)propyl)-4-methylbenzamide (IS)

To a stirred solution of L1 (5.0 mg, 0.0097 mmol) in DMF, EDC (3.0 mg, 0.019 mmol), DIEA (1.5 mg, 0.012 mmol) and Ispinesib (5.0 mg, 0.0097 mmol) were added and stirred overnight. The crude was purified by flash silica gel chromatography using 3:1 hexane/EA as running solvent so as to give a pale yellow compound IS (5.1 mg, 80%). ^1H NMR (500 MHz, CDCl$_3$) δ 8.33 (d, $J = 8.5$ Hz, 1H), 7.69 (d, $J = 1.8$ Hz, 1H), 7.53-7.46 (m, 1H), 7.39 (d, $J = 7.3$ Hz, 3H), 7.32 (t, $J = 7.4$ Hz, 3H), 7.25-7.19 (m, 3H), 6.12 (d, $J = 15.8$ Hz, 1H), 5.70 (d, $J = 10.5$ Hz, 1H), 5.20 (d, $J = 15.8$ Hz, 1H), 4.49 (s, 1H), 3.53-3.29 (m, 2H), 2.95 (d, $J = 12.4$ Hz, 1H), 2.89-2.77 (m, 1H), 2.78-2.63 (m, 2H), 2.41 (s, 3H), 2.08-1.92 (m, 2H), 1.88-1.77 (m, 2H), 1.74 (t, $J = 7.5$ Hz, 2H), 1.59 (dd, $J = 14.1$, 6.9 Hz, 2H), 0.95 (d, $J = 6.7$ Hz, 3H), 0.37 (d, $J = 6.3$ Hz, 3H); ^13C NMR (126 MHz, CDCl$_3$) δ 172.9, 170.8, 162.1, 155.9, 147.6, 140.9, 139.7, 136.7, 133.8, 129.3, 129.0, 128.7, 128.2, 127.7, 127.2, 126.8, 126.1, 82.6, 69.2, 59.6, 45.5, 41.8, 36.6, 32.4, 30.3, 30.0, 28.9, 28.0, 27.7, 21.4, 19.1, 18.3, 13.2. ESI-HRMS: m/z [M+Na]+, calcd: 687.2840, found: 687.2821.

Pyridin-3-ylmethyl (4-((2-(3-(3-(but-3-yn-1-yl)-3H-diazirin-3-yl)propanamido)phenyl) carbamoyl) benzyl) carbamate (EN)
To a stirred solution of L1 (2.6 mg, 0.016 mmol) in DMF, EDC (4.1 mg, 0.027 mmol), DIEA (2.1 mg, 0.016 mmol) and Entiostat (5.0 mg, 0.013 mmol) were added. The crude was purified by flash silica gel chromatography using 1:1 hexane/EA as running solvent so as to give a pale yellow compound EN (5.6 mg, 81%).

$^1$H NMR (500 MHz, CDCl$_3$) δ 9.16 (s, 1H), 8.61 (d, $J$ = 24.0 Hz, 2H), 8.36 (s, 1H), 7.90 (d, $J$ = 7.9 Hz, 2H), 7.74 (d, $J$ = 7.6 Hz, 1H), 7.58 (d, $J$ = 7.9 Hz, 1H), 7.40 (d, $J$ = 7.9 Hz, 2H), 7.36-7.31 (m, 1H), 7.19 (t, $J$ = 7.4 Hz, 2H), 7.09 (t, $J$ = 7.9 Hz, 1H), 5.39 (s, 1H), 5.18 (s, 2H), 4.47 (d, $J$ = 5.9 Hz, 2H), 2.09 (t, $J$ = 7.5 Hz, 2H), 1.98 (t, $J$ = 2.5 Hz, 1H), 1.94 (td, $J$ = 7.4, 2.6 Hz, 2H), 1.85 (t, $J$ = 7.5 Hz, 2H), 1.61 (t, $J$ = 7.4 Hz, 3H);

$^{13}$C NMR (75 MHz, CDCl$_3$) δ 167.9, 155.5, 151.6, 135.9, 132.2, 131.5, 127.7, 126.0, 121.5, 114.3, 114.0, 111.9, 82.5, 77.2, 69.4, 67.4, 33.9, 32.4, 32.3, 32.1, 29.7, 26.7, 16.3, 14.9, 13.1.

ESI-HRMS: m/z [M+Na]$^+$, calcd: 547.2064, found: 547.2077.

3. Biology

Recombinant Protein Expression and Purification

Recombinant TNKS1/2 proteins (catalytic domain only) tagged with a His6 tag was expressed by use of an E. coli strain. The plasmids containing TNKS1/2 were transformed into E. coli cells and plated on Luria-Bertani (LB) agar with ampicillin (100 mg/L) and chloramphenicol (34 µg/mL) and grown overnight at 37°C. The following day, a single colony was picked and re-suspended in LB medium supplemented with ampicillin (100 mg/L) and chloramphenicol (34 µg/mL). Starter cultures were grown to an OD600 of 0.6 at 37°C and stored overnight at 4°C. The starter culture was diluted 1:100 in LB media supplemented with ampicillin (100 mg/L) and chloramphenicol (34 µg/mL) and grown to an OD600 of 1.2 at 37°C. The culture was cooled for 1 h with shaking at 15°C prior to induction for 16 h at 15°C with isopropyl-β-D-thiogalactoside (IPTG, final concentration 1 mM). Cells were harvested by centrifugation (4,000 rpm, 15 min, 4°C) and stored at -80°C, or resuspended in chilled lysis buffer (25 mM HEPES, 300 mM NaCl, 2 mM MgCl$_2$, 5 mM β-mercaptoethanol, 10% glycerol) with 1 mM phenylmethyl sulfonyl fluoride (PMSF) for immediate purification by Ni-NTA affinity chromatography. The re-suspended pellet was lysed by sonication (6X pulses of 15s each at half maximal power) on ice. The solution was clarified by centrifugation at 10000 g (30 min, 4 °C) and loaded onto a column containing 200 µL of His-tagged resin (Amersham) pre-equilibrated with lysis buffer. Following incubation for 1 h at 4 °C, the resin was washed 6 times with wash buffer (25 mM HEPES, 300 mM NaCl, 2 mM MgCl$_2$, 20 mM imidazole) and the protein was finally eluted with elution buffer (25 mM HEPES, 300 mM NaCl, 2 mM MgCl$_2$, 200 mM imidazole). The protein was dialyzed with Microcon® centrifugal filter device (TNKS1: M. W. =28 kDa, TNKS2: M. W. =26 kDa), and stored at -20 °C with 20% glycerol.

Recombinant Protein Labelling
Either dose-dependent or competitive labelling of bacterial lysates (containing overexpressed TNKS1/2 proteins) was done by referring to the published procedure. To 50 µg of fresh bacterial lysates prepared above, the probes were added to fresh cell lysates (50 µg) in 50 µL HEPES buffer (25 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, pH 7.5, containing 50 µM PMSF) at various concentrations in the presence or absence of 10 times concentrated competitor (the wild-type bioactive compounds, WT). Samples were incubated for 2 h and UV irradiated (350 nm) for 20 min on ice. 4 µL of a freshly premixed click chemistry reaction cocktail in PBS containing 0.1 mM CuSO₄ (from a 100 mM freshly prepared stock solution in deionized water) 0.01 mM TBTA (from a 10 mM freshly prepared stock solution in deionized water), 1 mM TCEP (from a 100 mM freshly prepared stock solution in deionized water), and 10 µM Rh-N₃ (from a 50 mM stock solution in DMSO) were added. The reaction was incubated at room temperature for 2 h with gentle mixing. After 2 h of click reaction, 5X SDS loading dye was added and the mixture was heated to 95 °C for 10 min. The resulting proteins were resolved by 10% SDS-PAGE gel and the fluorescence signal visualised by in-gel fluorescence scanning on a Typhoon 9410 variable mode image scanner.

In vitro and in situ mammalian cell-based proteome labelling

The procedure is similar to our previously reported methods but with further optimizations as described below. For in vitro proteome labeling, the probe with or without WT (10X) was added to 50 µg fresh HepG2 cell lysates (prepared as previously described) in 50 µL of HEPES buffer (125 mM HEPES, 750 mM NaCl, 10 mM MgCl₂, 50 µM PMSF, pH 7.5) at various concentrations. Unless indicated otherwise, samples were incubated for 2 h at room temperature and then UV-irradiated (350 nm) for 20 min on ice. 4 µL of a freshly premixed click chemistry reaction cocktail in PBS containing 0.1 mM CuSO₄, 0.01 mM TBTA, 1 mM TCEP, and 10 µM Rh-N3 were added. The reaction was further incubated for 2 h with gentle mixing, before being terminated by addition of prechilled acetone (0.5 mL; 30 min incubation at -20 °C). Precipitated proteins were subsequently collected by centrifugation (13000 rpm × 10 min at 4 °C). The supernatant was discarded and the pellet was washed with 200 µL of prechilled methanol. After thoroughly drying the protein samples, 1X loading buffer was added to dissolve the proteins; the samples were then heated for 10 min at 95 °C. The protein samples were separated by SDS-PAGE (10% gel) and then visualized by in-gel fluorescence scanning.

For in situ labeling, cells were grown to 80-90% confluency in 6-well plates under conditions described above. The medium was removed, and cells were washed twice with cold PBS and then treated with 0.5 mL of the DMEM-containing probe in the presence or absence of excess competitor (diluted from DMSO stocks whereby DMSO never exceeded 1% in the final solution). After 3 h of incubation at 37 °C/5% CO₂, the medium was aspirated, and cells were washed gently with PBS twice to remove excessive probe, followed by UV irradiation for 20 min on ice. The cells were trypsinized and pelleted by centrifugation. Eventually, the cell
pellets were re-suspended in PBS (50 μL), homogenized by sonication, and diluted to 1 mg/mL with PBS. All subsequent procedures were similar to those from in vitro experiments.

**Cellular Imaging**

The general procedures are similar to what was reported earlier. Briefly, HepG2 cells were seeded in Cellview cell culture dishes (Greiner Bio-One, Ref. 627870) and grown until 30% confluence. Cells were then treated with 0.5 mL DMEM medium containing DMSO or 1 μM probes/NP. The competitive experiments were done simultaneously with the presence of 10X WT. After 3 h incubation in a 37 °C incubator, the medium was gently removed, and cells were gently washed twice with PBS. Then the cells were subsequently fixed for 30 min at 37 °C with 3.7% formaldehyde in PBS, washed twice with cold PBS again, and permeabilized with 0.1% Triton X-100 in PBS for 15 min at 37 °C. Cells were then treated with a freshly premixed click chemistry reaction solution in a 300 μL volume (final concentrations of reagents: 400 μM CuSO₄, 400 μM TCEP, 40 μM THPTA, and 10 μM Rh-PEG-N₃ in PBS) for 2 h at room temperature with vigorous shaking. Then cells were first washed with PBS twice, and then washed with EDTA (50 mM, 0.1% Tween-20) in PBS until no more copper left. The following Immunofluorescence (IF) and nuclear staining was carried out as previously described. Imaging was done with the Leica TCS SP5X confocal microscope system equipped with Leica HCX PL APO 63×/1.20 W CORR CS, 405 nm diode laser, white laser (470-670 nm, with 1 nm increments, with eight channels AOTF for simultaneous control of eight laser lines, each excitation wavelength provides 1.5 mV), and a photomultiplier tube (PMT) detector ranging from 410 to 700 nm for steady state fluorescence, a total of channels were employed including DIC channel, Probe-treated channel (upon clicked with TER-PEG-N₃, Probe), immunofluorescence (IF) and Hoechst-stained channel (NU). Images were uniformly processed with Photoshop CS6.

**Pull-down/Western Blotting**

Here, HepG2, HeLa, MCF-7, NIH3T3, HT29 cells were used accordingly. The general pull-down procedure was based on previously reported procedures. For in vitro pull-down, fresh cell lysates were prepared and their protein concentrations determined, as previously described. Cellular lysates (6 mg) were supplemented with 500 μL 1X HEPES buffer (125 mM HEPES, 750 mM NaCl, 10 mM MgCl₂, 50 μM PMSF, pH 7.5) and pre-incubated with WT (10X) for 1 h at room temperature. Subsequently, probes (5 μM) were added to corresponding cell lysates, and labeling was carried out for additional 1 h at room temperature. The CuAAC reaction was then performed with biotin-N₃ reporter under the conditions described in the labeling procedure. Subsequently, the proteins were acetone precipitated, washed with methanol for 2–3 times and re-solubilized in 1% SDS in PBS with brief sonication. These re-suspended samples were then incubated with avidin-agarose beads (100 μL/mg protein) for 5 h at room temperature. After centrifugation, the supernatants were removed and the beads were washed with 1% SDS three times and 1X PBS once. After washing, the beads were boiled in
1X SDS loading buffer (200 mM Tris pH 6.8, 400 mM DTT, 8% SDS) for 15 min to release the captured proteins. WB experiments were carried out by referring to the general procedure using corresponding antibodies (1:1000) to detect the target proteins. For in situ PD, briefly, the probes (5 µM) was directly added to live cells, followed by incubation at 37 °C/5% CO₂ for 3 h. DMSO should never exceed 1% in the final solution. The medium was aspirated, and cells were washed twice gently with PBS to remove the excessive probe, followed by UV irradiation (~350 nm) for 20 min on ice. The cells were then trypsinized and pelleted by centrifugation. Eventually, the cell pellets were resuspended in HEPES buffer (50 µL), homogenized by sonication, and diluted to 1 mg/mL with Tris buffer. The labeled lysates were then subjected to click reaction with biotin-azide, and all subsequent experiments were carried out as above described. Control PD experiments using DMSO and the negative probe (NP) were carried out concurrently both in vitro and in situ.

SILAC-based large-scale pull-down and LC-MS/MS

SILAC experiments were done by referring to the reported procedures. Briefly, the cells were seeded into two wells of a 6-well plate with a 20 % confluency and labelled with light or heavy SILAC medium respectively. Upon cell attachment, light/heavy cells were replaced with light or heavy SILAC medium immediately, and medium was changed for every 2-3 days during cell culture. Upon cells reached 90% confluency, the light/heavy cells were subdivided into 3 wells of a 6-well plate respectively. After 90% confluency, the 3-well light/heavy cells were detached and combined into one 10 cm culture dish. When they reached 90% confluency again, either light or heavy amino acids should be reasonably incorporated. The whole process may need to repeat if it is the first time for this cell line to incorporate heavy amino acids. Three wells of light/heavy cells 10 cm cell culture dishes were collected and lysed with 6M urea/2M thiourea buffer. Lysates were vortexed intermittently for 5 min and pelleted by centrifuge for 10 min at 16,000g, 18 °C. The lysates were incubated with 5 µM probe in the presence/absence of the WT (50 µM), and the light/heavy samples were mixed equally. The rest procedures were the same as the previous PD as described above. Finally, the protein samples were separated with SDS-PAGE and digested by trypsin to obtain the peptide fragment (in-gel digestion) for further MS analysis.

Reference

NMR Spectra

GW
XA