iScience, Volume *24*

Supplemental information

CLICK-enabled analogues reveal pregnenolone interactomes in cancer and immune cells

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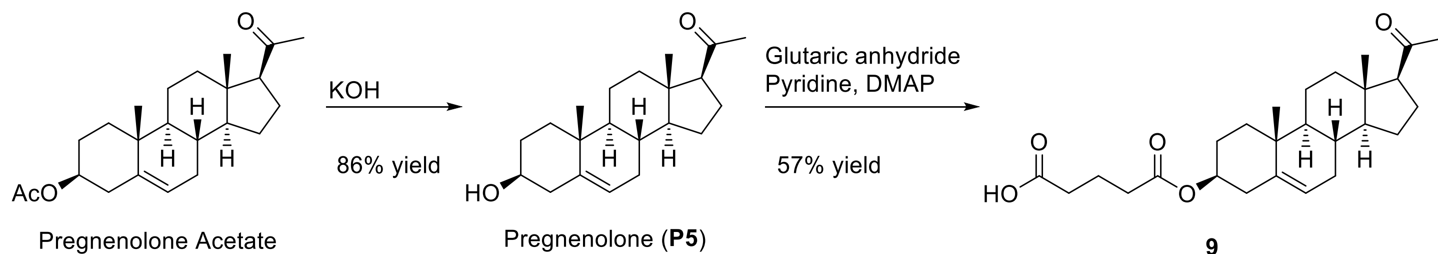
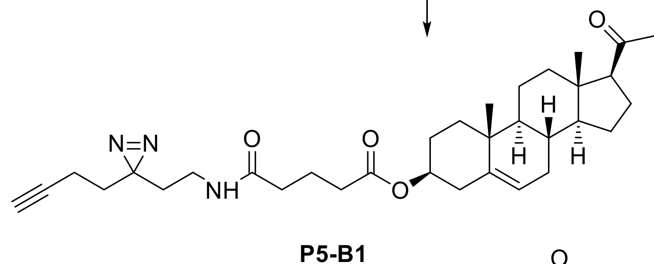
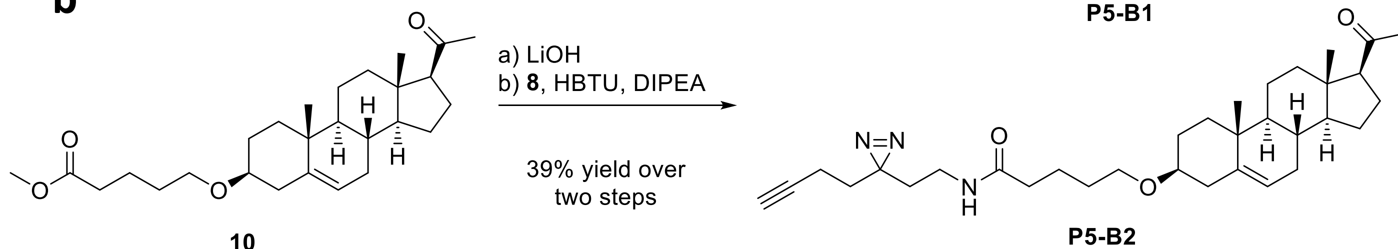
FigureS1

a

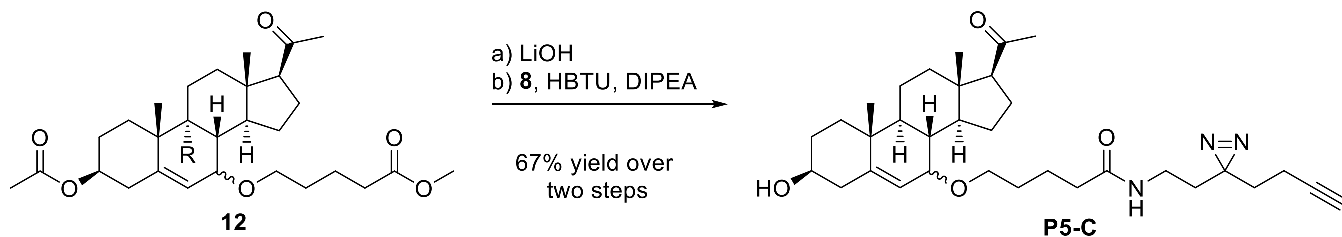




b



c



d

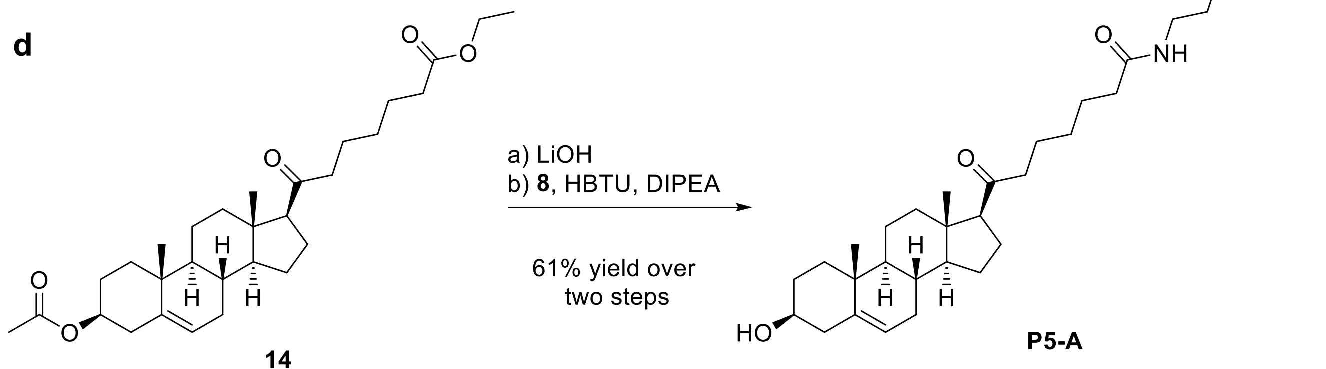


Figure S1. Chemical modification of P5, related to Figure 1

a. The details of the 8 steps photoactivatable and clickable linker synthesis with yields.

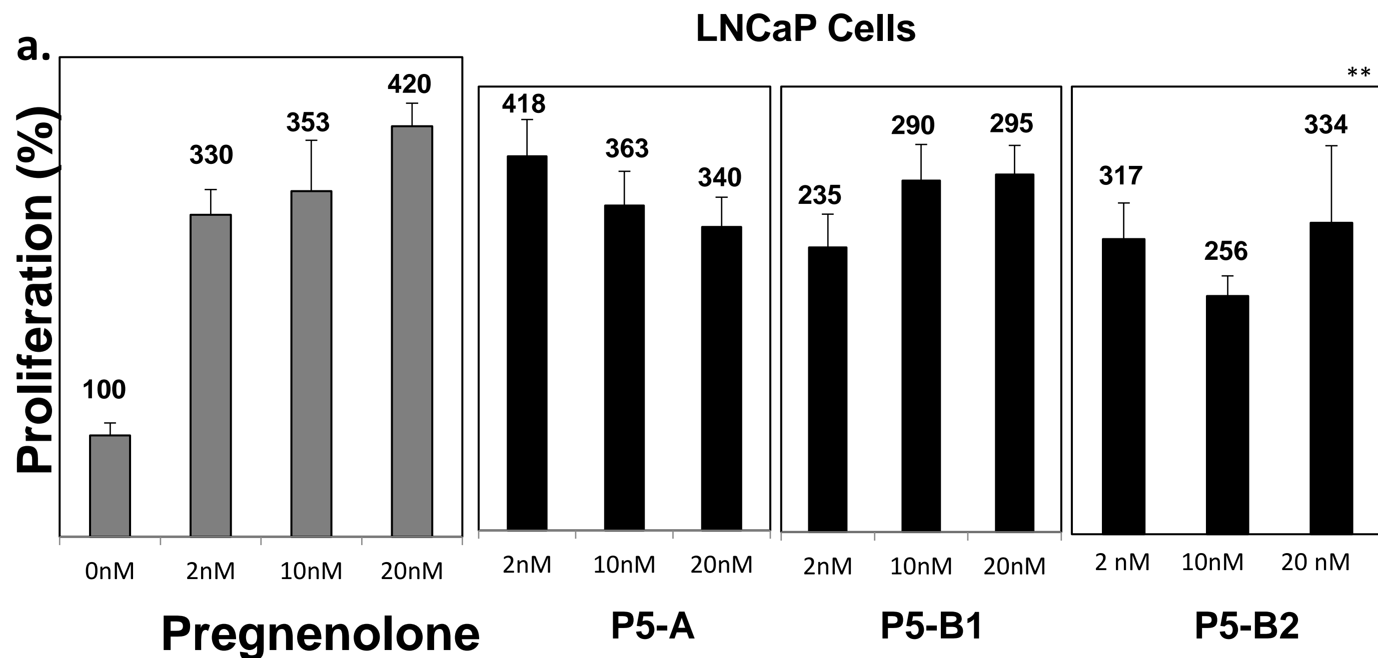
b. The details of chemical synthesis of **P5-B1**.

c. The details of chemical synthesis of probe **P5-B2**.

d. The details of chemical synthesis of probe **P5-C.**

e. The details of chemical synthesis of probe **P5-A**.

Figure S2



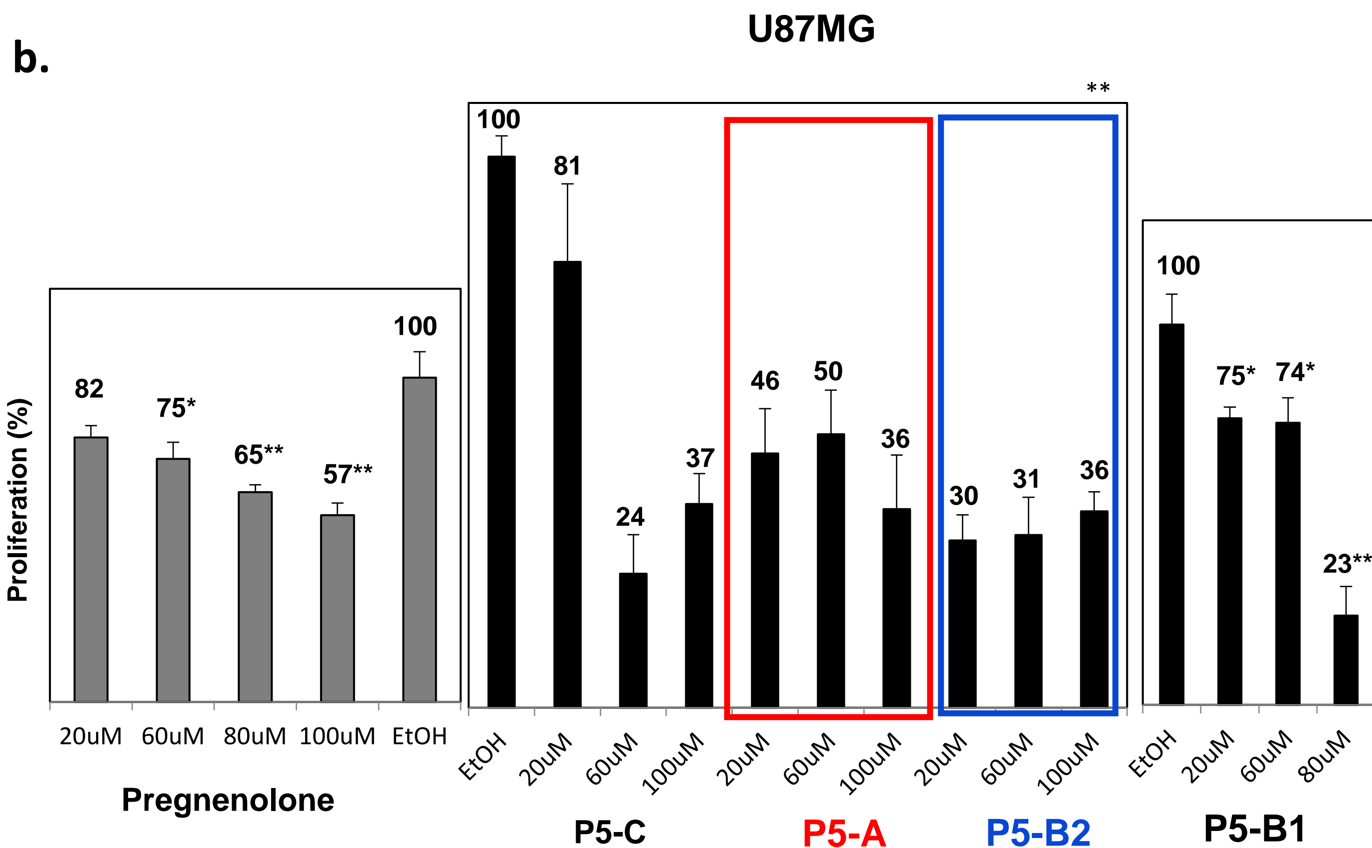


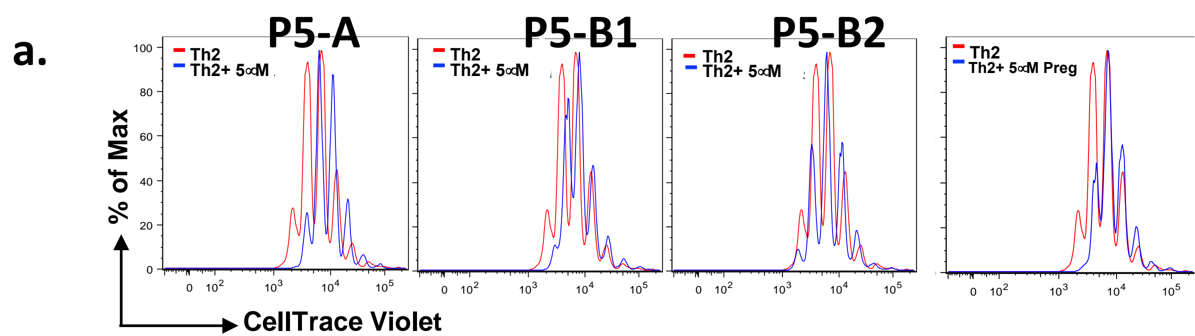
Figure S2. Bioactivity of Probes P5-A, P5-B1 and P5-B2, related to Figure 2

a. Probes **P5-B1**, **P5-B2** and **P5-A** mimic the biological activity of P5 in human prostate cancer cell line LNCaP. Equal number of LNCaP cells were cultured in a 96 well plate. The medium was replaced by charcoal stripped FBS containing medium with either the parent molecule P-5 or its chemical analogues P5-A, P5-B1 or P5-B2. XTT assay was used to check the viability of the cells treated with different concentration of the probes (2, 10 and 20 nM) respectively. Each concentration from individual probes had at least 5 independent replicates. Statistical test were done using Student's T test; * p<0.05, ** p<.0.01. Data are represented as mean +/- SEM.

b. Probes **P5-B1**, **P5-B2** and **P5-A** mimic the biological activity of P5 in human glioma cell line U87. After culturing equivalent number of U87 cells in 96 well plates the medium is replaced with charcoal stripped FBS containing medium. Subsequently the cells are treated with either 20, 60 and 80/100 μM of P5 or its analogues P5-A, P5-B1 or P5-B2. The XTT assay was used to determine the cell viability in at least 5 independent readings. Statistical test were done using Student's T test; * p<0.05, ** p<.0.01. Data are represented as mean +/- SEM.

Figure S3

a.



b.

0mM
15%
FSC-A
IgG1

10mM
5mM

0.45%
2.25%
P5-A

7.16%
7.39%
P5-B1

0.12%
6.57%
P5-B2

1.27%
5.58%
Preg

FSC-A
IgG1

Figure S3. Bioactivity of Probes P5-A, P5-B1 and P5-B2 in immune cells, related to Figure 2

a. Probe **P5-A**, **P5-B1**, and **P5-B2** mimic the biological activity of P5 in mouse primary Th2 immune cells and inhibit Th2 cell proliferation. Naive CD4+ T cells were stained with CellTrace Violet and activated under Th2 differentiation conditions for 72 hrs in the presence (red histogram) or absence (blue histogram) of pregnenolone or linker-tagged pregnenolene (P5-A, B1, and B2). We used two concentrations (10 μM and 5 μM). The cell proliferation profile was captured by flow cytometry-based dye decay assay.

b. Probes **P5-A**, **P5-B1**, and **P5-B2** mimic the class switching activity of P5 in mouse primary B immune cells and inhibit B cell immunoglobulin class switching. Naive resting B cells were induced with LPS and IL4 in the presence of different concentrations of P5 or P5-C (0, 5and 10 μM). Cell-surface expression of IgG1 was analyzed by flow cytometry on fifth day of stimulation. Data shown are representative of three independent experiments with three mice in each experiment.

Figure S4

a.

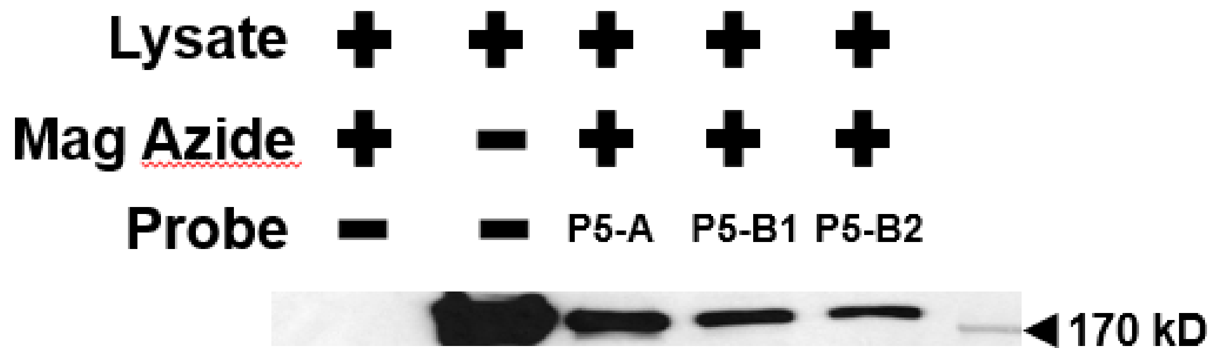


Figure S4. P5-A, P5-B1 and P5-B2 mimic native P5 – CLIP1 binding, related to Figure 2.

Binding capacity of the Probes **P5-B1**, **P5-B2** and **P5-A** with CLIP1 *in vitro*. HA-tagged CLIP1 was ectopically expressed in HEK cells. About 400 µg of whole cell lysate was used incubated with 50 nM P5-B1, P5-B2 and P5-A respectively. The azide-coated magnetic beads were clicked and pulled down using magnets. The magnetic-azide beads were incubated with cell lysate to capture any background binding of CLIP1 to the beads. SDS-PAGE and Western blotting followed by incubation with HA antibody revealed P5-B1, P5-B2 and P5-A binding to CLIP1.

Figure S5

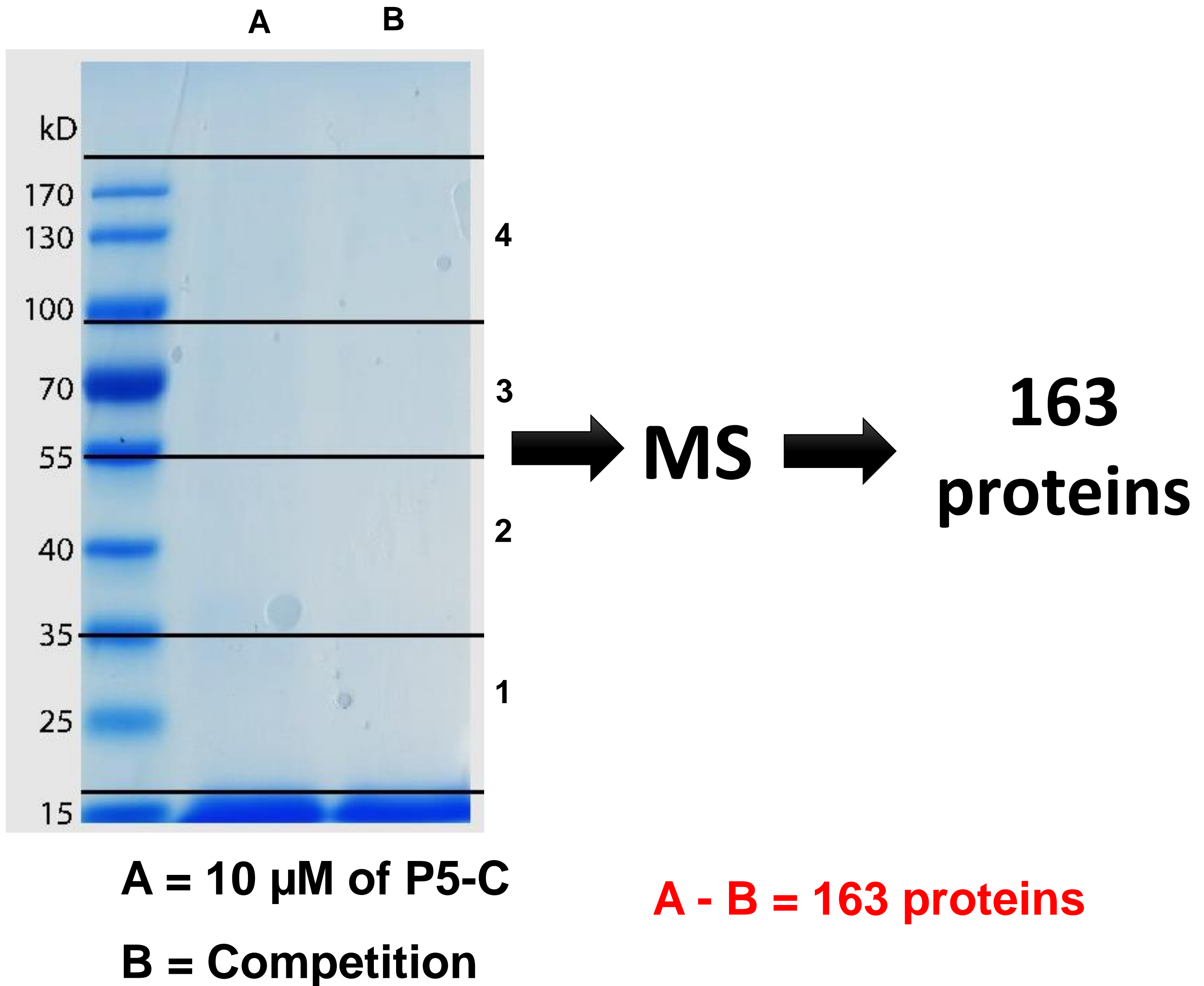


Figure S5. P5-C competition assay, related to Figure 3

CLICK proteomics after competition and subsequent mass spectrometry from gel band excision enriches 163 proteins. 10 million live LNCaP cells were incubated with P5-C alone or with competing amounts (10X) of P5. The P5-C protein complexes were CLICK-ed to biotin azide and pulled down using neutravidin beads. Subsequently, samples were loaded in lanes denoted A and B on a 10% SDS containing PAGE. After staining and destaining with colloidal blue the two lanes were cut into four equivalent parts and then sent for in-gel digestion followed by mass spectrometry.

Figure S6

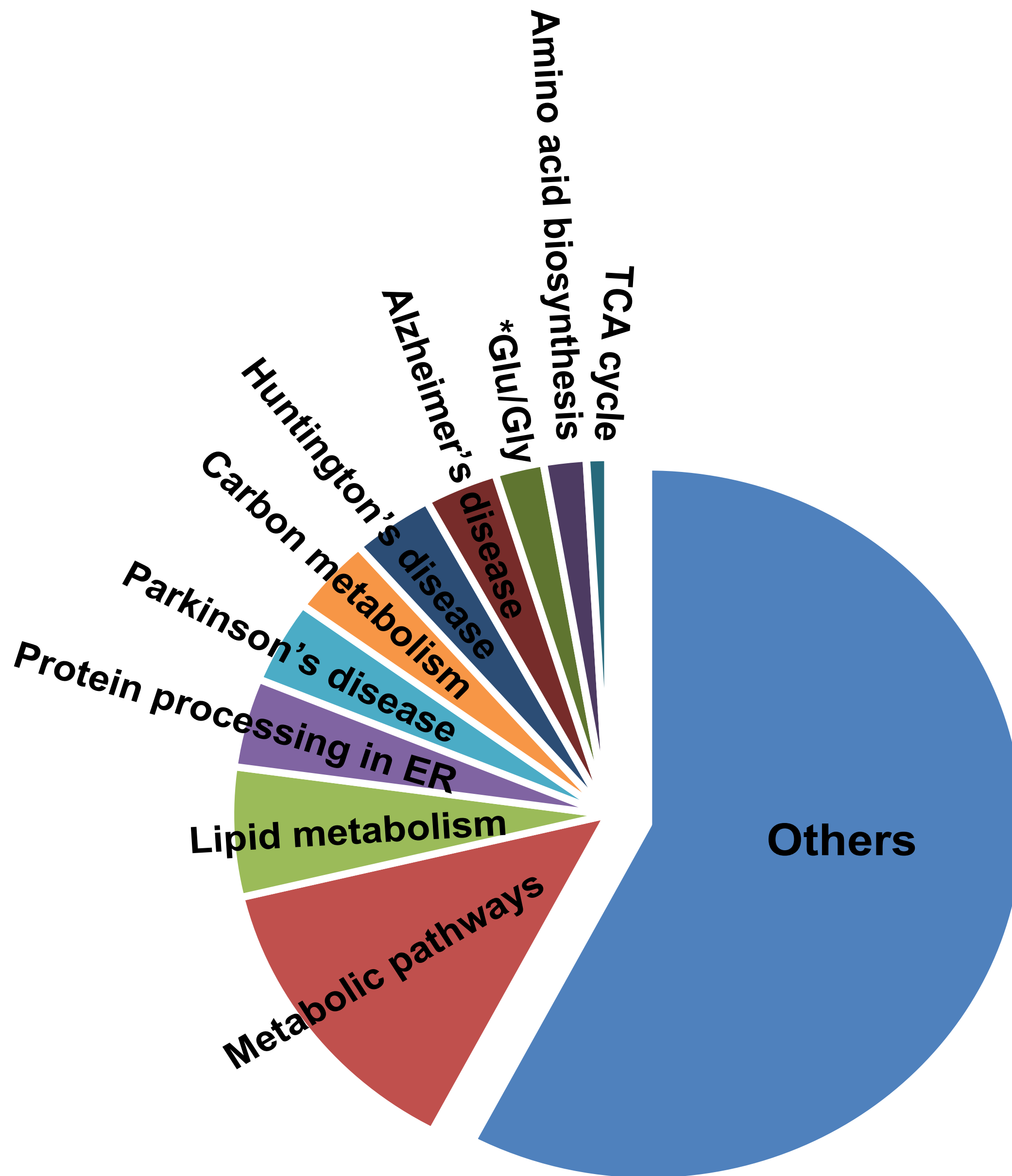


Figure S6. Functional categorization of P5 interactome from LNCaP cells, related to Figure 3.

Functional categorization of all the 442 proteins enriched from the LNCaP cells using two different mass spectrometry approach. Mass spectrometry after on-bead and in-gel digestion allowed us to enrich 441 proteins in the P5 interactome. DAVID functional classification analysis yielded the different categories of the enriched proteome.

Figure S7

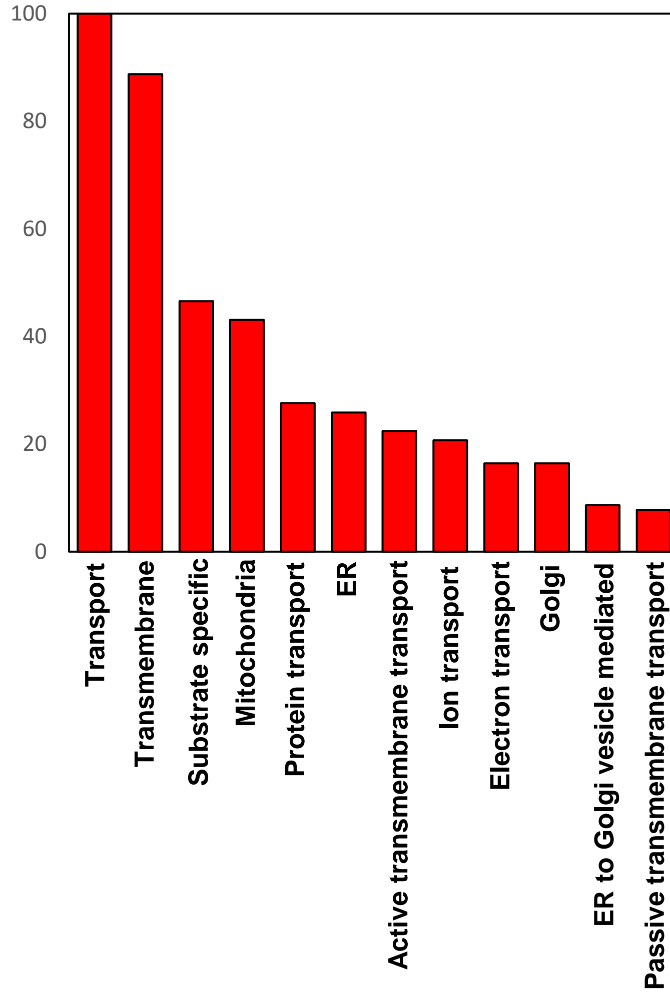


Figure S7. Transporter proteins from P5 interactome in LNCaP cells, related to Figure 3

P5 interacting with transporters proteins from 442 LNCaP proteins. Only those proteins that have proven role in transport were selected and classified.

Figure S8

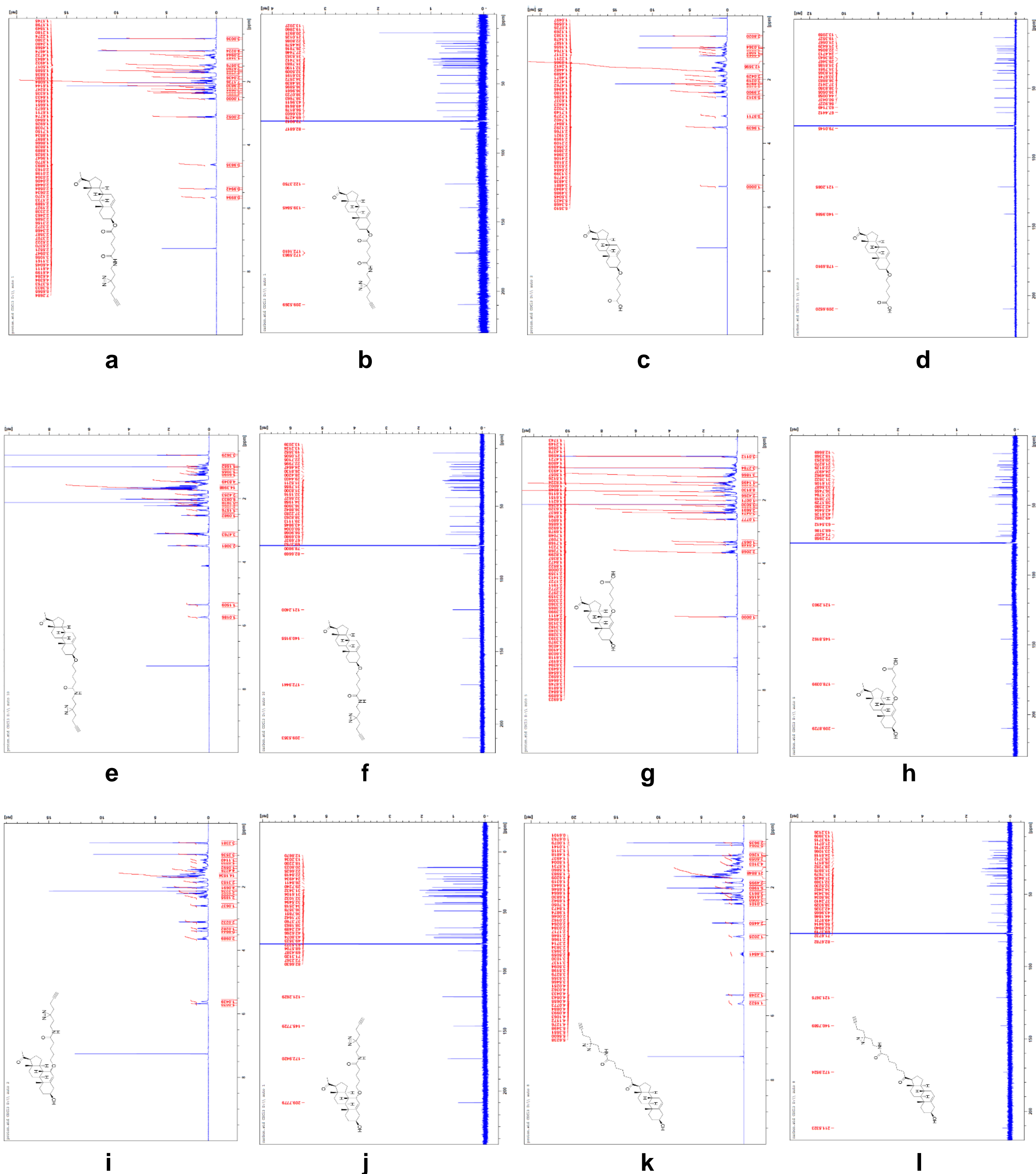


Figure S8 a-l. NMR spectra of the P5 analogues, related to figure 1

^{1}H NMR and ^{13}C NMR spectra of the intermediates and probes (**P5-A**, **P5-B1**, **P5-B2** and **P5-C**) along with their respective structures.

Figure S9

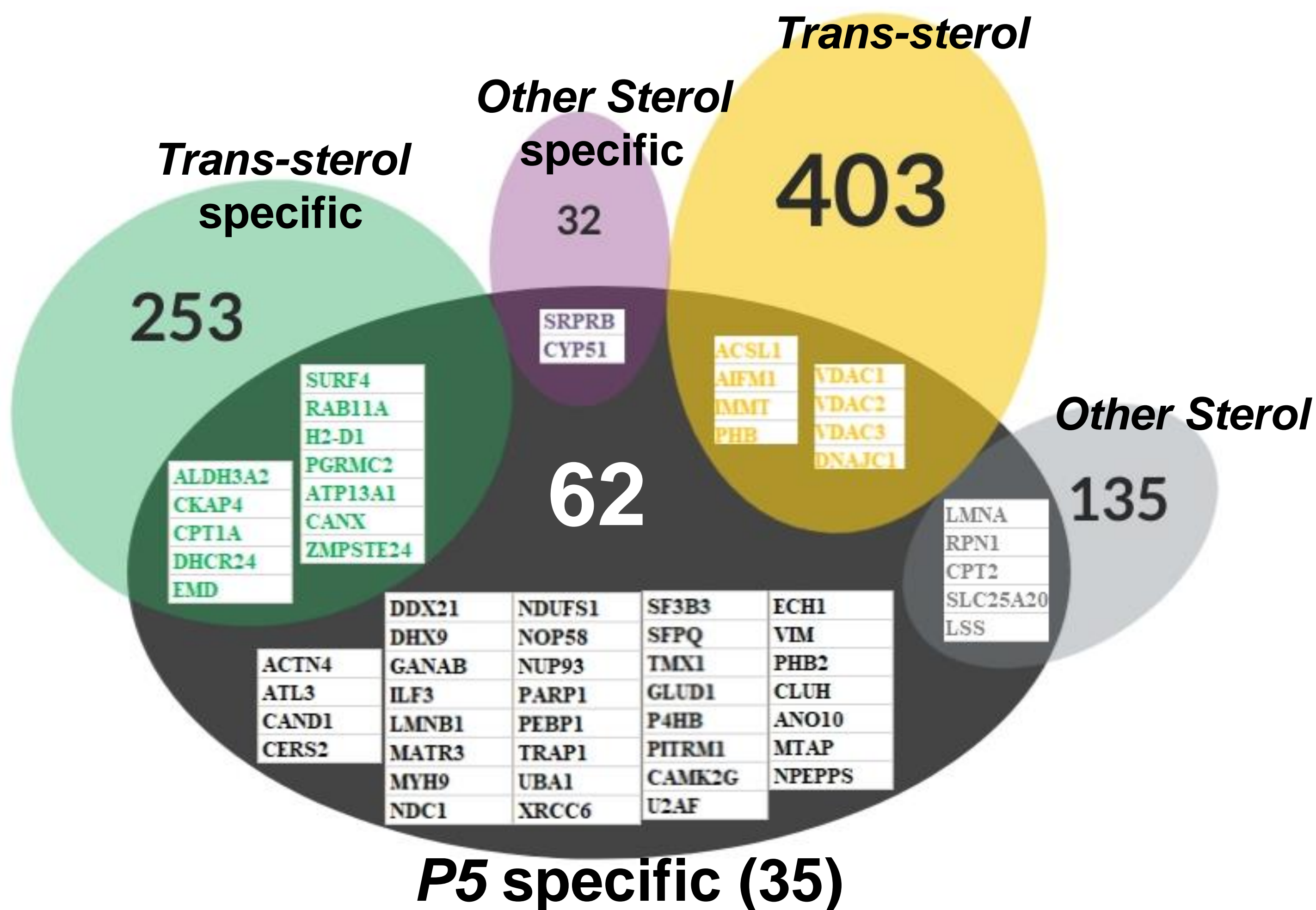


Figure S9. P5-specific interactome, related to Figure 4 and Table 1.

Comparison of our P5 interactome with different sterol probes from Hulce et al., Nature Methods 2013[18]. Based on the sensitivity to cholesterol and specificity to either trans-sterol or other sterol probes the sterol proteome is divided into 4 categories. Those that are termed ‘specific’ show cholesterol sensitivity in competition assay. The other two categories are not affected by cholesterol competition. Filtering them out we show the P5 specific interactome.

Figure S10

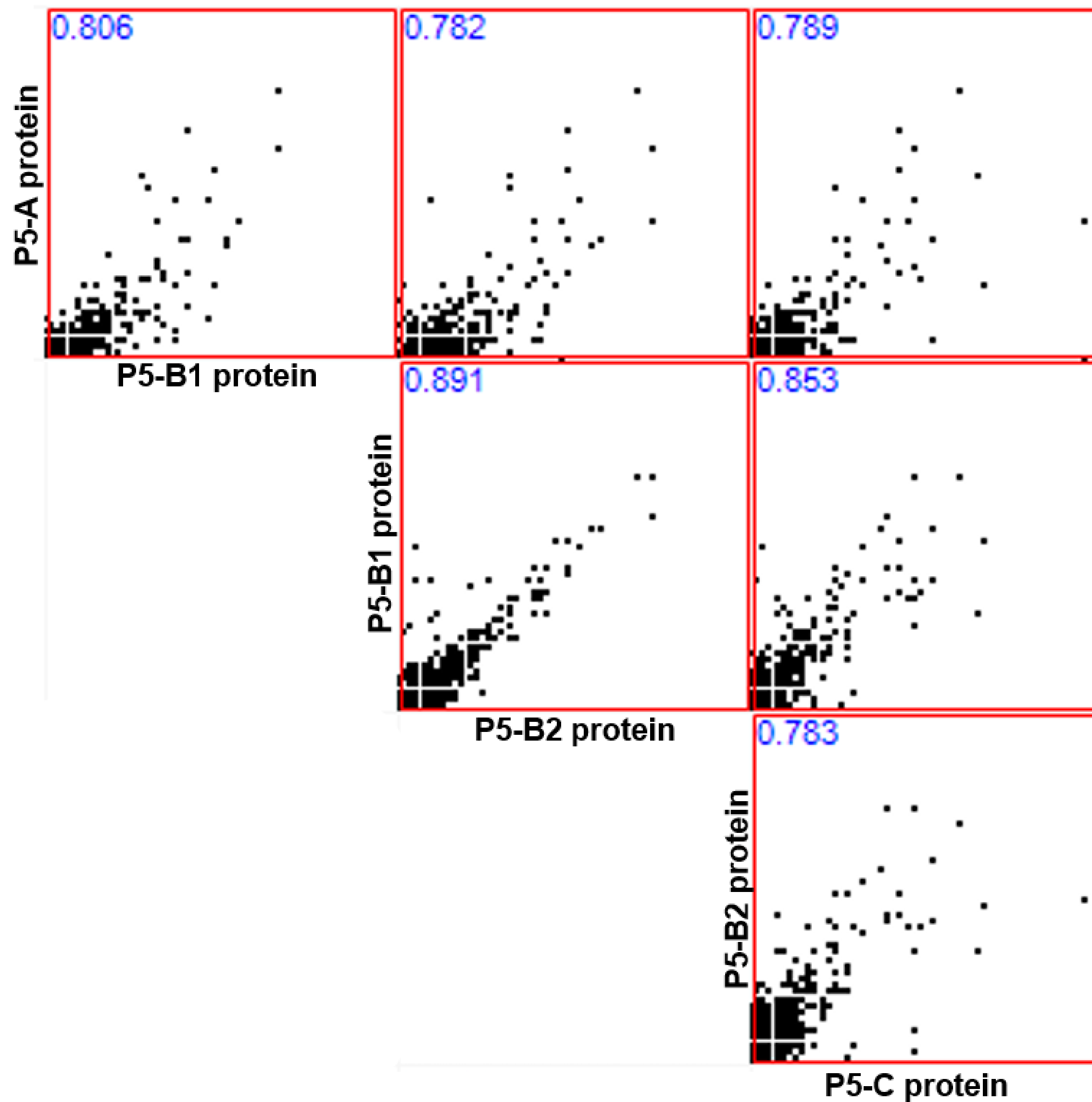


Figure S10. Correlation between protein interactomes captured by the different probes, related to figure 3.

The correlation coefficient of each pair is given on the top (in blue). To do this we used the in-built algorithm in the Perseus software[20].

Transparent Methods

Chemical synthesis

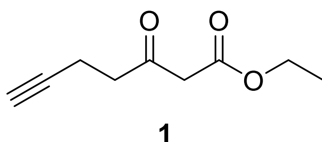
General Methods

Solvents were purified and dried using standard methods prior to use: THF and $E_{t2}O$ by distillation from calcium hydride and lithium aluminium hydride; CH_2Cl_2, toluene and acetonitrile by distillation from calcium hydride. Petroleum ether 40-60 refers to petroleum ether distillate (BP = 40-60 °C). All other solvents were used as supplied unless otherwise stated. All reagents were used as supplied or purified using standard procedures as necessary. *n*-BuLi was titrated in triplicate using menthol and 1,10-phenanthroline in THF at 0°C prior to use. Flash column chromatography was performed using Breckland Scientific silica gel 60, particle size 40-63 nm under air pressure. All solvents used for chromatographic purification were distilled prior to use. Analytical thin layer chromatography (TLC) was performed using silica gel 60 F_{254} pre-coated glass backed plates and visualised by ultraviolet radiation (254 nm), potassium permanganate or ninhydrin as appropriate. ^{1}H NMR spectra were recorded on Bruker DPX-400 (400 MHz) or Bruker DRX-600 (600 MHz) spectrometers. Chemical shifts are reported in ppm with the resonance resulting from incomplete deuteration of the solvent as the internal standard (CDCl3: 7.26 ppm). ^{13}C NMR spectra were recorded on Bruker DPX-400 (100 MHz) or Bruker DRX-600 (150 MHz) spectrometers with complete proton decoupling. Chemical shifts are reported in ppm with the solvent resonance as the internal standard ($^{13}CDCl_3$: 77.2 ppm, triplet). Data are reported as follows: chemical shift δ ppm (integration (^{1}H only), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, br = broad, app = apparent, m = multiplet or combinations thereof, coupling constants J = Hz, assignment). ^{13}C signals are singlets unless otherwise stated, where they are shown in

the same format as ^{1}H NMR.High resolution mass spectrometry (HRMS) was performed on a Waters Micromass LCT spectrometer using electrospray ionisation and Micromass MS software. HRMS signals are reported to 4 decimal places and are within 5 ppm of theoretical values.Infrared spectra were recorded neat as thin films on a Perkin-Elmer Spectrum One FTIR spectrometer andonly selected peaks are reported (s = strong, m = medium, w = weak, br = broad).

Chemical abbreviations: DIPA – diisopropylamine; DIPEA – *N*-*N*-diisopropylethylamine ; DMAP – 4-dimethylaminopyridine; DMF – *N*-*N*-dimethylformamide; HBTU - (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; PTSA – *para*-toluenesulfonic acid

Ethyl 3-oxohept-6-ynoate (1)

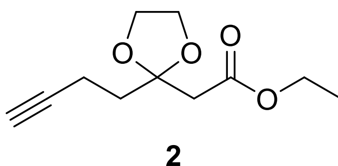


Diisopropylamine (42.0 mL, 300 mmol) was added to anhydrous THF (250 mL) under an atmosphere of argon and then cooled to 0 °C. *n*-BuLi (120 mL, 2.5 M in hexane, 300 mmol) was then added to the stirred solution dropwise *via* cannula syringe. After 15 min, ethyl acetoacetate (19.0 mL, 150 mmol) was added dropwise then left for 30 min. Propargyl bromide (16.7 mL, 150 mmol) was added dropwise to the solution and then stirred for 2 h at 0 °C. The solution was allowed to warm to room temperature then stirred for a further 15 min before quenching with glacial AcOH (9 mL) and the solution stirred for a further 15 min. The solution was diluted with Et_2O (500 mL), washed with H_2O (2 x 200 mL) and brine (200 mL) then dried over $MgSO_4$. The solvent was removed *in vacuo* and the crude mixture was purified

by distillation using a kugelrohr (1.7 Torr, 108 °C) to give **1** (10.8 g, 64.3 mmol, 43% yield) as a colourless oil.

IR (thin film, cm^{-1}): 3284 (w), 1741 (s), 1714 (s); ^{1}H NMR (600 MHz, $CDCl_3$) δ = 1.29 (3H, t, J = 7.1 Hz), 1.96 (1H, t, J = 2.3 Hz), 2.48 (2H, td, J = 7.2 Hz, 2.5 Hz), 2.82 (2H, t, J = 7.3 Hz), 3.47 (2H, s), 4.21 (2H, q, J = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 12.9, 14.2, 41.8, 49.5, 61.6, 69.2, 82.5, 167.3, 200.8; HRMS (*m/z*): $[M+Na]^+$calcd. for $C_9H_{12}NaO_3$, 191.0679; found, 191.0678.

Ethyl 2-(2-(but-3-yn-1-yl)-1,3-dioxolan-2-yl)acetate (2)

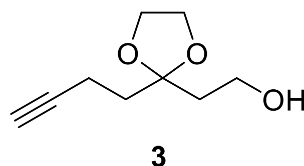


1(8.00 g, 47.6 mmol) was mixed with *para*-toluene sulfonic acid (0.24 g, 1.27 mmol) and ethylene glycol (13.3 mL, 238 mmol) in toluene (100 mL) in a dean stark apparatus and refluxed for 5 h. After this time the flask was cooled to room temperature then washed with sat. $NaHCO_3$ (3 x 80 mL) and brine (30 mL) then dried over $MgSO_4$. The solvent was removed *in vacuo*to give **2** (8.01 g, 37.8 mmol, 79% yield) as a pale yellow oil.

IR (thin film, cm^{-1}): 3292 (w), 1731 (s); ^{1}H NMR (600 MHz, $CDCl_3$) δ = 1.17 (3H, t, J = 7.1 Hz), 1.86 (1H, m), 2.00 (2H, m), 2.21 (2H, m), 2.55 (2H, m), 3.90 (4H, m), 4.06 (2H, q, J = 7.1 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 12.7, 14.1, 36.2, 42.5, 60.5,

65.1, 68.1, 83.8, 108.1, 169.1; HRMS (*m/z*): $[M+Na]^+$calcd. for $C_{11}H_{16}NaO_4$, 235.0941; found, 235.0955.

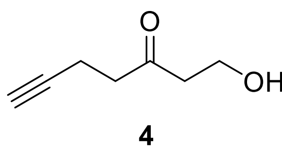
2-(2-(But-3-yn-1-yl)-1,3-dioxolan-2-yl)ethan-1-ol (3)



$LiAlH_4$ (1.43 g, 37.7 mmol) was suspended in anhydrous ether (250 mL) under an atmosphere of argon and the stirred suspension cooled to 0 °C. **2** (8.00 g, 37.7 mmol) was added dropwise to the flask and then left for 15 min. To quench the residual $LiAlH_4$, H_2O (1.43 mL) was added slowly followed by a 15% (w/v) solution of NaOH (1.43 mL) and then H_2O (4.29 mL). The mixture was stirred for 1 h until a white precipitate formed. $MgSO_4$ was added to the flask then the suspension filtered. Solvents were removed *in vacuo* to give **3** (6.25 g, 36.8 mmol, 97% yield) as a pale yellow oil.

IR (thin film, cm^{-1}): 3500-3200 (br w), 3288 (w); ^1H NMR (600 MHz, $CDCl_3$) δ = 1.93 (5H, m), 2.25 (2H, m), 2.71 (1H, br s), 3.72 (2H, m), 3.98 (4H, m); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 13.1, 35.8, 38.2, 58.6, 64.9, 68.3, 83.9, 111.0; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_9H_{15}O_3$, 171.1016; found, 171.1015.

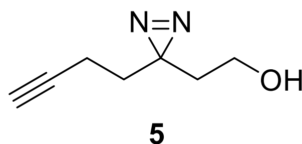
1-Hydroxyhept-6-yn-3-one (4)



3 (6.27 g, 36.8 mmol) was added dropwise to a solution of *para*-toluene sulfonic acid (1.75 g, 9.21 mmol) in acetone (100 mL) and H_2O (1.5 mL) and stirred at room temperature for 5 h. The reaction was quenched with sat. $NaHCO_3$ (150 mL), extracted with CH_2Cl_2 (5 x 150 mL) then the organic layer was washed brine (100 mL) and dried over $MgSO_4$. Solvents were removed *in vacuo* to give **4** (4.33 g, 34.3 mmol, 93% yield) as a pale yellow oil.

IR (thin film, cm^{-1}): 3550-3200 (br w), 3291 (w), 1708 (s); ^1H NMR (600 MHz, $CDCl_3$) δ = 1.94 (1H, t, J = 2.7 Hz), 2.43 (2H, td, J = 7.3 Hz, 2.7 Hz), 2.67 (5H, m), 3.83 (2H, t, J = 5.6 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 12.7, 41.7, 44.6, 57.6, 64.9, 82.8, 209.1. HRMS (*m/z*): $[M+H]^+$ calcd. for $C_7H_{11}O_2$, 127.0754; found, 127.0753.

2-(3-(But-3-yn-1-yl)-3H-diazirin-3-yl) ethan-1-ol (5)

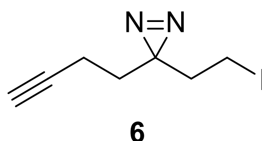


4 (2.80 g, 22.2mmol) was added to a flask and placed under an atmosphere of argon then ammonia (30 mL) was condensed into the flask at -78 °C. The solution was refluxed at -35 °C for 5 h then cooled to -78 °C and a solution of hydroxylamine sulfonic acid (2.90 g, 25.6 mmol) in MeOH (20 mL) was added dropwise. The reaction was then refluxed at -35 °C for 1 h. The reaction was then allowed to warm to room temperature and left open to the atmosphere and stirred overnight. The suspension was then filtered, washing with MeOH with the liquor retained. The solvent was then removed *in vacuo* and the resultant solid dissolved in CH_2Cl_2 (10 mL). The solution was cooled to 0 °C and iodine (5.60 g, 22.2 mmol) was added portion wise until the solution retained a brown colour. The reaction mixture was

diluted with CH_2Cl_2 (80 mL) then washed with 10% HCl solution (30 mL), sat. sodium thiosulfate (80 mL), H_2O (40 mL) and brine (40 mL) respectively. The solution was then dried over $MgSO_4$ and solvent removed *in vacuo* and the crude mixture purified using flash column chromatography (hexanes:EtOAc) to give **5** (0.965 g, 6.98 mmol, 32% yield) as a colourless oil.

IR (thin film, cm^{-1}): 3550-3200 (br w), 3294 (w); ^{1}H NMR (600 MHz, $CDCl_3$) δ = 1.69 (5H, m), 2.00 (1H, t, J = 2.7 Hz), 2.04 (2H, td, J = 7.4 Hz, 2.7 Hz), 3.49 (2H, t, J = 6.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 13.2, 26.6, 32.6, 35.5, 57.3, 69.2, 82.8; HRMS (*m/z*): $[M-H]^-$calcd. for $C_7H_9N_5O$, 137.0720; found, 137.0714.

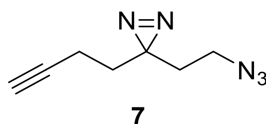
3-(But-3-yn-1-yl)-3-(2-iodoethyl)-3H-diazirine (6)



Iodine (1.54 g, 6.08 mmol) was added to a solution of PPh_3 (1.46 g, 5.58 mmol) and imidazole (1.04 g, 68.1 mmol) in CH_2Cl_2 (16 mL) in a flask protected from light and stirred for 10 min at 0 °C. **5** (0.700 g, 5.07 mmol) in CH_2Cl_2 (2 mL) was then added and the reaction stirred for 4 h. The excess iodine was quenched by sat. sodium thiosulfate solution (40 mL) and the mixture extracted with EtOAc (2 x 50 mL). The organic layer was washed with H_2O (40 mL) brine (40 mL) and dried over $MgSO_4$. The solvents were removed *in vacuo* and the crude mixture purified using flash column chromatography (hexanes: 3% EtOAc) to obtain **6** (0.907 g, 3.65 mmol, 72% yield) as a colourless oil.

IR (thin film, cm^{-1}): 3296 (w); ^{1}H NMR (600 MHz, $CDCl_3$) δ = 1.68 (2H, t, J=7.2 Hz), 2.02 (3H, m), 2.11 (2H, J=7.7 Hz), 2.88 (2H, t, J=7.7 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ = -4.0, 13.2, 28.6, 31.8, 37.5, 69.4, 82.4.

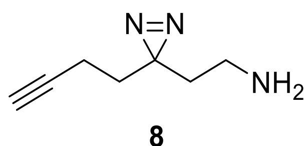
3-(2-Azidoethyl)-3-(but-3-yn-1-yl)-3H-diazirine (7)



6 (0.907 g, 3.65 mmol) was added to a solution of DMF (10 mL) and NaN_3 (0.286 g, 4.40mmol) under an atmosphere of argon, then the solution was heated to 75 °C with stirring for 2 h. The reaction was allowed to cool to room temperature then was diluted with H_2O (30 mL) and EtOAc (75 mL). The organic layer was retained and then washed with 5% (w/v) LiCl solution (2 x 30 mL), H_2O (30 mL) and brine (30 mL) then dried over $MgSO_4$. The solvent was removed *in vacuo* to give **7** (0.503 g, 3.07 mmol, 82% yield) as a pale yellow oil.

IR (thin film, cm^{-1}): 3299 (w), 2093 (s); ^{1}HNMR (600 MHz, $CDCl_3$)δ = 1.69 (4H, m), 2.02 (3H, m), 3.16 (2H, t, J= 6.8 Hz);^{13}CNMR (150 MHz, $CDCl_3$) δ =13.2, 26.4, 32.2, 32.4, 45.9, 63.4, 82.5;HRMS (*m/z*): $[M-H]^-$calcd. for $C_7H_8N_5$, 162.0785; found, 162.0783.

2-(3-(But-3-yn-1-yl)-3H-diazirin-3-yl)ethan-1-amine (8)

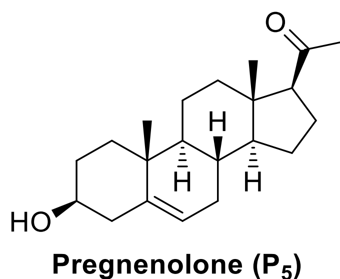


PPh_3 (0.734 g, 2.80mmol) and **7** (0.400 g, 2.45 mmol) were added to a 10:1 solution of THF:H_2O (5 mL) then stirred for 5 h at room temperature. 1 N HCl (10 mL) was added and the aqueous phase was washed withCH_2Cl_2 (2 x 15 mL). The solution was then made basic with 1 N NaOH (20 mL) and then extracted with CH_2Cl_2 (2 x 15 mL) and the organic layer dried over$MgSO_4$. The solvent was removed *in vacuo* to give **8** (0.245 g, 1.79 mmol, 73% yield) as a pale yellow oil.

^{1}HNMR (600 MHz, $CDCl_3$)δ =1.14 (2H, s),1.65 (4H, m), 2.01 (3H, m), 2.51 (2H, t, J = 7.1 Hz);^{13}C NMR (150 MHz, $CDCl_3$) δ =13.3, 26.9, 32.6, 36.2, 36.7, 69.1, 82.7; HRMS (*m/z*): $[M+H]^+$calcd. for $C_7H_{12}N_3$, 138.1026; found, 138.1024.

In **Scheme 2A**, starting from the pregnenolone acetate (obtained from Sigma Aldrich) the synthesis of the probe **P5-B1** has been shown.

Pregnenolone (P5)



Pregnenolone (P_5)

Pregnenolone acetate (1.00g, 2.80mmol) was added to *t*-BuOH (20 mL) which had been warmed to 42°C. To this an aqueous solution of KOH (1.71 mL, 1.06 g, 16.07 mmol) was added and the mixture was stirred overnight at 42 °C. The reaction was quenched with 5% HCl solution (30 mL) and extracted with Et_2O (2 x 30 mL). The organic phase was dried with $MgSO_4$, filtered and evaporated. The resultant white solid was recrystallized in methanol to obtain pregnenolone (0.761g, 2.40mmol, 86% yield).

IR (thin film, cm^{-1}): 3507 (m), 1683 (s); ^{1}H NMR (600 MHz, $CDCl_3$) δ =0.68 (3H, s), 0.99 (1H, m), 1.01 (3H, s), 1.09-1.19 (2H, m), 1.24 (1H, m), 1.44-1.56 (5H, m), 1.60-1.71 (3H, m),1.86 (2H, m), 1.99-2.07 (2H, m), 2.14 (3H, s), 2.15-2.27 (2H, m), 2.35 (1H, m), 2.54 (1H, t, J = 9.0 Hz), 3.54 (1H, m), 5.36 (1H, m); ^{13}C NMR (150MHz, $CDCl_3$) δ = 13.2, 19.4, 21.1, 22.8, 24.5, 31.5, 31.6, 31.8, 31.9, 36.5, 37.3, 38.8, 42.3, 44.0, 50.0, 56.9, 63.7, 71.7, 121.4, 140.7, 209.5; HRMS (*m/z*): $[M+Na]^+$calcd. for $C_{21}H_{32}NaO_2$, 339.2295; found, 339.2290.

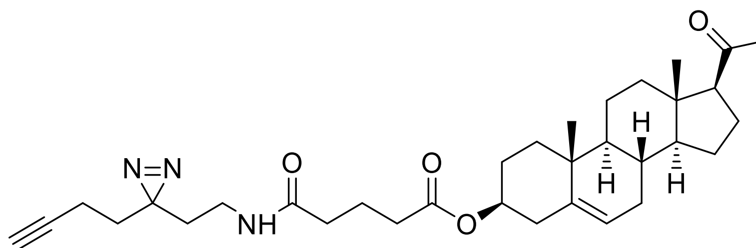
Compound 9

Pregnenolone (0.640 g, 2.02mmol), glutaric anhydride (0.920 g, 8.04mmol) and 4-(dimethylamino) pyridine (0.042 g, 0.34 mmol) were added to pyridine (6 mL) which had been placed under an atmosphere of argon. The solution was stirred overnight at

60°C then allowed to cool to room temperature.1 N HCl (15 mL) was added and then the solution was extracted with Et_2O (2 x 40 mL). The organic layer was then washed once with water (40 mL) and brine (40 mL) then dried over $MgSO_4$. The solvent was then removed *in vacuo* then purified by flash column chromatography (hexane:ethyl acetate, 4:1) to yield **9** (0.512g, 1.19 mmol, 57% yield) as an off white powder.

IR (thin film, cm^{-1}):3100-2850 (br), 1729 (s), 1667 (s); ^1H NMR (600 MHz, $CDCl_3$) δ= 0.60 (3H, s), 1.00-1.04 (4H, m), 1.12-1.28 (3H, m), 1.42-1.52 (3H, m), 1.55-1.72 (5H, m), 1.87 (2H, m), 1.94-2.06 (4H, m), 2.13 (3H, s), 2.18 (1H, m), 2.32 (2H, m), 2.38 (2H, t, J = 7.3 Hz), 2.44 (2H, t, J = 7.3 Hz), 2.54 (1H, t, J = 9.0 Hz), 4.63 (1H, m), 5.38 (1H, d, J = 5.0 Hz);^{13}C NMR (150MHz, $CDCl_3$) δ = 13.2, 19.3, 19.9, 21.0, 22.8, 24.5, 27.7, 31.5, 31.7, 31.8, 32.9, 33.5, 36.6, 37.0, 38.1, 38.8, 44.0, 49.9, 56.8, 63.7, 74.0, 122.4, 139.6, 172.3, 178.5, 209.7.; HRMS (*m/z*): $[M+Na]^+$calcd. for $C_{26}H_{38}NaO_5$, 453.2611; found, 453.2609.

Probe P5-B1



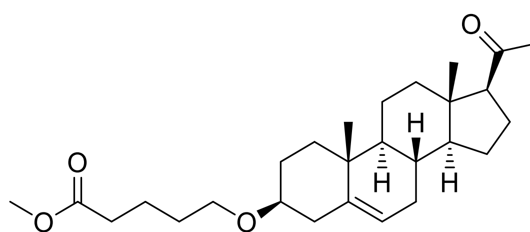
P5-B$_1$

HBTU (0.232 g, 0.632mmol) and **9** (0.112 g, 0.252 mmol) were added to a flask and placed under argon. DMF (6 mL), DIPEA (0.110 mL, 0.632 mmol) and **8** (0.040 g,

0.292 mmol) were added by syringe. The solution was stirred for 16 h at room temperature then H_2O (30 mL) was added to the solution then it was extracted with Et_2O (2 x 40 mL). This was washed with 5% (w/v) LiCl (40 mL), H_2O (3 x 30 mL), brine (40 mL) and then dried over Na_2SO_4. The solvent was removed *in vacuo* then purified by flash column chromatography (hexane:ethyl acetate: 7:3) to obtain **P5-B1** (0.053 g, 0.096 mmol, 38% yield). The product appeared approximately 90% pure by ^{1}HNMR.

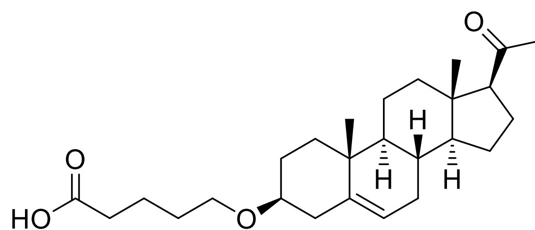
IR (thin film, cm^{-1}):3352 (w), 3299 (m), 1728 (s), 1702 (s), 1648 (s); ^{1}H NMR (600 MHz, $CDCl_3$) δ =0.64 (3H, s), 1.03 (4H, m), 1.12-1.19 (2H, m), 1.20-1.24 (1H, m), 1.43-1.52 (3H, m), 1.56-1.64 (3H, m), 1.64-1.68 (4H, m), 1.69-1.72 (2H, m), 1.83-1.87 (2H, m), 1.94-2.08 (7H, m), 2.14 (3H, s), 2.19 (1H, m), 2.25 (2H, t, J = 7.4 Hz), 2.32 (2H, m), 2.37 (2H, t, J = 7.2 Hz), 2.54 (1H, t, J = 9.0 Hz), 3.11 (2H, app q, J = 6.3 Hz), 4.62 (1H, m), 5.38 (1H, d, J = 4.9Hz), 5.66 (1H, br s); ^{13}C NMR (150 MHz, $CDCl_3$) δ =13.2 (2C, m), 19.3, 20.9, 21.0, 22.8, 24.5, 26.8, 27.8, 31.5, 31.76, 31.80, 32.1, 32.5, 33.6, 34.3, 35.5, 36.6, 37.0, 38.1, 38.8, 44.0, 49.9, 56.8, 63.7, 69.4, 73.9, 82.7, 122.4, 139.6, 172.2, 172.6, 209.5;HRMS (*m/z*): $[M+H]^+$calcd. for $C_{33}H_{48}O_4N_3$, 550.3639; found, 550.3632.

Scheme 2B depicts the synthesis of **P5-B2** starting from **10** which was synthesised by NewChem Technologies Ltd, Durham, UK.



10

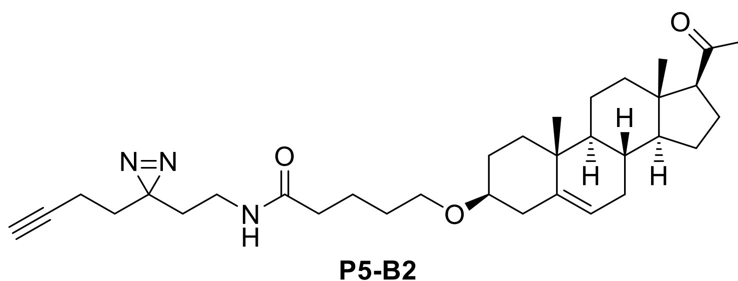
Compound 11



10 (0.101 g, 0.230mmol) was added to a mixture of MeOH:THF:H_2O1:1:1 (6 mL) LiOH.H_2O (0.041 g, 0.977mmol) was added and the solution was stirred at room temperature for 2 h. Subsequently the reaction was acidified with 1M HCl solution (10 mL) then extracted using Et_2O (3 x 40 mL). The organic layer was washed with H_2O (40 mL), brine (40 mL) and dried over Na_2SO_4. The solvents were removed *in vacuo* to give **11** (0.084g, 0.201 mmol, 88% yield) as a white powder.

IR (thin film, cm^{-1}):3300-2960 (br), 1731 (s), 1704 (s);^{1}H NMR (600 MHz, $CDCl_3$) δ = 0.60 (3H, s), 0.98 (4H, m), 1.04 (1H, m), 1.15 (1H, m), 1.21-1.27 (1H, m), 1.41-1.72 (12H, m), 1.88 (2H, m), 2.02 (2H, m), 2.12 (3H, s), 2.19 (2H, m), 2.38 (3H, m), 2.53 (1H, t, J = 9.0 Hz), 3.13 (1H, m), 3.49 (2H, m), 5.34 (1H, m); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 13.2, 19.4, 21.1, 21.7, 22.8, 24.5, 28.4, 29.4, 31.5, 31.8, 31.9, 33.7, 36.9, 37.3, 38.8, 39.1, 44.0, 50.1, 56.9, 63.7, 67.5, 79.0, 121.2, 141.0, 178.7, 209.6; HRMS (*m/z*): $[M+Na]^+$calcd. for$C_{26}H_{40}NaO_4$, 439.2819; found, 439.2811.

Probe P5-B2

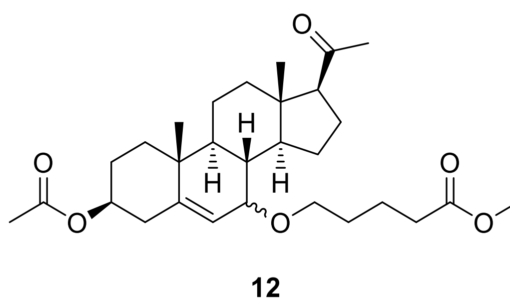


DMF (6 mL) was added to a flask containing **11** (0.080 g, 0.192mmol) and HBTU (0.174 g, 0.458mmol) that had been placed under an atmosphere of argon. DIPEA (0.08 mL, 0.459mmol) and **8** (0.032 g, 0.233mmol) were then added to the flask and it was stirred for 16 h. The reaction was then diluted with H_2O (40 mL) then the solution extracted with Et_2O (3 x 40 mL). The organic layer was washed with 5% (w/v) LiCl solution (60 mL), H_2O (40 mL) and brine (40 mL) then dried over Na_2SO_4. The crude mixture was concentrated *in vacuo* then purified by flash column chromatography (hexane:ethyl acetate: 7:3) to give **P5-B2** (0.045 g, 0.084 mmol, 44% yield) as a colourless gum.

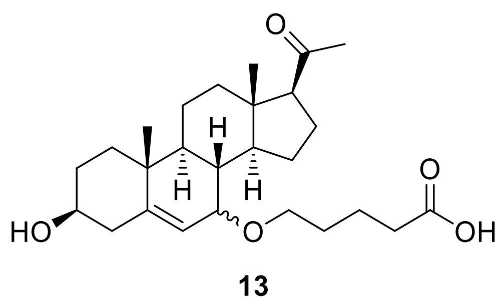
IR (thin film, cm^{-1}): 3311 (m), 1702 (s), 1646 (s); ^{1}H NMR (600 MHz, $CDCl_3$)δ =0.62 (3H, s), 0.97 (1H, m), 0.99 (3H, s) 1.04 (1H, m), 1.11-1.18 (1H, m), 1.20-1.28 (2H, m), 1.41-1.52 (4H, m) 1.55-1.74 (13H, m), 1.84-1.91 (2H, m), 1.97-2.05 (5H, m), 2.11 (3H, s), 2.14-2.32 (4H, m), 2.36 (1H, m), 2.52 (1H, t, J = 9.0 Hz), 3.08-3.15 (3H, m), 3.45-3.50 (2H, m), 5.33 (1H, m), 5.73 (1H, br s);^{13}C NMR (150 MHz, $CDCl_3$) δ = 13.22, 13.23, 19.4, 21.1, 22.7, 22.8, 24.5, 26.8, 28.4, 29.5, 31.5, 31.78, 31.83, 32.2, 32.6, 34.2, 36.4, 36.9, 37.2, 38.8, 39.1, 44.0, 50.0, 56.9, 63.7, 67.7, 69.4, 79.0, 82.7, 121.2, 140.9, 172.9, 209.5; HRMS (*m/z*): $[M+H]^+$calcd. For$C_{33}H_{50}N_3O_3$, 536.3847; found, 536.3854.

Please note that within the proton NMR for this compound we have reported 51 proton peaks, whilst it contains only 49. It is believed this is due to a minor impurity within the sample. Due to the clustering of signals from the steroidal core, these cannot be isolated and therefore we have reported the spectra that are observed as we cannot determine where the extra protons are within the spectra. It should be noted that all other data is consistent for the reported product.

Scheme 3 shows the synthesis of **P5-C** which starts from compound **12** which was synthesised by NewChem Technologies Ltd, Durham, UK.



Compound 13



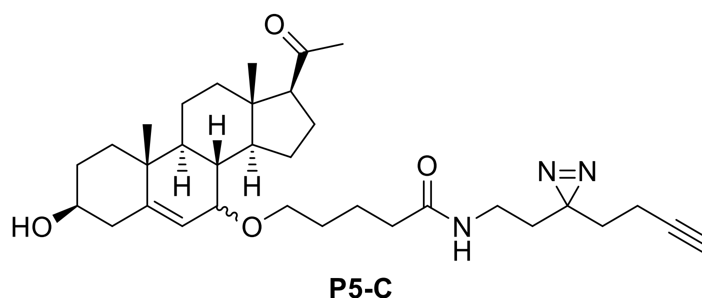
LiOH.H_2O (0.052 g, 1.20mmol) was added to solution of **12** as a 9:1 mix of diastereomers (0.101 g, 0.204 mmol) in a solution of MeOH:THF:H_2O 1:1:1 (6 mL) and stirred for 4 h. The solution was acidified with 1M HCl (15 mL) then the solution extracted with Et_2O (3 x 30 mL). The organic layer was washed with H_2O (40 mL)

and brine (40 mL) then dried over Na_2SO_4. The solvents were removed *in vacuo* to give **13** (0.085g, 0.196 mmol, 98% yield) as colourless gum, as a mixture of the two diastereomers, with one predominant isomer (approximately 9:1 by ^{1}HNMR based on protons at position 6 of the steroid core).

IR (thin film, cm^{-1}):3550-2990 (br), 1701 (s, with shoulder); ^{1}H NMR (600 MHz, $CDCl_3$) δ = 0.62 (3H, s), 0.98 (3H, s), 1.14-1.26 (3H, m), 1.41 (1H, m), 1.45-1.55 (4H, m), 1.59-1.76 (9H, m), 1.83-1.88 (2H, m), 2.00 (1H, m), 2.14 (3H, s), 2.18 (1H, m), 2.27-2.36 (2H, m), 2.39 (2H, t, J = 7.4 Hz), 2.61 (1H, t, J = 8.9 Hz), 3.32 (1H, dt, J = 9.1 Hz, 6.3 Hz), 3.40 (1H, m), 3.58-3.67 (2H, m), 5.68 (1H, dd, J = 4.9 Hz, 1.6 Hz); ^{13}CNMR (150 MHz, $CDCl_3$) δ = 12.9, 18.3, 20.8, 21.8, 22.8, 24.5, 29.5, 31.4, 31.6, 33.6, 36.8, 37.2, 37.4, 38.2, 42.3, 42.6, 43.8, 49.3, 63.6, 68.3, 71.4, 72.3, 121.3, 145.8, 178.0, 209.9; HRMS (*m/z*): $[M+Na]^+$calcd. For $C_{26}H_{40}NaO_5$, 455.2768; found, 455.2752.

Due to diastereotopic mixture the NMR cannot be comprehensively assigned. As such the peaks described here are those observed.

Probe P5-C



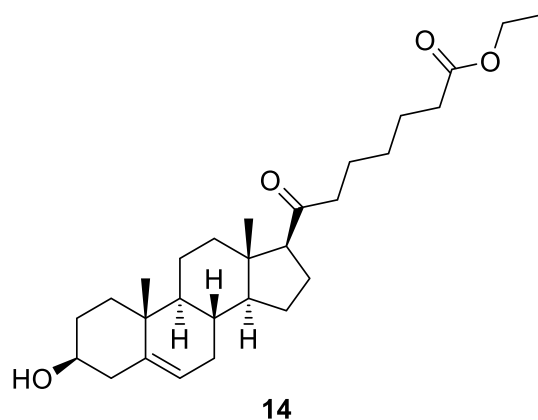
P5-C

A flask containing **13** (0.085 g, 0.196mmol) and HBTU (0.182 g, 0.480mmol) was placed under an atmosphere of argon then DMF (6 mL), DIPEA (0.084 mL, 0.482mmol) and compound **8** (0.033 g, 0.242mmol) were added. The solution was stirred for 16 hat room temperature. The reaction was diluted with H_2O (40 mL) then extracted with Et_2O (3 x 40 mL). The organic layer was washed with 5% (w/v) aqueous LiCl solution (60 mL), H_2O (40 mL) and brine (40 mL) respectively then dried over Na_2SO_4. The solvents were removed *in vacuo* and then purified by flash column chromatography (hexane:ethyl acetate: 7:3) was performed to give**P5-C** (0.075 g, 0.136 mmol, 68% yield) as a yellow gum.

IR (thin film, cm^{-1}) : 3305 (m), 1697 (s), 1649 (s); ^1H NMR: (600 MHz, $CDCl_3$)δ = 0.62 (3H, s), 0.98 (3H, s), 1.15 (1H, m), 1.22-1.31 (1H, m), 1.36-1.41 (1H, m), 1.42-1.54 (4H, m), 1.55-1.74 (14H, m),1.83-1.90 (2H, m), 1.99-2.05 (4H, m), 2.14 (3H, s), 2.15-2.24 (3H, m), 2.27-2.36 (2H, m), 2.60 (1H, t, J = 9.0 Hz), 3.11 (2H, appq, J = 6.4 Hz), 3.30 (1H, m), 3.40 (1H, m), 3.57-3.67 (2H, m), 5.62 (1H, brm), 5.69 (1H, m); ^{13}C NMR (150 MHz, $CDCl_3$) δ = 12.9, 13.2, 18.2, 20.8, 22.7, 22.9, 24.5, 26.9, 29.7, 31.4, 31.6, 32.1, 32.6, 34.3, 36.4, 36.8, 37.2, 37.4, 38.2, 42.3, 42.6, 43.8, 49.4, 63.5, 68.6, 69.4, 71.3, 72.2, 82.7, 121.3, 145.8, 172.9, 209.8; HRMS (*m/z*): $[M+H]^+$calcd. for$C_{33}H_{50}N_3O_4$, 552.3796; found, 552.3790.

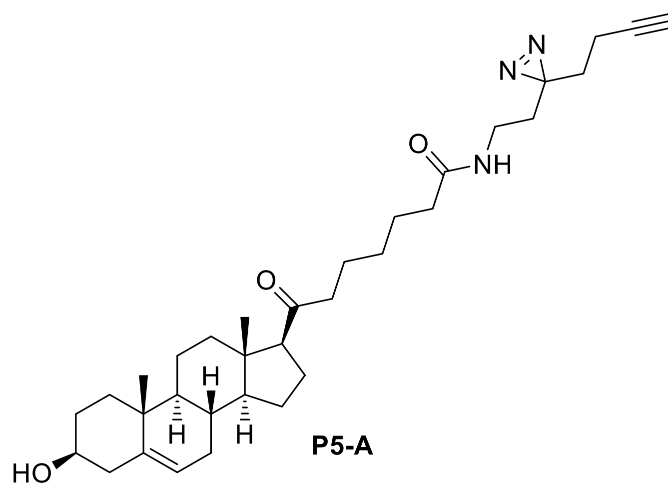
Due to diastereotopic mixture the NMR cannot be comprehensively assigned. As such the peaks described here are those observed.

Scheme 4 is the diagrammatic representation for the synthesis of **Probe P5-A** from **14** which was obtained from NewChem Technologies Ltd, Durham, UK.



Compound 15

Probe P5-A.



LiOH.H_2O (0.052 g, 1.24mmol) was added to solution of**14** (0.100 g, 0.224 mmol) in a solution of MeOH:THF:H_2O 1:1:1 (6 mL) and stirred for 4 h. The solution was acidified with 1 N HCl (15 mL) then the solution extracted withEt_2O (3 x 30 mL). The organic layer was washed with H_2O (40 mL) and brine (40 mL) then dried over Na_2SO_4. The solvents were removed *in vacuo* to give**15** as a white powder that was used immediately in the next step. HRMS (*m/z*): $[M+H]^+$calcd. for $C_{26}H_{41}NO_4$, 417.2999; found, 417.3004.

A flask containing the crude solid of **15** and HBTU (0.182 g, 0.483mmol) was placed under an atmosphere of argon then DMF (6 mL), DIPEA (0.084 mL, 0.483mmol) and **8** (0.033 g, 0.242mmol) were added. The solution was stirred for 16 h at room temperature. The reaction was diluted with H_2O (40 mL) then extracted with Et_2O (3 x 40 mL). The organic layer was washed with 5% (w/v) aqueous LiCl solution (60 mL), H_2O (40 mL) and brine (40 mL) respectively then dried over Na_2SO_4. The solvents were removed *in vacuo* and then purified by flash column chromatography (hexane:ethyl acetate: 7:3) was performed to give **P5-A** (0.073 g, 0.136 mmol, 61% yield over two steps) as a colourless gum.

IR (thin film, cm^{-1}): 3308(m), 1701 (m), 1647 (s); ^{1}H NMR: (600 MHz, $CDCl_3$) δ = 0.62 (3H, s), 0.67 (0.7H, s), 0.99 (1H, m), 1.01 (3.7H, s), 1.08-1.17 (2.6H, m), 1.18-1.35 (4.3H, m), 1.42-1.77 (21.8H, m), 1.85-1.89 (2.6H, m), 1.99-2.05 (6.2H, m), 2.18-2.26 (3.9H, m), 2.32-2.36 (2.6H, m), 2.39 (2H, t, J = 7.1 Hz), 2.51 (1H, t, J = 8.8 Hz), 3.11 (2.4H, app q, J = 6.4 Hz), 3.52 (1.2H, m), 4.06 (0.4H. m), 5.40 (1.2H, m), 5.60 (1.2H, br s);^{13}C NMR (150 MHz, $CDCl_3$) δ = 13.2, 13.4, 19.4, 21.1, 23.0, 23.1, 24.5, 25.4, 26.8, 28.7, 31.6, 31.76, 31.84, 32.2, 32.5, 34.2, 36.4, 36.5, 37.3, 38.9, 42.2, 44.0, 44.2, 50.0, 57.0, 62.9, 69.4, 71.7, 82.7, 121.4, 140.8, 172.9, 211.5; HRMS (*m/z*): $[M+H]^+$calcd. for$C_{33}H_{50}N_3O_3$, 536.3847; found 536.3859.

The product is a 5:1 mixture of the reported product and an unknown impurity. As such the peaks described here are those observed with partial integrals included. **P5-A** was used in subsequent experiments despite this known impurity and the appropriate controls were put in place to ensure data obtained with this probe was valid.

Cell Culture and reagents

The human androgen sensitive prostate cancer cell line LNCaP, glioblastoma cell line U87MG (Gift from Dr Mathew Garnett, Wellcome Sanger institute) were first checked for mycoplasma (Surrey Diagonostics). The mycoplasma free LNCaP, U87MG and HEK293T cells were cultured in RPMI 1640 (supplemented with 10% FBS and 1% penicillin-streptomycin), DMEM/F-12 (supplemented with 10% charcoal stripped FBS and 1% penicillin-streptomycin) and DMEM (supplemented with FBS and 1% penicillin-streptomycin) respectively. Pregnenolone and ethanol, were obtained from Sigma. Stock solutions, 100mM of A, B_1, B_2, and C and 50mM of pregnenolone in ethanol were prepared. For LNCaP cells, 5000 cells per well were cultured in 100 μL of complete medium in a 96 well flat-bottom clear poly-L-lysine coated plates for 24 hrs at 37°C. After a day, the medium was replaced with phenol-red free RPMI 1640, supplemented with charcoal stripped FBS and 1% penicillin-streptomycin. The cells were grown either in presence of 2 to 20nM of indicated probes or the vehicle, ethanol. The cultures were maintained for 6 days and replacing the medium every 48 hours. For U87MG cells, 5000 cells were cultured in 100μL of medium in 96 wells flat-bottomed clear plates. The next day the medium was replaced and appropriate amount of either the probes or the vehicle ethanol was added. The culture was maintained for 4 days with medium replacement every 48 hrs.

Cloning and expression of CLIP1

The sequence verified cDNA clone of human CLIP1 (from Dharmacon) was PCR amplified with primers CLIP FW – TTTTGGCCCACAGGGCCTTAGTATGCTAAAGCCAAGTGG, CLIP RW - TTTTGGCCGATATGGCCTCAGAAGGTTTCGTCGTCATTGC and subcloned in

frame into a mammalian expression vector (kind gift from Martin Beck, EMBL) with triple tag (HA, StrepII and His) after digestion with Sfi I (NEB) and extraction from agarose gel (using Qiagen kit). The correct incorporation was verified by sequencing and then transfected into HEK293T cells using Lipofectamine (Invitrogen) as per the manufacturer's protocol. The transfected cells were harvested after 48 hours, washed once with PBS and stored at -80°C until further use.

Protein Binding Assay and proteomics

To test the *in vitro* binding of the probes (**P5-A, P5-B1, P5-B2** and **P5-C**) to CLIP1, the protein was transiently expressed in HEK cells. The transfected HEK cells were homogenized in 10 mM Tris pH 8, 1.5 mM EDTA, 10% glycerol, 1 µl Phosphatase and 1 µl Protease inhibitor (from ThermoFisher) using a 26G ½ needle fitted in a 1mL syringe. Roughly 400 µg of clear whole cell lysate (WCL) (measured in a NanoDrop spectrophotometer) was mixed with 50 nM probes and incubated at 37°C for 1 hour. The mixture was then irradiated at 365nm with a lamp (from UVP) on ice and then clicked with 100 nmoles magnetic azide (Turbobeadsazide, Sigma) in the presence of 1 mM $CuSO_4$ (Sigma), 0.1 mM TBTA (Sigma) and 1mM TCEP (Sigma) for 1 hour at 37°C in 500 µL of PBS/0.1% SDS. A blank assay without the probe was carried out with all the above steps to ascertain the background binding to the magnetic beads. In a parallel competition assay, pregnenolone was added to the WCL and incubated at 37°C for 30 minutes before adding the probe and then the above procedure was followed. After clicking the beads were pulled down washed thoroughly 3 times with 1 mL PBS/0.1% SDS and then 3 times with 1 mL PBS. Subsequently, the beads were suspended in the SDS buffer and boiled for 10 minutes at 95°C. The samples were loaded on a 7.5% TGX gels (BioRad) and after electrophoresis the proteins were

transferred to a nitrocellulose membrane using a wet transfer unit (BioRad). The membrane was blocked for 4 hours in 5% non-fat dry milk in 1X PBS and then incubated overnight at 4°C in 1X PBS with 5% non-fat dry milk and HA primary antibody (Abcam). After 3 washes with 1X PBS-0.05% Tween-20 the membrane was incubated with anti-rabbit secondary antibody (Santa Cruz Biotechnology) for 1 hour at RT. After 4 five minutes washes the membrane was incubated with SuperSignal West Dura (ThermoFisher) for five minutes and the resulting chemiluminescence was captured using the LAS4000 Image Quant (GE).

To test the global binding efficiency and specificity, **P5-C** was used with LNCaP cells extracts. Three samples were prepared in parallel where 400 µg of protein, measured in a NanoDrop spectrophotometer was incubated with 10 µM of **P5-C** at 37°C for 30 minutes, irradiated with 365 nm UV for 30 minutes at 4°C and then clicked with 0.5 mM Biotin Azide (Sigma) in presence of 1 mM $CuSO_4$ (Sigma), 0.1mM TBTA (Sigma) and 1mM TCEP (Sigma) for 1 hour at 37°C in 500µL of PBS/0.1 % SDS. In the second one 100 µM (10X) pregnenolone was added to extract and incubated for 1 hour at 37 °C before adding the probe, and the third sample was prepared without the UV irradiation. Another sample was prepared without the probe to determine the background binding of the beads. To get rid of the excess probes and biotin, 1 volume of the protein samples were washed with chloroform:methanol:water (1:4:3 volumes) and after removal of the aqueous top layer the protein containing interface was precipitated with 4 volumes of methanol. The precipitate was dried for 10 minutes and then resuspended in 200µL of PBS/1%SDS at 70°C for 10 minutes. Once the protein pellet was completely dissolved, the SDS concentration was diluted to 0.2% with PBS. The solution was centrifuged at 3000 X g for 3 minutes at RT to remove any undissolved particles and then the supernatant was mixed with 100 µl of 50 %

slurry of neutravidin beads (ThermoFisher) pre-equilibrated in PBS/0.2% SDS. The mixture was incubated with slow rotation at RT for 1 hour. The beads were collected at 100 X g for 1 minute and then washed 3 times with 1 mL PBS/0.1% SDS, and then 3 times with only PBS. 25 µL of 2 X Laemmli buffer was added to beads and boiled at 95 °C for 5 minutes, the supernatant was then loaded on to a 10% PAGE with SDS. The gel was stained with silver (ThermoFisher) as per manufacturer's protocol and imaged LAS4000 Image Quant (GE).

For proteomic experiments, on the one hand 10 µM of all 4 probes were incubated with 10 million LNCaP cells in phenol red free RPMI1640 for 1 hour in a 24 well plate alongside cells with no probes. The media was removed, cells washed once with cold PBS and then the cells were irradiated at 365nm for 15 minutes in 200 µL of cold PBS at 4°C. After removing the PBS the cells were washed once with PBS and then were extracted for proteins with PBS containing 0.2% SDS. After centrifugation, the clear lysates were used for copper-click and affinity purification. Following 3 times PBS/0.1% SDS, the beads were washed 3 times with 1 mL of PBS and then LNCaP cells were sent to the Wellcome Sanger Institute proteomics facility for on-bead digestion and MS sequencing. In parallel, to complement the above experiment, two samples, one with only 10 µM **P5-C** and another competition assay, where live cells were fed with 100µM (10X) P5 before adding the probe, were treated identically as above. After washing the beads with PBS/0.1%SDS the beads were boiled in Laemmli buffer at 95 °C for 5 minutes. The samples were loaded in adjacent lanes of an SDS-PAGE gel. After completion of the run the gel was stained with colloidal Coomassie Brilliant Blue. Each lane was cut into 4 pieces and from each piece and proteins were extracted, digested and sequenced in the Wellcome Sanger Institute's proteomics facility.

10 million murine CD8$^+$ T cells were used in three different experiments with two replicates for each. In the first experiment, CD8$^+$ T cells in phenol-free RPMI were incubated with only the vehicle (without probe), the second experiment had 10 µM **P5-C** and the third was incubated with 100 µM (10X) of P5 before 10 µM of **P5-C** incubation. The following protocol was the same for all the experiment and its replicates. The cells were centrifuged, washed twice with ice cold PBS and then exposed to UV and the subsequent cell lysate preparation, click reaction and pull-down procedure was same as for LNCaP cells in the above section. The washed neutravidin beads were sent to EMBL proteomics core facility for TMT labelling, peptide fractionation and mass spectrometry.

Cell Viability Assay

XTT assay (Cayman Chemical) was used for assessing the effect of the probes on cell proliferation. For LNCaP cells, after 6 days a background absorbance, a reading was taken at 485 nm before adding 50 µL of activated XTT reagent to each of the wells. The cells were incubated at 37°C in an incubator for 5-6 hours before measuring the absorbance at 485nm. For U87MG cells, after 4 days of culture, the medium was replaced by sterile 100 µL of phosphate buffered saline, and then a background reading was measured at 485nm. 50 µL of activated XTT reagent was then added to each well and the plate was incubated at 37°C for 6 hours. The absorbance was measured at 485nm in a plate reader (Biotek).

Mass Spectrometry

LNCap Cell proteomics

For gel-LC/MS samples, the gel lane was excised to 4 bands, and destained with 50% CH_3CN/50% of 50mM ammonium bicarbonate, then digested by trypsin (Roche) overnight. Peptides were then extracted by 50% CH_3CN/50% of 0.5% formic acid, then dried in SpeedVac.

For on-bead digestion samples, beads were washed with 100mM TEAB twice, then resuspended in 250 µl of 100mM TEAB buffer. 2µl of 500mM TCEP and 2µl of 500mM iodoaceamide were added then incubated at 25°C for 45minutes. Then 0.4 µg of trypsin was added and the mixture was incubated at 37°C for 18 h. Beads were then transferred to a spin column, centrifuged at 400 X g for 1 min to collect the flow through. 100 µl of 1M TEAB was added to the beads again and sand collect as above, and both collections were pooled, then dried in SpeedVac.

Peptides were resuspended in 0.5% formic acid before LC-MS/MS analysis on an Ultimate 3000 RSLC nano System (Dionex) coupled to an LTQ Orbitrap Velos (Thermo Fisher) mass spectrometer equipped with a nanospray source.

The peptides were first loaded and desalted on a PepMap C18 trap (0.1 mm id x 20 mm, 5µm) then separated on a PepMap 75 µm id x 25 cm column (2 µm) over a 60 min linear gradient of 5– 42% B / 90 min cycle time, where B is 80% CH_3CN/0.1% FA for gel band sample, but 5-40%B in 120min /150min cycle time for on-bead digestion sample. The LTQ Orbitrap Velos was operated in the “top 10” data-dependant acquisition mode while the preview mode of FT master scan was enabled. The Orbitrap full scan was set at m/z 380 – 1500 with the resolution at 30,000 at m/z 400 and AGC at $1x10^6$ with a maximum injection time at 200 msec. The 10 most abundant multiply-charged precursor ions, with a minimal signal above 2000 counts, were dynamically selected for CID fragmentation (MS/MS) in the LTQ ion trap,

which has the AGC set at 5000 with the maximum injection time at 100ms.The dynamic exclusion was set at ± 10 ppm for 45 sec.

Data from gel bands were processed in Proteome Discoverer 1.4 (Thermo) using the Mascot (V 2.5) search engine against a Uniprot human database (version May 2013) combined with the common contaminate database. The precursor mass tolerance is set at 20 ppm, and fragments at 0.5 Da. Trypsin with full specificity and 2-missed cleavage sites was used. The dynamic modifications are set as acetyl (protein N-term), carbamidomethyl (C), deamidated (NQ) and Oxidation (M). The FDR setting used q-value, where the strict is at 0.01, and relaxed is at 0.05. Only peptides at high confidence were selected for protein groups.

Data from on-bead digestion were processed by MaxQuant (version 1.5.3.30) (http://www.coxdocs.org/). The human protein database was downloaded from Uniprot (version June 2016) and a common contaminate database was used as well. The parameters were the default except the carbamidomethyl (C) was set as variable as above. Other variable modifications were also the same as above. Both PSM and protein FRD were set at 0.01.

For CD8+ cell proteomics

Sample preparation and TMT labeling

The supernatant was removed from the beads and 20 µl of 4x Laemmli buffer was added to the ~ 60 µl beads, vortexed, and kept shaking for 15 minutes at 95°C, vortexed again and subsequently kept shaking for additional 15 minutes at 95°C. The samples were cooled to room temperature and filtered using Mobi columns with a 90 µm filter.

The resulting samples (25 µl) were diluted with 50 µl 50 mM HEPES solution and treated with 2 µl 200 mM dithiothreitol in 50 mM HEPES at pH 8.5 for 30 min at

56°C to reduce disulfide bridges. The accessible cysteine residues were carbamidomethylated for 30 min in the dark after addition of 4 µl 400 mM 2-chloroacetamide in 50 mM HEPES at pH 8.5.

Protein clean up and digestion was done by SP3(Hughes et al., 2014). Two microliter of a 1:1 mixture of hydrophilic and hydrophobic Sera-Mag SpeedBeads (ThermoFisher) prewashed with water and at a concentration of 10 µg/µl were added to each sample. After addition of 83 µl acetonitrile, the suspensions were kept for 8 min prior to putting the vials on the magnet for 2 more minutes. The supernatants were discarded and the beads were washed twice with 200 µl 70% ethanol and once with 180 µl acetonitrile. After removing the acetonitrile, the beads were dried on air. After addition of 150 µg trypsin in 10 µl 50 mM HEPES buffer, the bound proteins were digested overnight. On the next day, the bead suspensions were sonicated for 5 minutes and vortexed prior to putting the vials on the magnet. The supernatants containing the peptides were transferred to new vials. The beads were rinsed with 10 µl 50 mM HEPES buffer and the resulting supernatants were combined with the first 10 µl. The individual samples were labeled by addition of 4 µl TMT-6plex reagent (ThermoFisher) in acetonitrile and incubated for one hour at room temperature. The reactions were quenched with a 5% hydroxylamine solution and acidified with 50 µl 0.05% formic acid. The 6 samples of each replicate were combined and the resulting two 6-plex samples were cleaned using an OASIS HLB µElution Plate (Waters). The wells were first washed twice with 0.05% formic acid in 80% acetonitrile and twice with 0.05% formic acid in water. The samples were loaded on the wells and washed with 0.05% formic acid in water. After elution with 0.05% formic acid in 80% acetonitrile the samples were dried and reconstituted in 4% acetonitrile and 1% formic acid in water.

Peptide fractionation

After adjusting the pH of the samples to pH 10 with ammonium hydroxide, the TMT-labeled peptides were fractionated on an Agilent 1200 Infinity HPLC system equipped with a degasser, quaternary pump, autosampler, variable wavelength UV detector (set to 254 nm), and fraction collector. Separation was performed on a Phenomenex Gemini C18 (100 x 1.0 mm; 3 µm; 110 Å) column using 20 mM ammonium formate pH 10 in water as mobile phase A and 100% acetonitrile as mobile phase B. The column was used in combination with a Phenomenex Gemini C18, 4 x 2.0 mm SecurityGuard cartridge. The flow rate was 0.1 ml/min. After 2 min isocratic separation at 100% A, a linear gradient to 35% B at minute 59 was used, followed by washing at 85% B and reconstitution at 100% A. In total 32 two minute fractions were collected and pooled to result in 6 samples. These were dried and reconstituted in 4% acetonitrile and 1% formic acid.

Mass spectrometry data acquisition

The fractionated samples were analyzed on an UltiMate 3000 nano LC system (Dionex) coupled to a QExactive plus (Thermo) mass spectrometer via a Nanospray Flex source (Thermo) using a Pico-Tip Emitter (New Objective; 360 µm OD x 20 µm ID; 10 µm tip). The peptides were first trapped on a C18 PepMap 100 µ-Precolumn (300 µm x 5 mm, 5 µm, 100 Å) prior to separation on a Waters nanoEase C18 75 µm x 250 mm, 1.8 µm, 100 Å column. The applied flow rates were 30 µl/min for trapping and 300 nl/min for separation. The mobile phase A was 0.1% formic acid in water and the mobile phase B was 0.1% formic acid in acetonitrile. After an initial isocratic step at 2% B for 2.9 minutes the multi-step gradient started with a gradient to 4% B at minute 4 followed by a linear increase to 8% B at minute 6. Subsequently, a shallow

gradient to 28% B at minute 43 was followed by a steep gradient to 40% B at minute 52, a washing step at 80% B, and reconstitution at 2% B.

All spectra were acquired in positive ion mode. Full scan spectra were recorded in profile mode in a mass range of 375-1200 *m/z*, at a resolution of 70,000, with a maximum ion fill time of 10 ms and an AGC target value of $3x10^6$ ions. A top 20 method was applied with the normalized collision energy set to 32, an isolation window of 0.7, the resolution at 17,500, a maximum ion fill time of 50 ms, and an AGC target value of $2x10^5$ ions. The fragmentation spectra were recorded in profile mode with a fixed first mass of 100 *m/z*. Unassigned charge states as well as charge states of 1, 5-8, and >8 were excluded and the dynamic exclusion was set to 30 seconds.

Th2 Cell Proliferation Assay

We followed the method as described previously in Ref(Pramanik et al., 2018). Negatively purified splenic naive $CD4^+$ T cells were stained with CellTrace Violet following the CellTrace Violet Cell Proliferation Kit (Invitrogen) protocol and cultured under Th2 activation/differentiation conditions as described previously in the presence or absence of pregnenolone and linker tagged pregnenolene (**P5-A, P5-B1, P5-B2** and **P5-C**) for 3 days. The cell proliferation profile was captured by a flow cytometry-based dye decay assay on BD Fortessa. Data were analyzed in FlowJo.

Immunoglobulin Class Switch Recombination Assay

The detailed method is described previously[4]. Splenic naïve B cells from 8 -12 week-old mice were purified by depletion of $CD43^+$ cells using anti-CD43-coupled magnetic microbeads (Miltenyi Biotec) and seeded into 96-wellplates in RPMI supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, 25 ng/ml recombinant

mouse IL4 (R&D Systems), and 40µg/ml LPS (Sigma-Aldrich). On day 5 of stimulation, B cell Fc receptors were blocked with PBS containing 2% rat serum and 10 mM EGTA and stained with FITC-conjugated anti-IgG1 (BD Biosciences). Flow cytometry was performed using a Fortessa (BD). Data were analyzed with FlowJo software.

Analysis of the MS results

After the LC-MS/MS data was obtained it was filtered to classify the proteins. The on-bead protein pull-down experiments were done in parallel with four samples (**P5-A, P5-B1, P5-B2** and **P5-C**) and were treated as four replicates and only those proteins with 2 or more unique peptides in at least three samples were selected for downstream analysis. The control experiment was used to eliminate the non-specifically bound proteins from the pool. This yielded a total of 442 proteins. The gel band analysis experiment was used to crosscheck and find the common proteins from the on-bead digestion experiment. The binding of the probe to LNCaP proteins was done in parallel to the probe binding in the presence of the competitor, P5. The expectation was that pull-down in the presence of cold P5 binding will lower the peptide count of specific binding proteins. 163 proteins were found to satisfy the criteria and among them, only 38 proteins were found to be common across both experiments, and were classified as the 'P5 binding proteins'. The remainder of the 404 proteins from the on-bead digestion experiment were classified as 'potential P5 binding proteins'. We did not analyze the 125 proteins from the in-gel digestion experiment.

MS data analysis for murine $CD8^+$ T cells

IsobarQuant (Franken et al., 2015) and Mascot (v2.2.07) were used to process the acquired data, which was searched against the Uniprot reference database of *Mus musculus* after the addition of common contaminants and reversed sequences. The following modifications were included into the search parameters: Carbamidomethyl (C) and TMT10 (K) (fixed modification), Acetyl (N-term), Oxidation (M) and TMT10 (N-term) (variable modifications). A mass error tolerance of 10 ppm was applied to full scan spectra and 0.02 Da to fragmentation spectra. Trypsin was selected as protease with an allowance of maximum two missed cleavages, a minimum peptide length of seven amino acids, and at least two unique peptides were required for protein identification. The false discovery rate (FDR) on peptide and protein level was set to 0.01.

The protein.txt output files from IsobarQuant were further processed using the R language. As quality filters, only proteins that were quantified with at least two unique peptides and have been identified in both biological replicates (analyzed in separate MS analysis) were used for further downstream analysis (416 proteins). The 'signal_sum' columns were used and potential batch-effects were removed using the respective function from the limma package(Ritchie et al., 2015). Subsequently, the data was normalized using a variance stabilization normalization (vsn)(Huber et al., 2002). The limma package was employed again to test for differential abundance between the various experimental conditions. T-values of the limma output were pasted into fdrtool (Strimmer, 2008) in order to estimate false discovery rates (q-values were used as FDR).

Hierarchial clustering of all the peptides from five different samples was done using cluster 3.0 (de Hoon et al., 2004) and the results viewed with java treeview 3.0(Keil et al., 2018). For functional annotation DAVID 6.8 software (Huang et al., 2009a,

2009b, 2007) was used. For analysis we chose only those characters with P value > 0.05.

Microscopy

About 20000 LNCaP cells were grown on 1.5 coverslips in 24 well plates in complete RPMI 1640 medium with 10% FBS. After 48 hours the medium was aspirated and the cells were washed twice with PBS. 500 µL of phenol free RPMI 1640 supplemented with 10% charcoal stripped FBS was added to each well and incubated for an hour. 1 µM of **P5-C** was added to each well and incubated in the dark for 2 hours. Then media was removed from the wells and washed with cold PBS (special care was taken to avoid too much light exposure). 200 µL of cold PBS was added to each well and irradiated at 365 nm for 30 minutes at 4°C. After crosslinking PBS was removed and the cells were fixed in 0.5 mL of cold MeOH for 15 minutes at -20°C. After 6 one minute extraction with (10:55:0.75) chloroform:methanol:acetic acid the cells were clicked with 0.1 µM Alexa Fluor 488 azide (A10266, Thermofisher) in 200 µL PBS with freshly prepared 1mM TCEP, 100 µM TBTA and 1mM $CuSO_4$ for 1 hour at RT with shaking. The coverslips were washed thrice in wells with PBS followed by two washes with water. Another parallel experiment with similar treatments but no probe was carried out as a control. The coverslips were mounted on slides with Prolong diamond anti-fade mountant (P36965, Thermofisher) with DAPI and visualized under Leica DM50000B fluorescent microscope equipped with narrow band-pass filters for DAPI-FITC with an ORCA-03G CCD camera (Hamamatsu). Digital images were captured using SmartCapture (Digital Scientific, UK).

Supplemental References

de Hoon, M.J.L., Imoto, S., Nolan, J., Miyano, S., 2004. Open source clustering software. Bioinformatics 20, 1453–1454. https://doi.org/10.1093/bioinformatics/bth078

Franken, H., Mathieson, T., Childs, D., Sweetman, G.M.A., Werner, T., Tögel, I., Doce, C., Gade, S., Bantscheff, M., Drewes, G., Reinhard, F.B.M., Huber, W., Savitski, M.M., 2015. Thermal proteome profiling for unbiased identification of direct and indirect drug targets using multiplexed quantitative mass spectrometry. Nat Protoc 10, 1567–1593. https://doi.org/10.1038/nprot.2015.101

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009a. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 4, 44–57. https://doi.org/10.1038/nprot.2008.211

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009b. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37, 1–13. https://doi.org/10.1093/nar/gkn923

Huang, D.W., Sherman, B.T., Tan, Q., Kir, J., Liu, D., Bryant, D., Guo, Y., Stephens, R., Baseler, M.W., Lane, H.C., Lempicki, R.A., 2007. DAVID Bioinformatics Resources: expanded annotation database and novel algorithms to better extract biology from large gene lists. Nucleic Acids Res 35, W169-175. https://doi.org/10.1093/nar/gkm415

Huber, W., von Heydebreck, A., Sültmann, H., Poustka, A., Vingron, M., 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 18 Suppl 1, S96-104. https://doi.org/10.1093/bioinformatics/18.suppl_1.s96

Hughes, C.S., Foehr, S., Garfield, D.A., Furlong, E.E., Steinmetz, L.M., Krijgsveld, J., 2014. Ultrasensitive proteome analysis using paramagnetic bead technology. Mol Syst Biol 10, 757. https://doi.org/10.15252/msb.20145625

Keil, C., Leach, R.W., Faizaan, S.M., Bezawada, S., Parsons, L., Baryshnikova, A., 2018. Treeview 3.0 (beta 1) - Visualization and analysis of large data matrices. Zenodo. https://doi.org/10.5281/zenodo.1303402

Pramanik, J., Chen, X., Kar, G., Henriksson, J., Gomes, T., Park, J.-E., Natarajan, K., Meyer, K.B., Miao, Z., McKenzie, A.N.J., Mahata, B., Teichmann, S.A., 2018. Genome-wide analyses reveal the IRE1a-XBP1 pathway promotes T helper cell differentiation by

resolving secretory stress and accelerating proliferation. Genome Med 10, 76. https://doi.org/10.1186/s13073-018-0589-3
Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., Smyth, G.K., 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 43, e47. https://doi.org/10.1093/nar/gkv007
Strimmer, K., 2008. fdrtool: a versatile R package for estimating local and tail area-based false discovery rates. Bioinformatics 24, 1461–1462. https://doi.org/10.1093/bioinformatics/btn209