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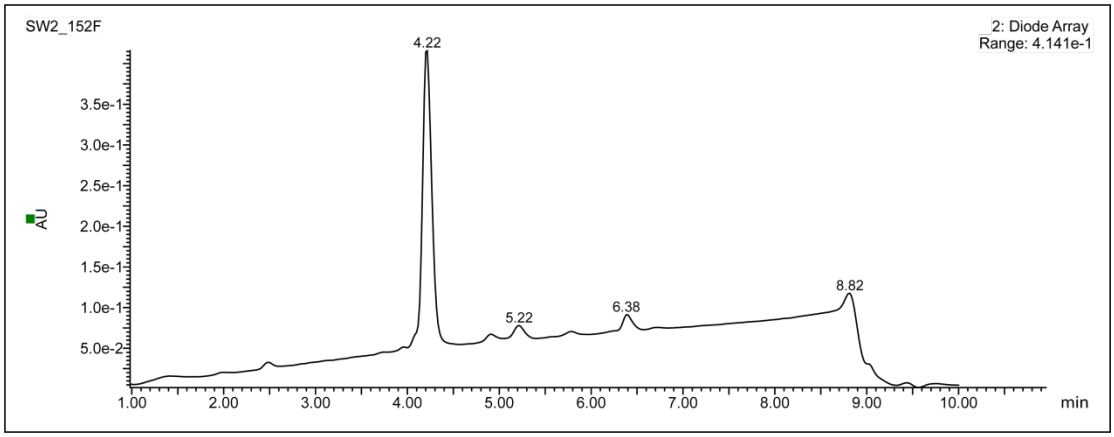
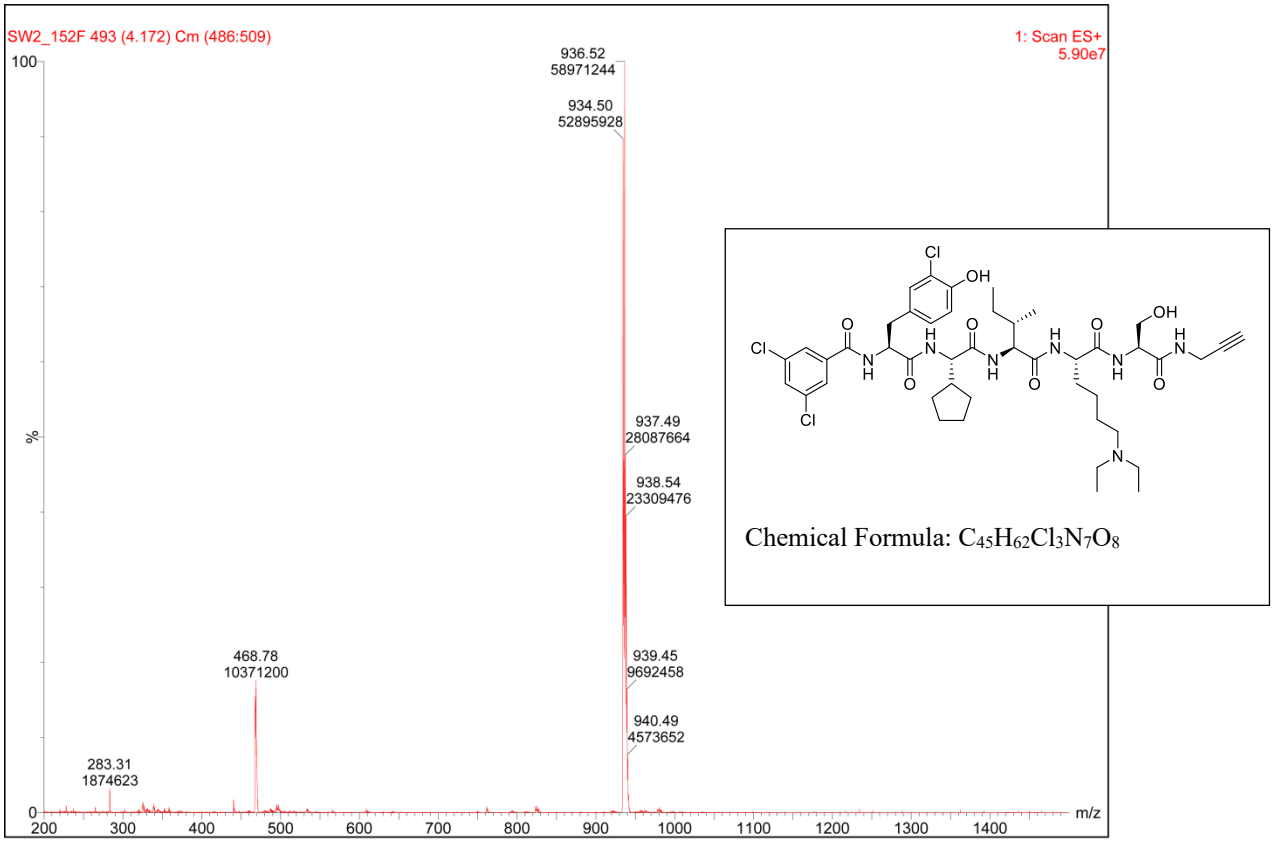
EXPERIMENTAL

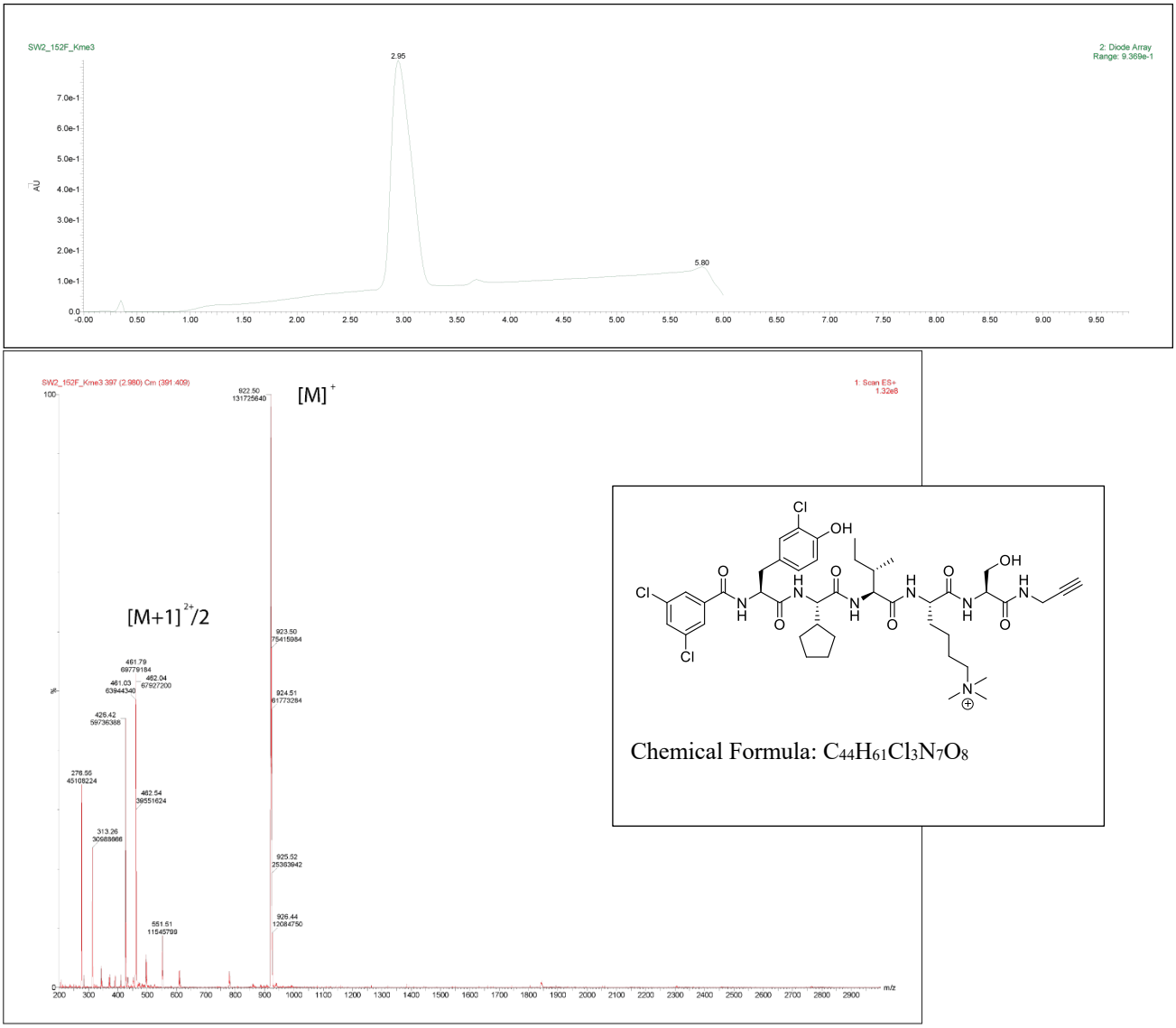
CBX ChD Protein Expression and Purification: Protein was expressed and purified as previously described.[1]

Synthesis of C-terminal Alkyne Peptide Using Solid Phase Peptide Synthesis (SPPS): Off-DNA peptides were prepared using traditional SPPS methods similarly as previously described.[1]





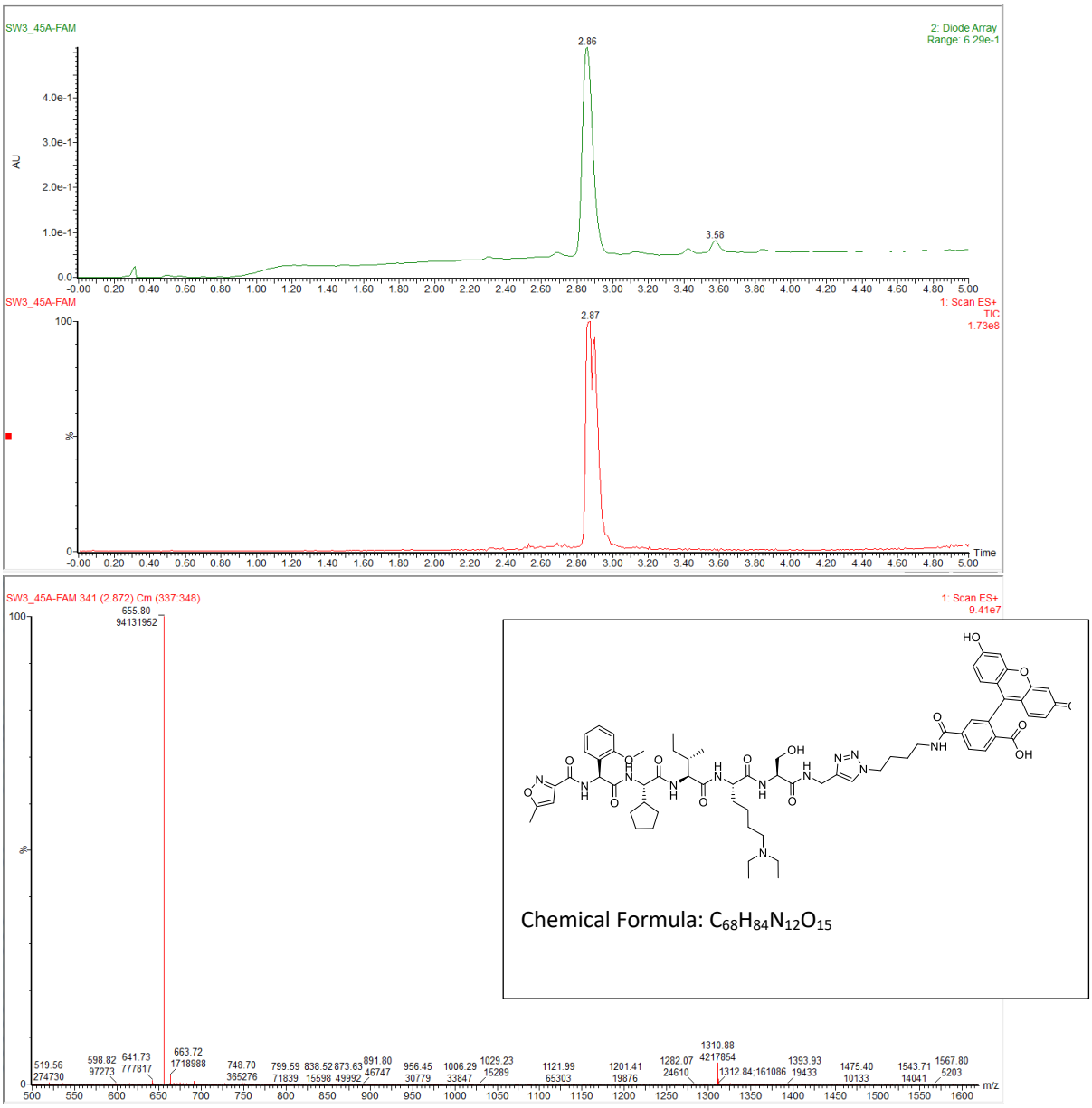
Characterization Data for compound SW2_152F A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{45}H_{62}Cl_3N_7O_8$: 934.37/936.37; found: 934.50/936.52



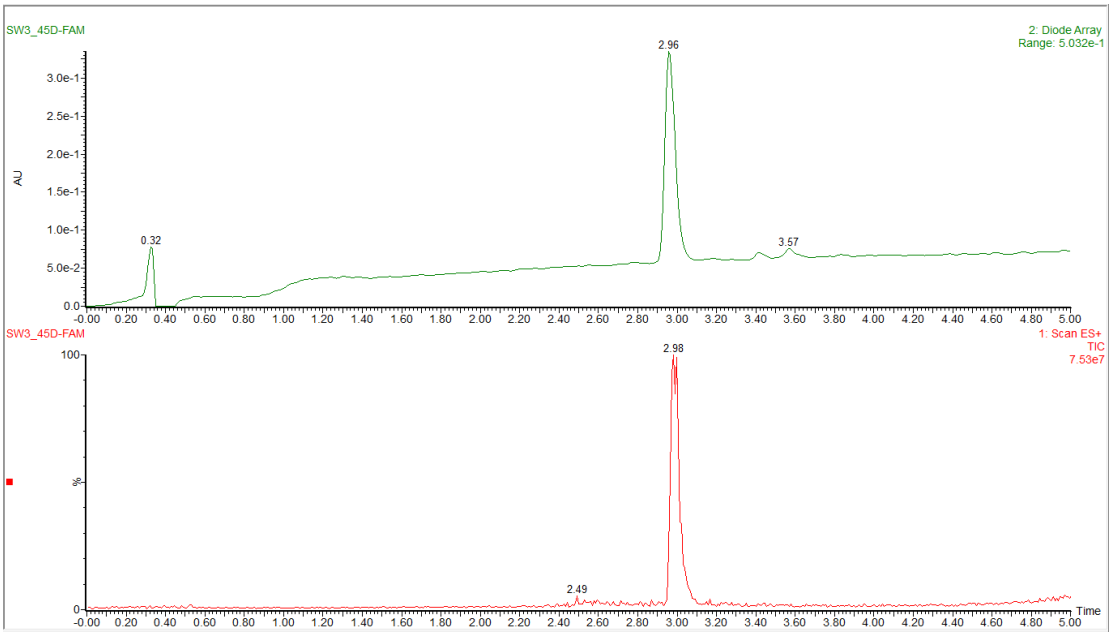
Characterization Data for compound SW2_152F_Kme3 A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{44}H_{61}Cl_3N_7O_8$:920.36/922.36; found: 922.50

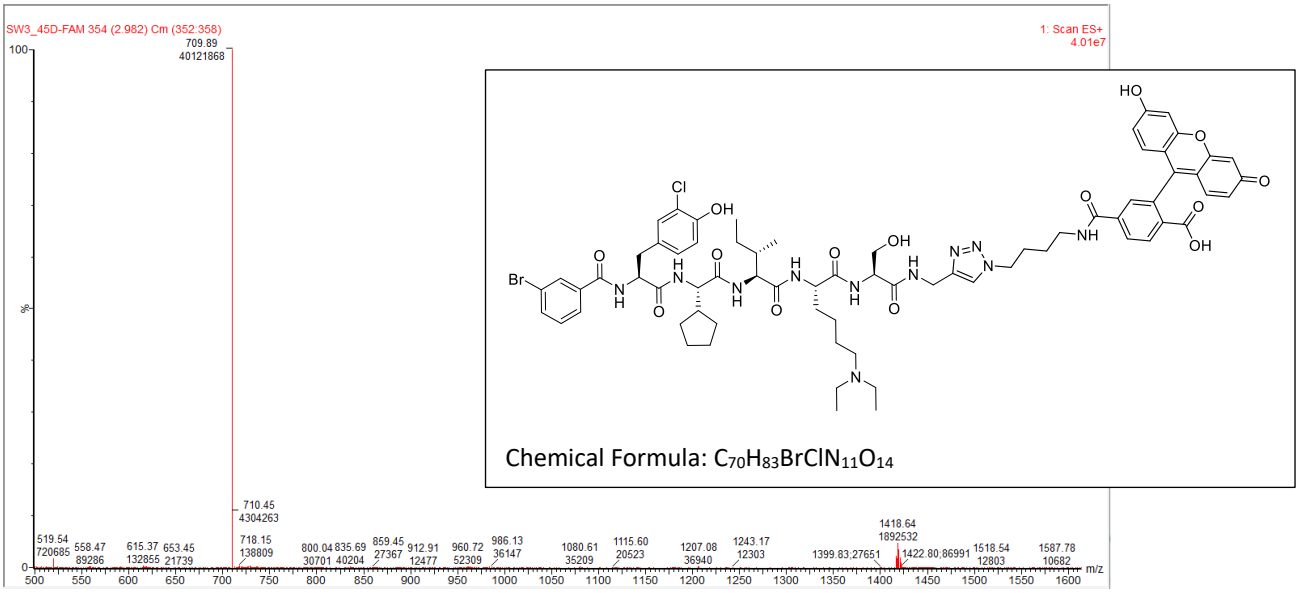
Synthesis of 5-/6-FAM: Synthesis was done as previously described.[1]

Synthesis of FAM-peptide Conjugates: Fluorescein conjugates were synthesized as previously described.[1]

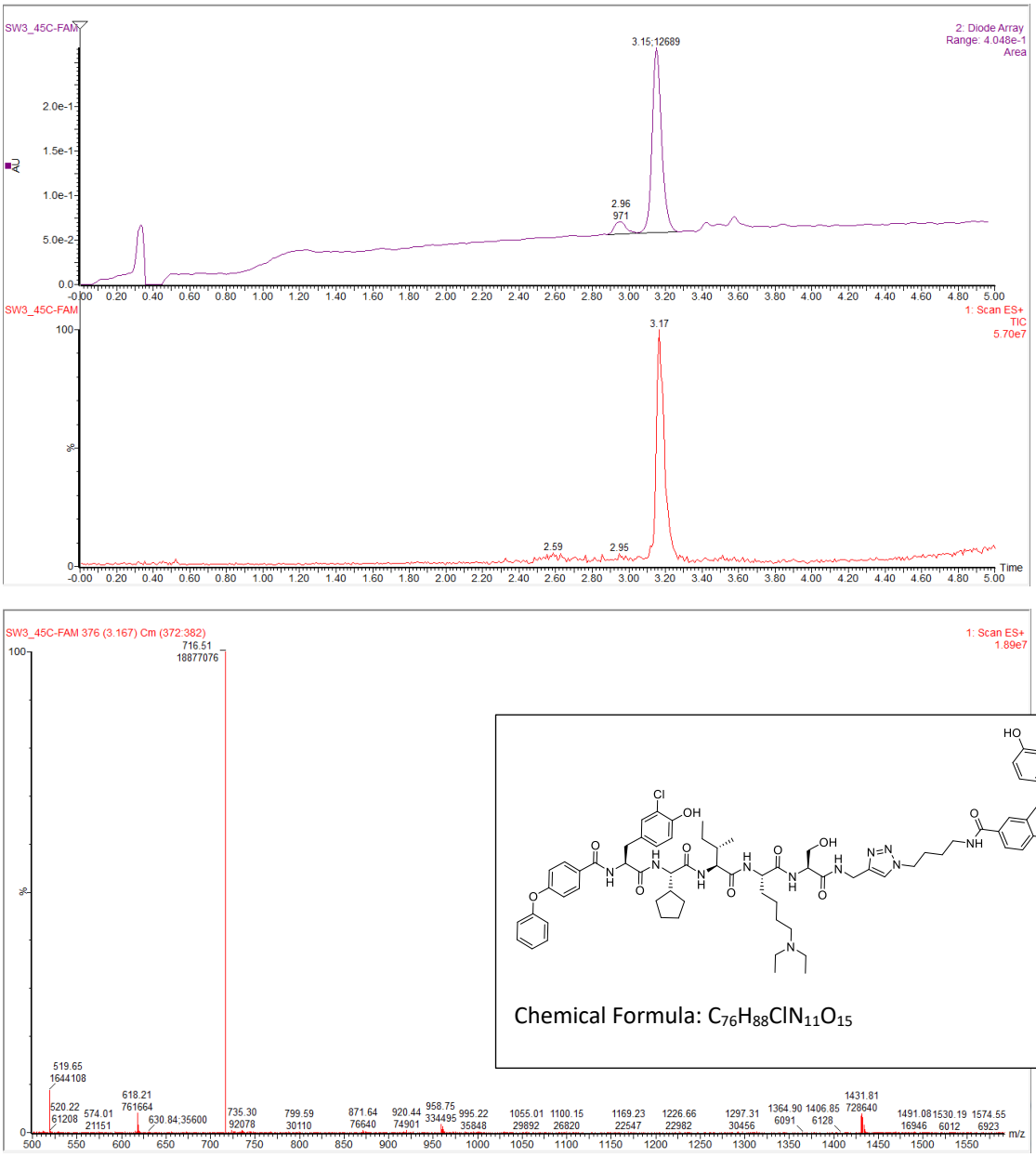


Characterization Data for compound SW3_45A_FL A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{68}H_{84}N_{12}O_{15}$: 1310.49; found: 1310.88

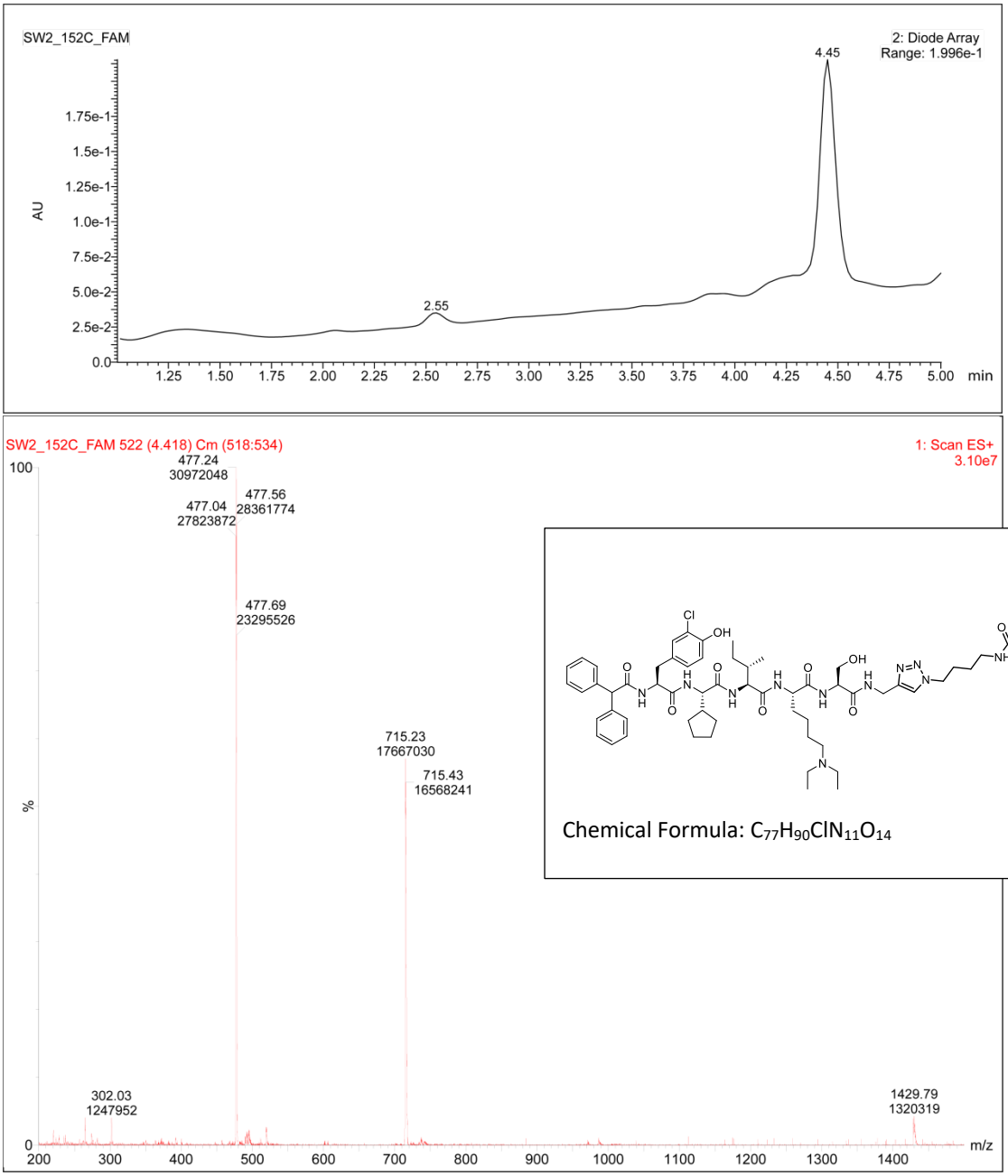




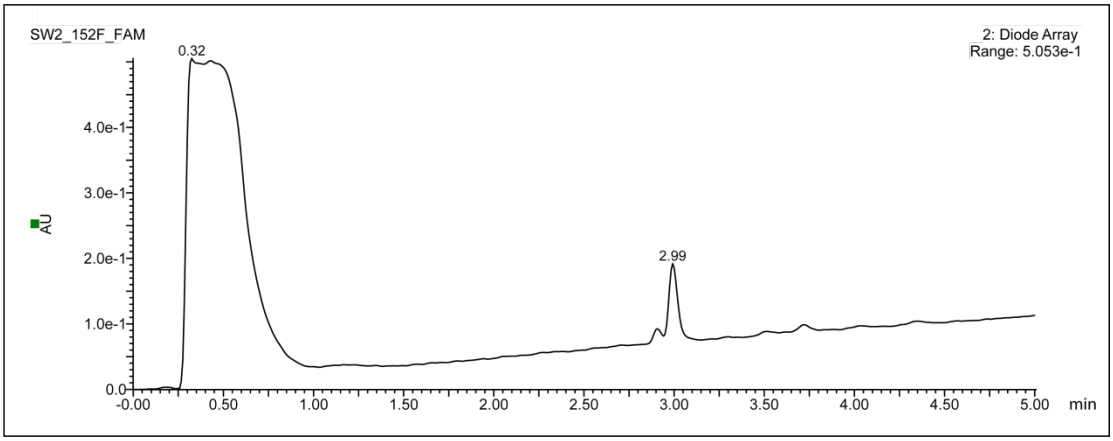
Characterization Data for compound SW3_4D_FL A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{76}H_{88}ClN_{11}O_{15}$: 1418.85; found: 1418.64

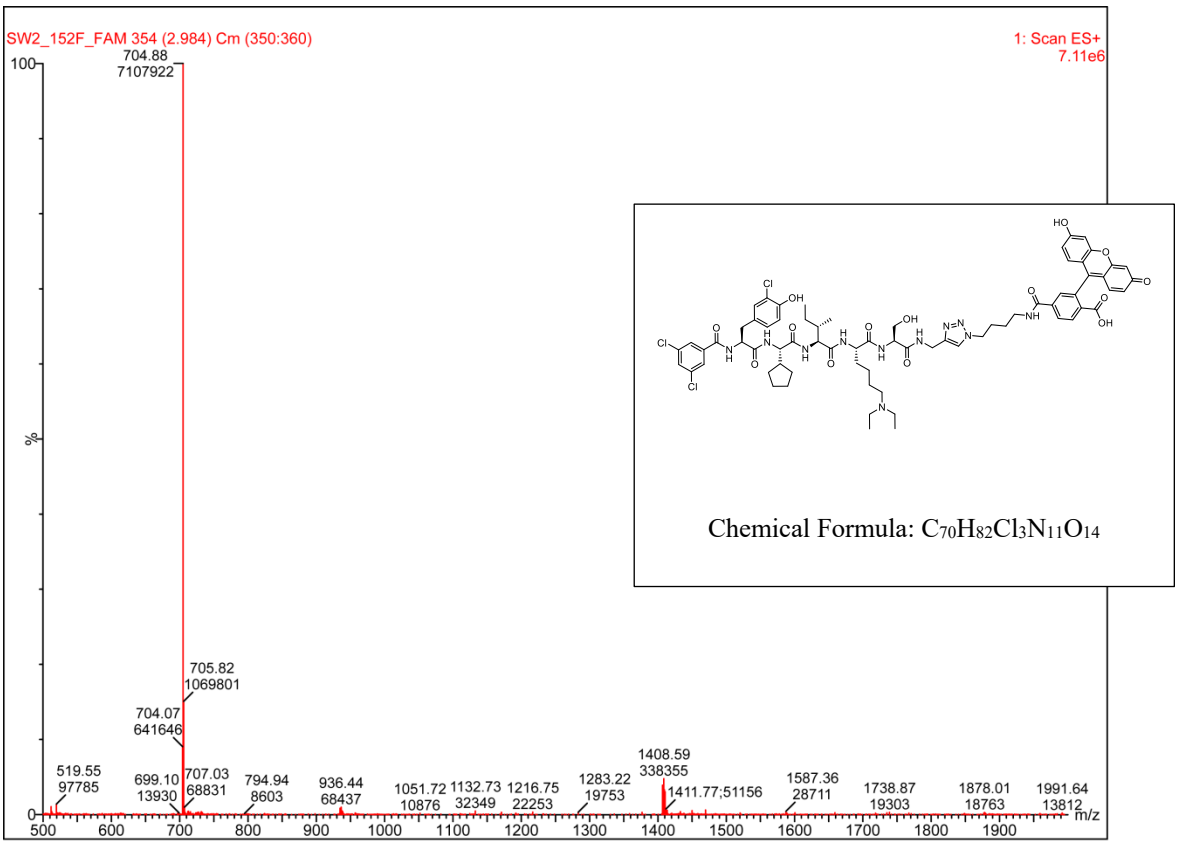


Characterization Data for compound SW3_45C_FL A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{76}H_{88}ClN_{11}O_{15}$: 1432.05; found: 1431.81



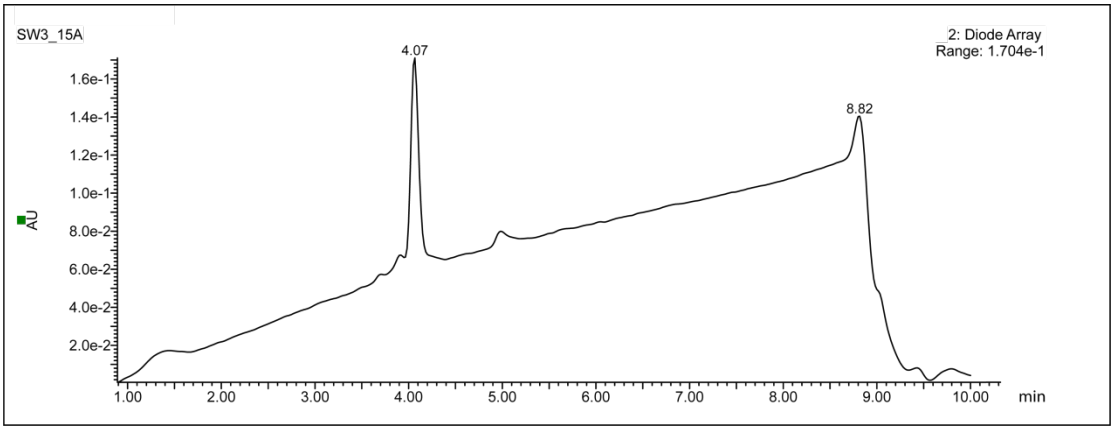
Characterization Data for compound SW2_152C-FL A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{77}H_{90}ClN_{11}O_{14}$: 1429.64; found: 1429.79

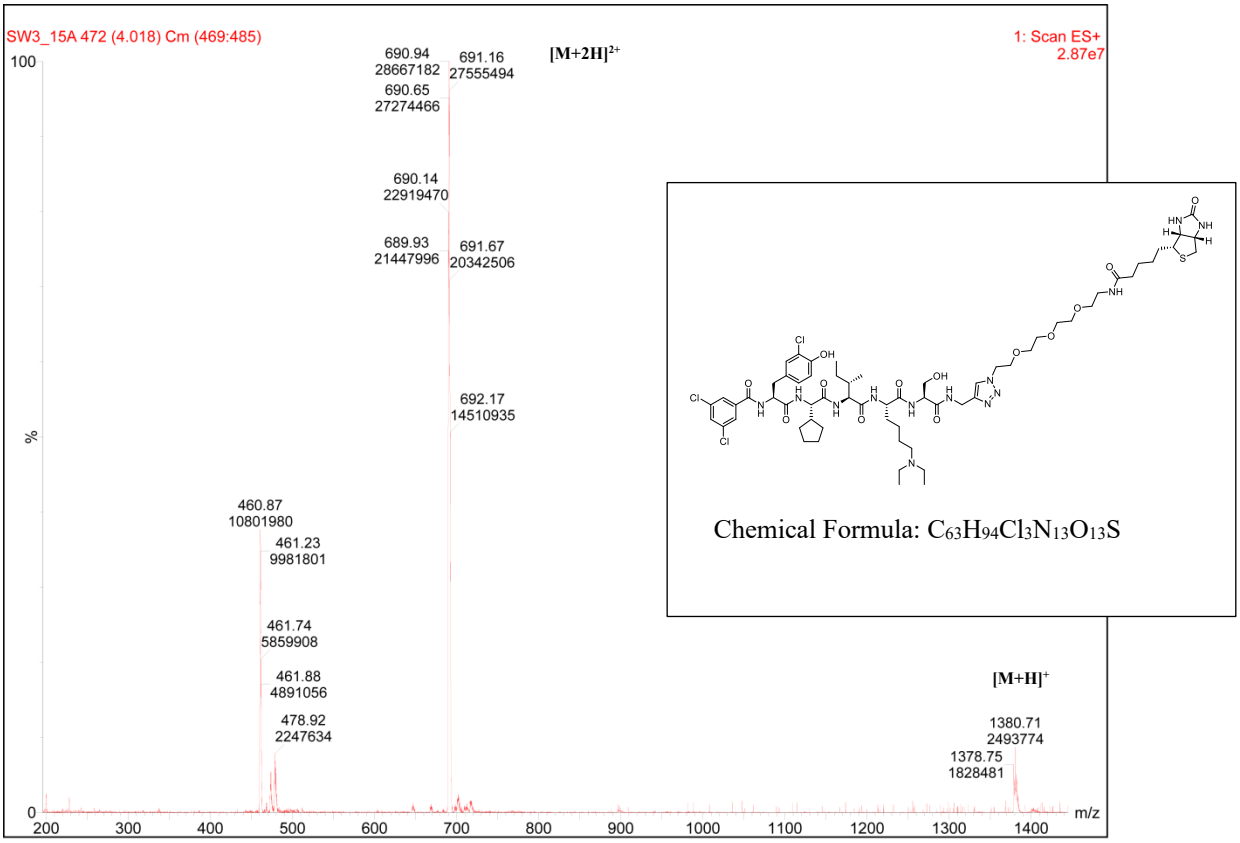




Characterization Data for compound SW2_152F_FL A) LC/MS UV traces (DMSO peak at 0.5 min) B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{70}H_{82}Cl_3N_{11}O_{14}$: 1406.51/1408.84; found: 1406.81/1408.59

Synthesis of Biotin-peptide Conjugates: Biotinylated peptides were synthesized as previously described.[1]



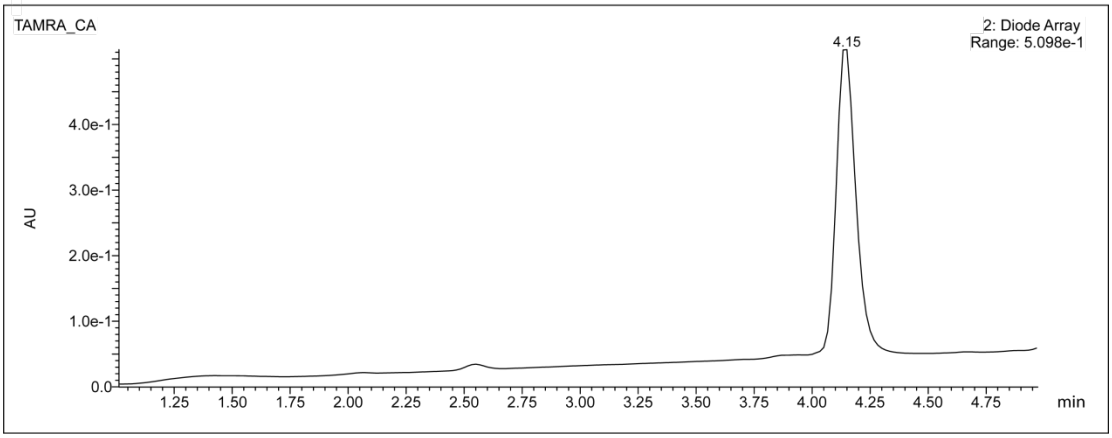


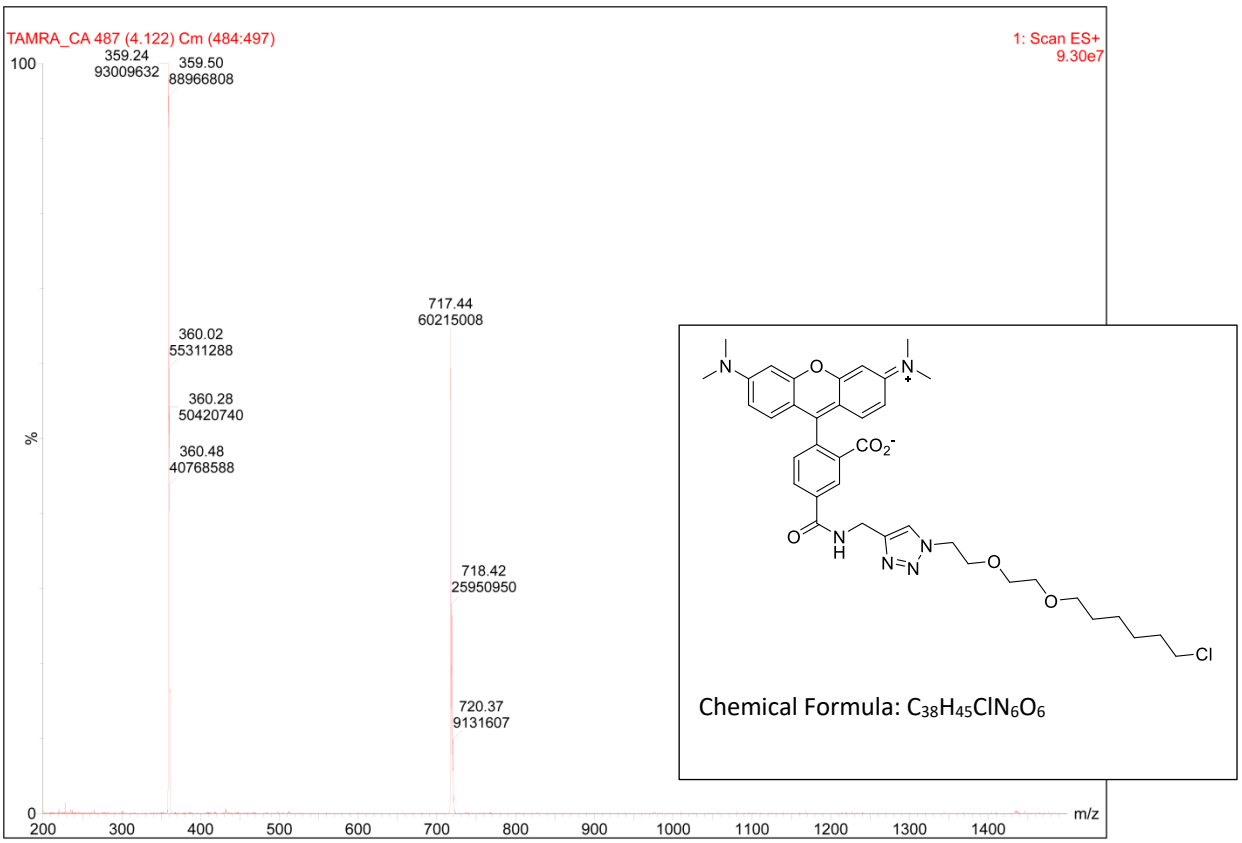
Characterization Data for compound SW2_152F-B A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{63}H_{94}Cl_3N_{13}O_{13}S$: 1378.6/1380.6; found: 1378.75/1380.71

Synthesis of Chloroalkane Linker: 2-(2-azidoethoxy)ethanol was prepared as previously described with minor modifications.[1,2]

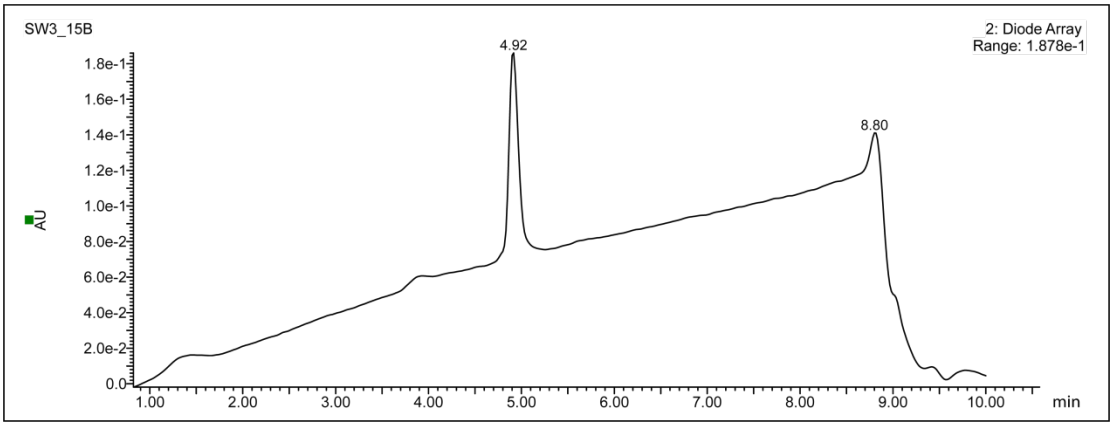
Synthesis of HT-TAMRA (TAMRA-chloroalkane): To a 150 µL of 100 mM alkyne TMARA (Lumiprobe, #

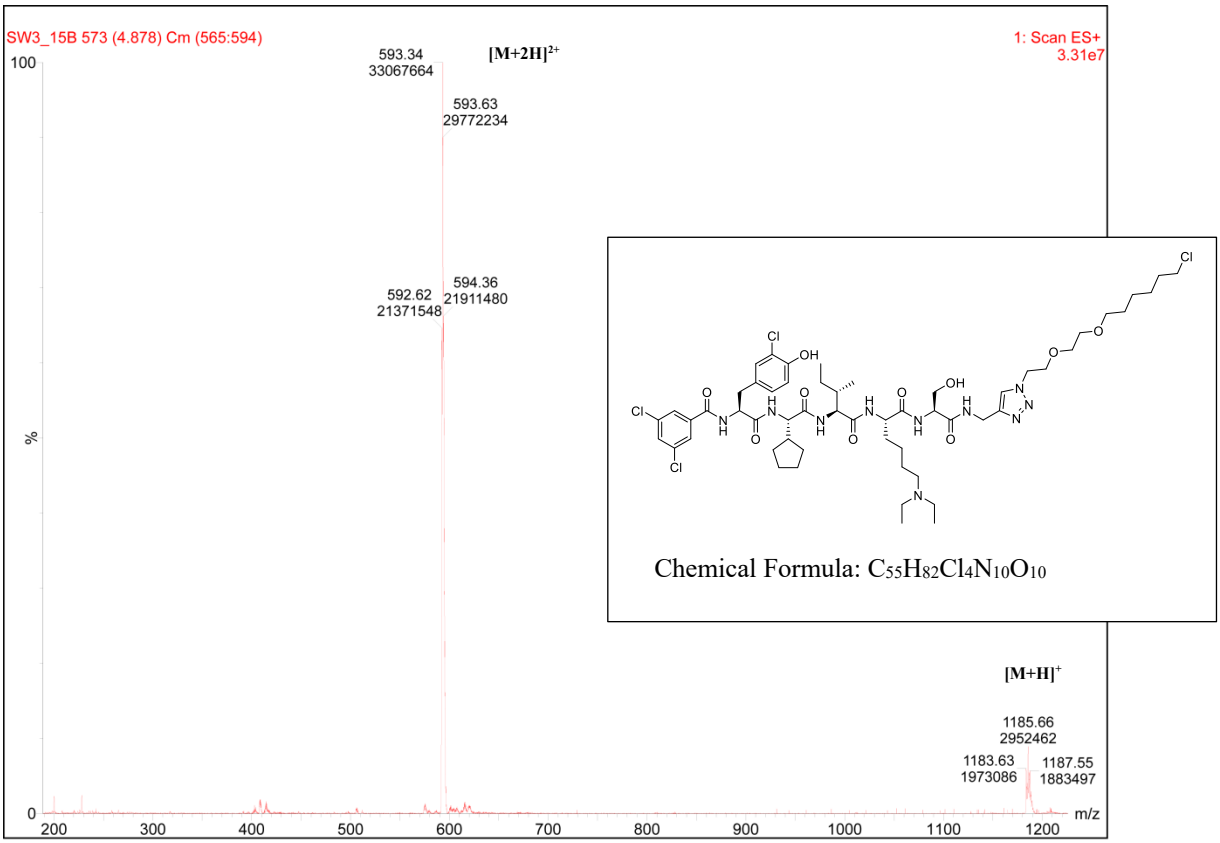
B71B0) in DMSO, 2.0 eq. of azide chloroalkane linker was added. To this, 5.0 µL of 2 M TEAA, pH 5.5, and 10 µL 0.1 M aminoguanidinium-HCl was added. Separately, 25 µL of CuBr saturated DMSO was suspended in 50 µL of 50 mM THPTA and then added to the azide/alkyne mixture. The mixture was incubated at RT for overnight and then 10 µL of 0.5M EDTA, pH 8 was added. The TAMRA-chloroalkane conjugate was purified as described above. Purity was confirmed to be > 95% by HPLC.





Characterization Data for compound TAMRA-Chloroalkane A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{38}H_{45}ClN_6O_6$: 717.31; found: 717.44





Characterization Data for compound SW2_152F-CA A) LC/MS UV traces B) Low Resolution mass spectrum. LR-ESI-MS: [M+1] calcd. For $C_{55}H_{82}Cl_4N_{10}O_{10}$: 1183.5/1185.5; found: 1183.63/1185.66

Direct Fluorescence Polarization (FP) Binding Assay of Fluorescein Labeled Peptides against PcG CBX ChDs: FP assays were conducted as previously described with slight modifications. The FAM-labeled peptide was kept constant at 100 nM. The CBX ChD proteins were titrated by 2-fold series dilutions in the assays with varying protein concentrations, depending on the binding affinity of the ligands. Four replicates were used for each ligand. Raw data were analyzed for determinations of K_d, using GraphPad Prism 8 following a "one-site" total binding model with any outliers (95% confidence interval) being excluded.

Competitive Fluorescence Polarization (FP) Binding Assay of unlabeled Peptides against CBX2 ChD: FP assays were conducted as previously described with slight modifications. The FAM-labeled peptide was kept constant at 100 nM andhe CBX2 ChD was kept constant at 500 nM, while varying concentrations of unlabeled ligand was added. Four replicates were used for each ligand. Raw data were analyzed for determinations of IC_{50} using GraphPad Prism 8 following a "one-site" total binding model with any outliers (95% confidence interval) being excluded.

Thermal Shift Analysis (TSA): The TSA was performed according to a previous reported protocol.[3] In brief, the reaction was run in 20 µL using a standard Real-Time qPCR machine with a ROX passive reference (Applied Biosystems). The reaction was run in the following reaction buffer: 10 mM HEPES 7.0, 150 mM NaCl, 8X SYPRO™ Orange protein gel stain S6651 Invitrogen (5000X stock), 0.2 mg/mL CBX2 ChD, 5% DMSO, containing SW2_152F at designated concentrations. Melt curves were obtained using a temperature gradient of 25-75 °C in 40 minutes with readings every 0.5 °C. Melt curves for CBX2 ChD were obtained for six replicates at each ligand concentration and the T_m values were calculated using nonlinear least squares fit on Prism 9. The approximate K_d was calculated from T_m values using nonlinear least squares fit on Prism 9.

Cell Culture: HEK293T cells were cultured in Dubecco's Modified Essential Media (DMEM), 10% fetal bovine serum (FBS, JR Scientific), 1% glutagro (Corning), 1% penicillin/streptomycin (Corning), 1% sodium pyruvate (Corning). Human LNCaP cells were cultured in (RPMI (Gibco), 10% FBS (J R Scientific), 1% Sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), 1% Glutamax (Thermo Scientific) LNCaP_NED cells were induced in (RPMI, no phenol red (Gibco), 10% CSS FBS (J R Scientific), 1% Sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), 1% Glutamax (Thermo Scientific). Halo-GFP-Mito Hela Cell were cultured in (DMEM (Gibco), 10% FBS (J R Scientific), 1% Sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), 1% Glutamax (Thermo Scientific), 1 µg/mL puromycin). Human Hs68 cells were cultured in (DMEM (Gibco), 10% FBS (J R Scientific), 1% Sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), 1% Glutamax (Thermo Scientific). Human K562 cells were cultured in (RPMI (Gibco), 10% FBS (J R Scientific), 1% Sodium pyruvate (Invitrogen), 1% Pen/Strep (Invitrogen), 1% Glutamax (Thermo Scientific). All cells were grown at 37 °C and 5% CO_2.

Chemoprecipitation (peptide pull-down): Peptide pull-down assay protocol was similar as previously described.[1] 293T Cells were grown to confluency in a 15 cm cell culture dish, washed with PBS and trypsinized. Cells were lysed with 2 ml Buffer A (25mM HEPES, 5mM KCl, 25mM MgCl2, 0.05mM EDTA, 10% glycerol, 0.1% NP-40, plus protease inhibitors) and lysed on ice for 15 minutes. The nuclei were pelleted and re-suspended in 1 mL peptide pulldown buffer (20 mM Hepes pH 7.9, 150 mM NaCl, 0.3% NP-40, protease inhibitor and 0.5 mM DTT). Benzonase (200U) was added and the lysate was incubated at 37 ℃ for 10 minutes and room temperature for 10 minutes with agitation. The samples were centrifuged at 13,000 rpm at 4 ℃ for 10 min and lysates were transferred to separate tubes. BCA assay was used to quantify the lysate concentration. Vehicle control (DMSO) or SW2_152F were added to the equal amount of lysate. Meanwhile, 30 µL streptavidin M-270 Dynabeads (Solulink, San Diego, CA) was washed three times with peptide pulldown buffer. Biotinylated ligands SW2_152F-B in DMSO (0.3 µL of 10 mM stock), were incubated with pre-equilibrated beads at room temperature for an hour. Extra unbound ligands were removed by washing 2× with peptide pulldown buffer and 300 µL (~300 µg) of nuclear lysate supernatant (vehicle control or SW2_152F treated) was added to immobilized biotinylated peptide (SW2_152F-B) or beads alone. The mixture was rotated at 4 ºC overnight, the depleted lysate was removed, and the beads were washed with

peptide pulldown buffer 3×5 minutes at room temperature. The bound proteins were eluted from the beads with 1× Bolt LDS Sample Buffer (Invitrogen) with 10% β-mercaptoethanol (AMRESCO LLC, Solon, OH). The samples, along with 10% input samples were heated at 95℃ for 5 min and loaded onto a 4–12% gradient gel (Invitrogen) for immunoblotting analysis of the proteins of interest. Image Studio was employed to quantitate the protein bands.

Immunoblot and Antibodies: Lysates were boiled and loaded on a 4-12% SDS-page gel (Invitrogen). Gels were transferred to PDVF membranes (Millipore) and incubated in 5% bovine serum albumin (BSA) in PBS-t (PBS with 0.1% Tween-20) prior to primary antibody. Blots were incubated at 4 °C overnight in primary antibody. Blots were washed with PBS-t and incubated for an hour at room temperature in goat anti-rabbit or mouse conjugated to IRDye® 800CW or IRDye® 680 (LI-COR) secondary antibody. Blots were imaged on the Licor Odyessy®. Primary antibodies used: CBX8 (rabbit, 1:1000, Bethyl Cat# A300-882A), CBX7 (rabbit, 1:2000, Bethyl Cat# A302-525A), CBX2 (rabbit, 1:1000, Bethyl Cat# A302-524A), CBX6 (mouse, 1:400, Santa Cruz Cat# sc-86354), BAF155 (mouse, 1:200, Santa Cruz Cat# sc-48350), H3K27me3 (rabbit, 1:1000, Diagenode Cat# C15410195), N-myc (mouse, 1:200, Santa Cruz Cat# sc-53993), ENO2 (mouse, 1:200, Santa Cruz Cat# sc-271384), AR (rabbit, 1:1000, Active Motif Cat# 39781), TBP (mouse, 1:1000, Abcam Cat# ab818).

Sequential Salt Extraction (SSE): SSE was performed similarly as previously described.[1] 2.5 × 10^6 293T cells were seeded in 10 cm cell culture dish (#353003, Corning) overnight. Next day, media was removed and cells were washed with PBS. Then, cells were pretreated with peptides SW2_152F (10 μM, 1% DMSO) or 1% DMSO on plate in media for 4 hours in 37 ºC. After the 4-hour pretreatment, media was removed and cells were washed again with PBS. Cells were harvested and washed with PBS. (Note: it is critical to equalize the cell numbers between the peptide treated group and the DMSO treated control group.) Cells were resuspended in 1 mL Buffer A (25 mM HEPES, 25 mM KCl, 5 mM $MgCl_2$, 0.1% NP-40, 10% Glycerol, 0.05 M EDTA, pH 7.8, plus protease inhibitor) post centrifugation and rotated at 4 ℃ for 10 minutes. Cells were spun down at 6500 × g for 5 min at 4 ºC. Supernatant was removed and cell pellet was resuspended with 500 μL of mRIPA (Modified Radioimmunoprecipitation Assay) Buffer (50 mM Tris, 1% Nonidet P-40, 0.25% Sodium deoxycholate, plus protease inhibitors) by pipetting up and down 15 times and incubated on ice for 5 minutes. (Note: it is critical to maintain the consistence for all washing steps with the subsequent NaCl containing mRIPA buffers) Then the sample was centrifuged for 3 min at 6500 × g. The supernatant was saved in a separate tube, labeled as “0 mM fraction”- 0 mM sequential salt extraction washing supernatant. (Notes: DMSO or the peptide SW2_152F was also added to the mRIPA washing buffers, in order to maintain the peptide in binding to the protein through the assay) The pellet was sequentially resuspended in 500 μl of mRIPA Buffer with increasing NaCl concentrations (100, 200, 300, 400 mM). The procedures for 0mM were repeated for each salt concentrations in the subsequent washes. supernatants after each wash were saved and labeled. 4× Bolt LDS Sample Buffer (Invitrogen) with 40% β-mercaptoethanol (AMRESCO LLC, Solon, OH) was added to each sample, and 40 μl of each fraction was loaded onto a 4–12% gradient gel (Invitrogen) for immunoblotting analysis of the proteins of interest. Image Studio was employed to quantitate the protein bands.

Cell Proliferation Assays: LNCaP cell lines were seeded at 80,000 cells/well with charcoal-stripped serum (CSS) containing media in 6-well flat bottom cell culture plate for 5 days (#353046, Corning). At day 6, control vehicle (0.5% DMSO) or inhibitor SW2_152F (2 μM or 10 μM in 0.5% DMSO) was added. Cells were grown for 5 days post compound treatment. Cells were counted every other day and the media was replenished with control vehicle DMSO or SW2_152F.

Cell Titer-Glo Luminescent Dose-Dependent Cell Viability Assay: The effect of enzalutamide on LNCaP or LNCaP_NED cell viability was determined using a Cell Titer-Glo ATP detection system (#G7573, Promega). LNCaP or LNCaP_NED cells were seeded in 10,000 cells/mL density in 96-well clear bottom white microplate (#655098, Greiner Bio-One). Cells were treated with 0.5% DMSO or 10 μM enzalutamide (in 0.5%

DMSO) for 5 days, with fresh compound replenishment at day 3. At day 5, Cell Titer-Glo reagent was added to cells, and incubated with gentle shake for 15 minutes in dim light at R.T. Luminescence was read on a GloMax® microplate reader at both day 0 and day 5.

Lentiviral Transduction LNCaP cell lines were seeded at 200,000 cells/well with charcoal-stripped serum (CSS) containing media in 6-well flat bottom cell culture plate for 5 days (#353046, Corning) as LNCaP_NED cells. HEK293T cells were co-transfected with pLKO.1 constructs and viral packaging vectors (pMD2.G and psPAX2). Short hairpin constructs for knockdown are below: CBX2 (TRCN0000020324). Viral supernatant was harvested 72 hrs after transfection and concentrated by ultracentrifugation at 17,500 rpm for 2h. Virus was resuspended in 100 µL PBS and 5 µL was added to LNCaP_NED cells. Twenty-four hours after transduction, cells were selected with puromycin (2 µg/mL) for 72h.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR): To evaluate the transcriptional changes in AR target gene and NED marker genes upon androgen deprivation, LNCaP cell line was seeded at 80,000 cells/well in charcoal-stripped serum (#353046, Corning) containing media in 6-well flat bottom cell culture plate for 9 days. Cells were harvested after 0, 3, 6, 9 days for RNA extraction. After homogenization of LNCaP_NED cells using TRIzol reagent (Thermo Scientific), RNA was extracted from the aqueous phase in the phase separation step. RNA pellet was washed with 75% ethanol and concentrated for subsequent reverse transcription. 500ng of RNA was converted into cDNA using Verso cDNA synthesis kit (Thermo Scientific). SYBR Green Mastermix (Thermo Scientific) was used for quantitative PCR. Primers below were used for qPCR.

To evaluate the transcriptional changes in AR target gene and NED marker gene upon CBX2 knockdown, 200,000/well in 6-well plate with charcoal-stripped serum containing media in 6-well flat bottom cell culture plate for 5 days (#353046, Corning) as LNCaP_NED cells. At day 6, shCBX2 lentivirus (generated as above) was added to cells for 24h, and puromycin was then added for 72h selection. After selection, cells were harvested for RNA extraction as above.

To quantitate the effect of SW2_152F on AR target gene and NED marker gene expression, LNCaP cell line was seeded at 200,000 cells/well in 6-well plate with charcoal-stripped serum containing media in 6-well flat bottom cell culture plate for 5 days (#353046, Corning) as LNCaP_NED cells. At day 6, control vehicle (0.5% DMSO) or inhibitor SW2_152F (10 µM in 0.5% DMSO) were added to the cells. Cells were harvested after 48 h for RNA extraction. 500ng RNA was then converted into cDNA using Verso cDNA synthesis kit (Thermo Scientific). SYBR Green Mastermix (Thermo Scientific) was used for quantitative PCR. Primers below are used in the qPCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
YWHAZ	TGCTTGCATCCCACAGACTA	AGGCAGACAATGACAGACCA
PSA	AAGACACAGGCCAGGTATTTC	CGATTCTTCAGGAGGCTCATATC
TMPRSS2	CAGGAGTGTACGGGAATGTGATGGT	GATTAGCCGTCTGCCCTCATTTGT
ENO2	CTGATCCTTCCCGATACATCAC	CTGGTCAAATGGGTCCTCAA
CHGA	CTGAACACACAGGCAGCTTTCTA	CAGTCAGGAGTTCTCAGCTTTC

CBX2	GCAAGCTGGAGTACCT GGTC	GGCTCCCAGCTGTTATG TTT

Chromatin Immunoprecipitation-qPCR (ChIP-qPCR): ChIP was performed as previously described with slight modifications.[1]

Hs68 ChIP: Cells were grown to confluency in 100 mm cell culture plates (~4 × 10^6 cells). Hs68 cells were treated with 100 µM SW2-152F (0.5% DMSO) or 0.5% DMSO for four hours. Briefly, cells were washed with PBS and fixed with 1% formaldehyde in PBS for 10 min at room temperature. Crosslinking was quenched with 0.125 M glycine for 5 min at 4 °C. Cells were washed once with PBS and resuspended in CiA NP Rinse buffer 1 (50 mM HEPES pH 8.0, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X) for 10 min. Cells were pelleted at 1200 x g for 5 min at 4 °C. The supernatant was removed, and the cells were resuspended in CiA NP rinse buffer 2 (10 mM Tris pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Cells were collected by centrifugation, 1200 x g for 5 min. Supernatant was removed and the cells were washed twice with shearing buffer (0.1% SDS, 1 mM EDTA, 10 mM Tris HCl, pH 8.0). Cells were resuspended in 2 mL shearing buffer and sonicated with a probe sonicator (Branson) to obtain 200~600 bp DNA fragments. Lysate was centrifuged at 21,000 x g for 15 min to remove debris. For immunoprecipitation, 250 µL cell lysate was incubated with 2 µg of antibody overnight and 10% input was saved. The IPs were washed 3 x 3 min at RT with IP buffer (50 mM HEPES/KOH pH 7.5, 300 mM NaCl, 1 mM EDTA, 1% Triton X, 0.1% DOC, 0.1% SDS) followed by 1x 3 min with DOC (10 mM Tris pH 8.0, 0.25M LiCl, 0.5% NP-40, 0.5% DOC, 1 mM EDTA) and 1x with TE. Protein was eluted from beads with 300 µL elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 20 minutes at RT with agitation. Samples, including saved input, were treated with RNase A (100U) at 37 °C for 30 min followed by proteinase K (2 µg) digestion for 3 hours at 55 °C. Samples were reverse crosslinked overnight at 65°C, and extracted with phenol chloroform followed by isopropanol precipitation. The isolated DNA was resuspended in 80 µL water for qPCR analysis (4 µL used per well). Antibodies used for IP were IgG (CST Cat# 2729, rabbit), CBX8 (Bethyl Cat# A300-882A, rabbit), CBX7 (Bethyl Cat# A302-525A, rabbit).

K562 ChIP: ChIP was performed as described above. Cells were grown in 150 mm cell culture plates to confluency. 20-25 million Cells were transferred and treated with 10 µM SW2_152F (0.5% DMSO) or 0.5% DMSO for four hours in 10 cm plates. Cells were collected after 4h and washed with PBS and CiA fixation buffer. Cells were crosslinked in 100 mm plates using 1% formaldehyde in PBS with shaking for 10 min at room temperature. After fixing, cells were washed, and lysed to extract the nuclei, which was sheared to generate DNA fragments between 200~600 bp. The chromatin was immunoprecipitated overnight at 4°C with antibodies against IgG (CST Cat# 2729, rabbit), H3K27me3 (Diogenade Cat# C15410069, rabbit), CBX2 (Bethyl Cat# A302-525A, rabbit), CBX8 (Bethyl Cat# A300-882A, rabbit), and captured by Protein A+G Dynabeads (Invitrogen) in IP buffer. ChIP DNA was resuspended in 80 uL water for qPCR analysis.

LNCaP_NED ChIP: ChIP was performed as described above. LNCaP_NED cells were grown in charcoal stripped serum containing media to confluency (~10 × 10^6) in 100 mm culture plates. Cells were treated with 10 µM SW2-152F (0.5% DMSO) or 0.5% DMSO for four hours. ChIP was performed as described above. Antibodies against IgG (CST Cat# 2729, rabbit), CBX2 (Bethyl Cat# A302-525A, rabbit), and H3K27me3 (Diogenade Cat# C15410069, rabbit) were used for immunoprecipitations. Primers below are used in the qPCR.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
LMNB2	CCGAATCTCTGAAATGA AAGTCCATGC	TTAAAGATCTGAGGGACTCCT CAGTC

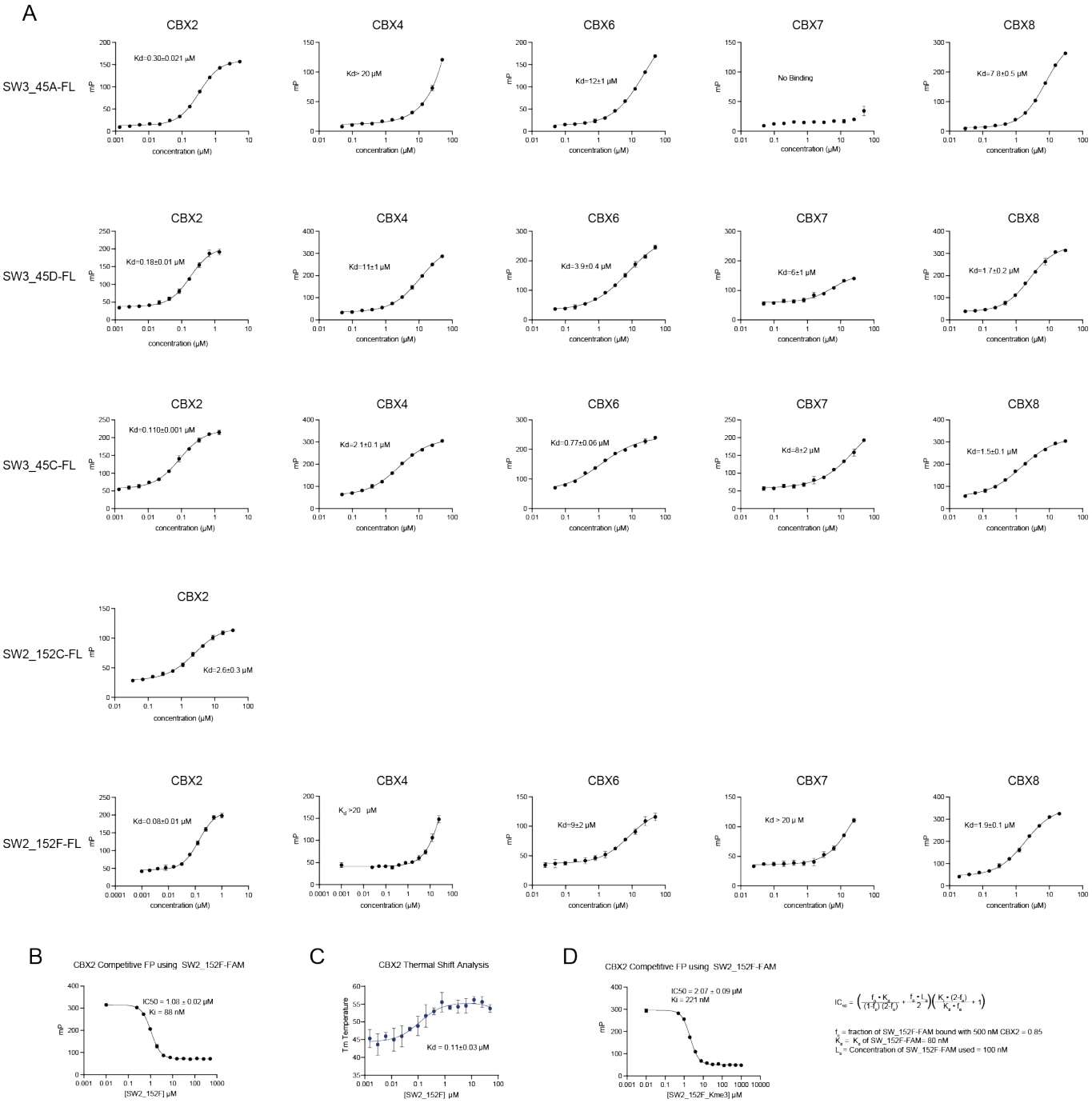
CCND2	ACTGTCTGAAATGAAG GTGAAGC	GATTTGATGGACACTTGGTTTG T
RUNX3	TCAAAAGGCATCCGCC TCTCCGT	AAGGATGCACCTGCCGGGAAT TG
FYN	AAAGCACATTTGCCTCT TGG	TGAGTTCATGGTGGACTGTCTT
TCF21	CCCCGACACCAAGCTC TCCAAG	AGTGGGCGATGTAGCTGGACG
PAX7	GCATCAGCCCGCACAA CTTCTG	ACTCCAACCCCAAGTCCGCC
Tm9SF4	TAGTGCCGCATCCCTTT TTA	TGGCTCAAACCCAAGTAACC
KLK3e	TTTGTATCCCAGACCAA ATGCC	TCTCGATGCTTCCCTACAGGAT
KLK3p	TCTGTTTCATCCTGGGC GTGT	TCTGCAATTACTAGATCACCCT GG
TMPRSS2e	CCACCTGGTGAAGTGC AGAT	TGGAGCTAGTGCTGCATGTC
TMPRSS2p	CAGGGTCGAGGGCGA CA	CTTATGGGTCCCTCCGCAGC

Chloroalkane Penetration Assay (CAPA): CAPA was performed as previously described.[1] Briefly, Halo-GFP-Mito Hela cells were cultured and seeded at a 1× 10^5 cells/well in a 24-well plate the day before experiments. Cells were rinsed by PBS and treated with chloroalkane conjugated peptidomimetic ligands SW2_110A-CA or SW2_152F-CA in acidified Opti-MEM (0.15% 6N HCl) for 4 h. Next, media was removed and cells were washed by phenol red-free Opti-MEM for 30 minutes, followed by incubation with 10 μM HT-TAMRA (synthesized as above) for another 30 minutes. Then, cells were washed for 15 minutes by phenol red-free DMEM +10% FBS +1% pen/strep, followed with PBS wash and trypsin incubation. Cells were transferred to a new microcentrifuge tube and pelleted by centrifuge, 2X PBS wash. Cell pellets were resuspended in 250 μL PBS, and 200 μL was used for flow cytometry analysis. Live cells were gated and 10,000 cells were measured per sample. Mean fluorescence intensity was calculated from raw data, and these values were normalized to the samples with no dye (0% red signal) and with dye but no HT-molecule (100% red signal).

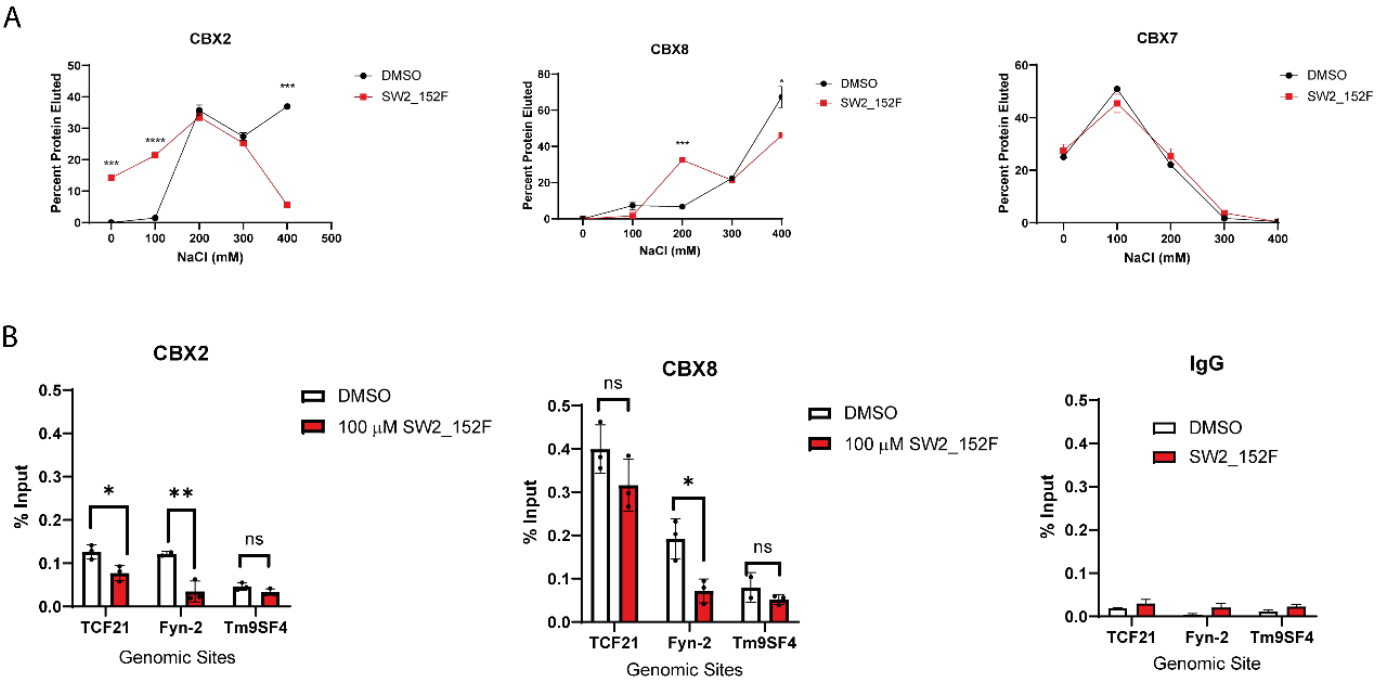
Bioinformatic Analysis using Public Clinical Prostate Cancer Patient Data and Patient-Derived Xenograft Data: Multiple clinical prostate cancer patient corhorts (683 samples in total) from public datasets were used for *AR* and AR-target gene expression changes in NEPC/PCa as plotted in Figure2A.[4] Published clinical patient dataset,[5] and patient-derived xenografts (13 PDX samples in total) were used for analyzing CBX paralogs and EZH2 transcriptional changes in NEPC/PCa as in Figure2B.[6] All PDX samples used for analysis were validated by the Living Tumor Lab upon their histological features of their respective subtypes.[6] The gene expression data for PDX cell lines were extracted from NCBI Gene Expression

Omnibus (GEO) under the accession number (GSE41193). The average transcriptional fold changes in all NEPC samples over prostate cancer adenocarcinoma samples were plotted using both clinical and PDX datasets. The disease-free survival analysis of *CBX2* and *EZH2* in PRAD were accomplished using GEPIA2 public database (Group Cutoff: median, 95% confidence interval). All statistical analyses were carried out with the Graphpad Prism software (version 8.0) using a statistical threshold of p-value ≤ 0.05 unless otherwise stated.

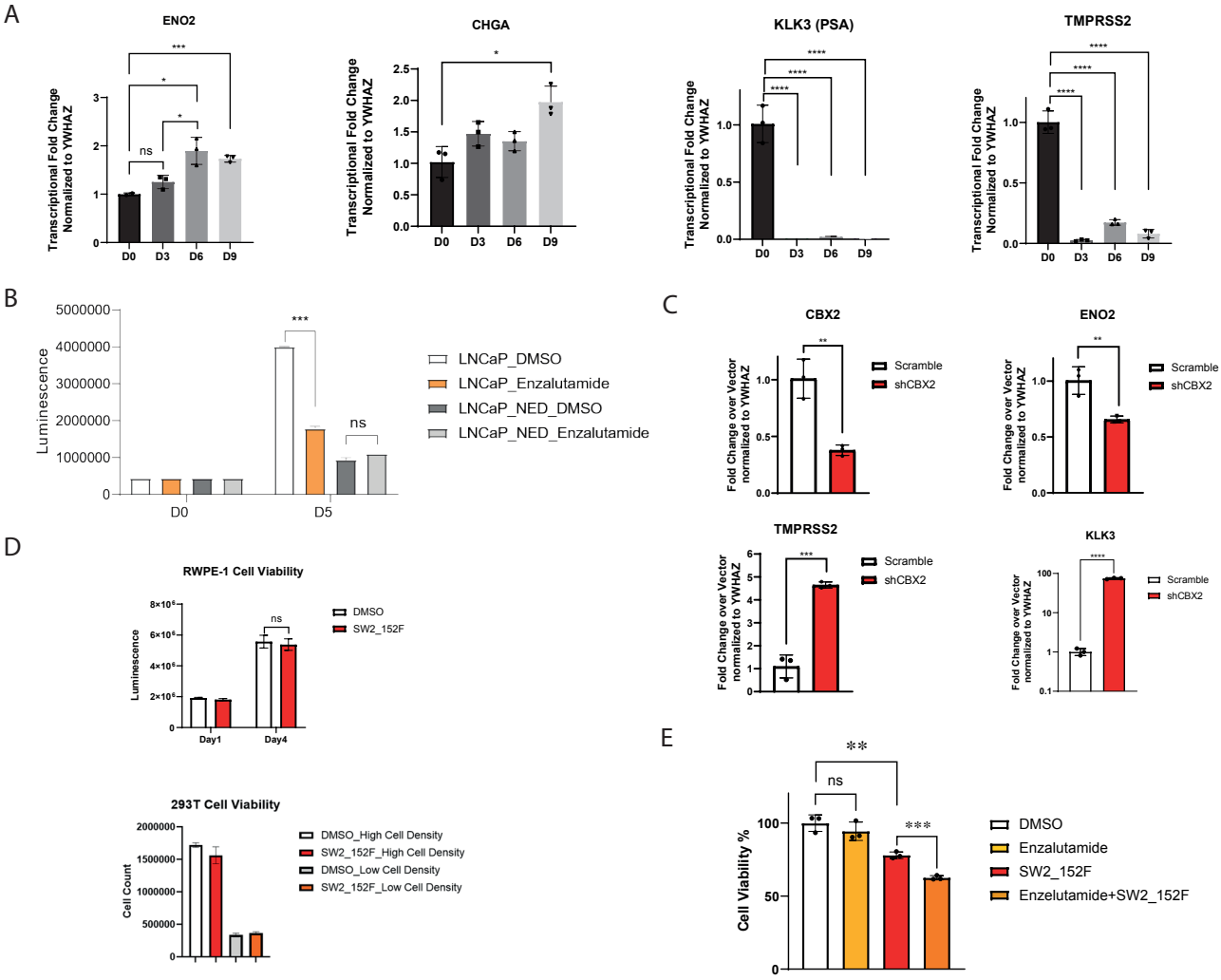
SUPPLEMENTARY FIGURES



SI1 A) Direct binding Fluorescence Polarization Assay Quantitative analysis of fluorescent ligand binding to CBX chromodomains. Assays used 100 nM of fluorescein-conjugated ligands with increasing concentrations of recombinant CBX chromodomains. Values are reported as the average of quadruplicates ± s.d. NB: No binding. Curves were fit using "One site - Total" mode in GraphPad 8. **B) Competitive FP assay for SW2_152F** Assays used 100 nM of SW2_152F_FL and 500 nM recombinant CBX2 CD with increasing amounts of competitive ligand. Values are reported as the average of quadruplicates ± s.d. NB: Curves were fit using "Inhibitor vs variable response" mode in GraphPad 8. **C) Thermal Shift Assay** T_m values for the CBX2 chromodomain were calculated in the presence of increasing concentrations of SW2_152F using a thermal shift assay. T_m values for six replicates were obtained for each compound concentration and the approximate K_d was calculated using nonlinear least squares fit on Prism 8. **D) Competitive FP assay for SW2_152F_Kme3**. Performed as in SI 1B. Ki calculated from IC50 using derived equation.[7] Related to **Table 1**.



SI 2 A) Sequential Salt Extraction Analysis and quantification of SW2_152F in abrogating bulk binding affinity of endogenous CBX proteins to chromatin in HEK293T cells by Sequential Salt Extraction. Quantitation of amount of CBX in each fraction as a percent of total CBX proteins. n=3 independent biological replicates, errors bars represent standard error of the mean (SEM), P-values were calculated using Student's two-tailed t-test: *p < 0.05 ***p < 0.001, ****p<0.0001. **B) ChIP-qPCR** Chromatin immunoprecipitation (ChIP) followed by quantitative PCR of genomic regions at *Tm9SF4* (negative locus), *TCF21*, *Fyn-2*, and *PAX2* with H3K27me3, CBX2 and CBX8 binding in K562 cell line. Cells were treated with 100 μM SW2_152F for 4h prior to harvest. For all qPCR, error bars represent SEM n=3 biological replicates, p-values were calculated using two-tailed Student's t-test, * = p < 0.05, ** = p <0.01, *** = p < 0.001, **** = p <0.0001. Related to **Figure 4**.



SI 3 A) Transcriptional fold changes of NED markers (*ENO2* and *CHGA*) and AR target genes (*KLK3* and *TMPRSS2*) by RT-qPCR at different (0/3/6/9) days of androgen deprivation. Fold changes were quantified normalized to control gene *YWHAZ*. For all qPCR, error bars represent SEM n=3 biological replicates, p-values were calculated using two-tailed Student's t-test, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$. **B)** Cell proliferation of LNCaP and LNCaP_NED following 5 days incubation of 10 μM enzalutamide were quantified by Cell Titer-Glo assay. DMSO was used as negative vehicle to enzalutamide. **C**) Transcriptional Fold Changes upon CBX2 knockdown. qRT-PCR analysis of *CBX2*, *ENO2, TMPRSS2* and *KLK3* gene expression in LNCaP_NED cells with knockdown of CBX2. **D**) Cell viability of RWPE-1 and 293T following 4 days incubation of 10 μM SW2_152F were quantified by Cell Titer-Glo assay (RWPE-1) or cell counting with trypan blue (HEK293T). **E**) Cell viability of LNCaP_NED following 4 days incubation of 10 μM enzalutamide, 10 μM SW2_152F or both were quantified by Cell Titer-Glo assay. DMSO was used as negative vehicle.

REFERENCES

[1] S. Wang, K. E. Denton, K. F. Hobbs, T. Weaver, J. M. B. McFarlane, K. E. Connelly, M. C. Gignac, N. Milosevich, F. Hof, I. Paci, C. A. Musselman, E. C. Dykhuizen, C. J. Krusemark, *ACS Chem. Biol.* **2020**, *15*, 112–131.

[2] L. Peraro, Z. Zou, K. M. Makwana, A. E. Cummings, H. L. Ball, H. Yu, Y. S. Lin, B. Levine, J. A. Kritzer, *J. Am. Chem. Soc.* **2017**, *139*, 7792-7802

[3] M. Vivoli, H. R. Novak, J. A. Littlechild, N. J. Harmer, *J. Vis. Exp.* **2014**, *91*, 51809.

[4] H. Beltran, D. Prandi, J. M. Mosquera, M. Benelli, L. Puca, J. Cyrta, C. Marotz, E. Giannopoulou, B. V. S. K. Chakravarthi, S. Varambally, S. A. Tomlins, D. M. Nanus, S. T. Tagawa, E. M. Van Allen, O. Elemento, A. Sboner, L. A. Garraway, M. A. Rubin, F. Demichelis, *Nat. Med.* **2016**, *22*, 298–305.

[5] H. Beltran, D. S. Rickman, K. Park, S. S. Chae, A. Sboner, T. Y. MacDonald, Y. Wang, K. L. Sheikh, S. Terry, S. T. Tagawa, R. Dhir, J. B. Nelson, A. de la Taille, Y. Allory, M. B. Gerstein, S. Perner, K. J. Pienta, A. M. Chinnaiyan, Y. Wang, C. C. Collins, M. E. Gleave, F. Demichelis, D. M. Nanus, M. A. Rubin, *Cancer Discov.* **2011**, *1*, 487–495.

[6] P. L. Clermont, D. Lin, F. Crea, R. Wu, H. Xue, Y. Wang, K. L. Thu, W. L. Lam, C. C. Collins, Y. Wang, C. D. Helgason, *Clin. Epigenetics* **2015**, *7*, 1–13.

[7] X. Huang, *J. Biomol. Screen.* **2003**, *8*, 34–38.