Supporting Information

Characterizing the Covalent Targets of a Small Molecule Inhibitor of the Lysine Acetyltransferase P300

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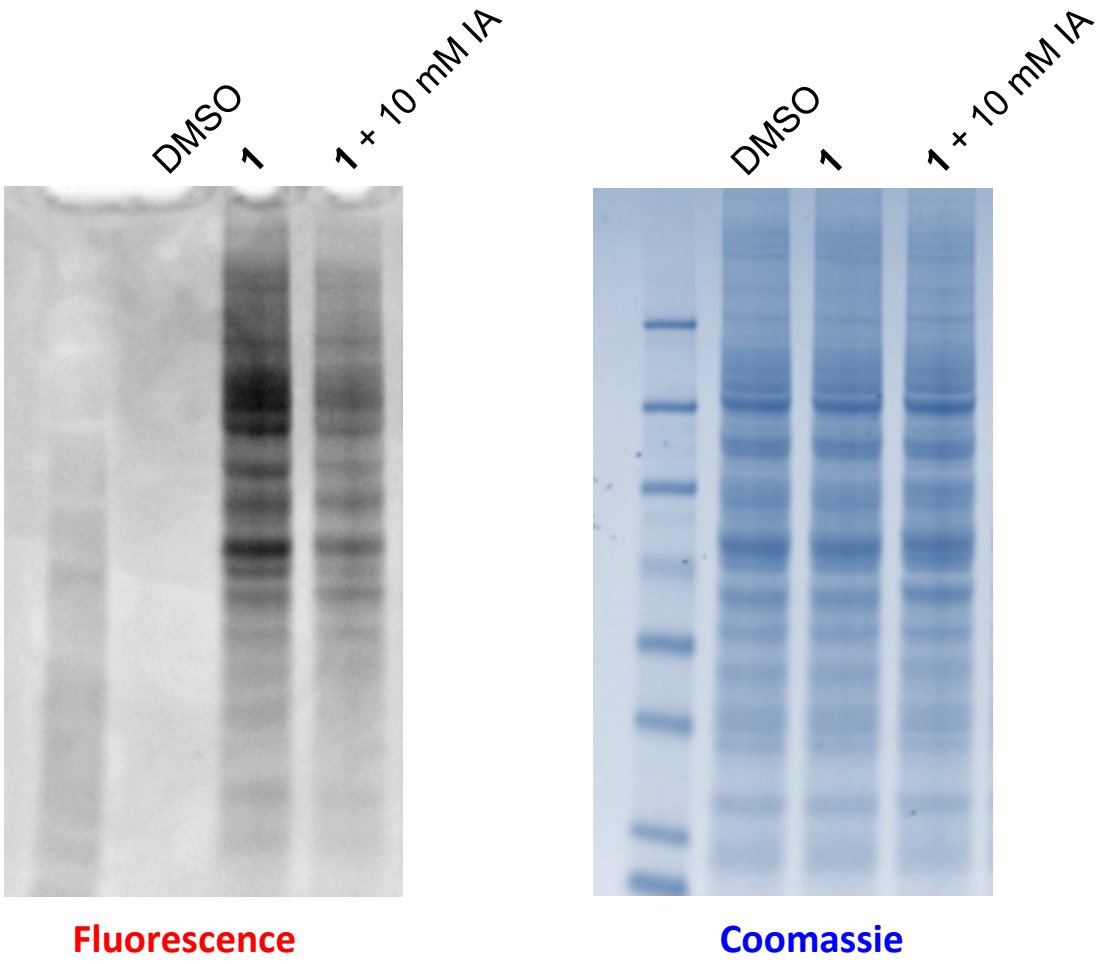


Figure S1: C646-yne labeling of HEK-293 proteomes is sensitive to iodoacetamide modification. Conditions: 20 µM C646-yne, 1 mg/mL HEK-293 proteome, overnight. Samples denoted "+ 10 mM IA" were pre-incubated with 10 mM iodoacetamide for 1 hour prior to C646 addition. C646-yne itself is not fluorescent under the conditions used to excite TAMRA (data not shown).

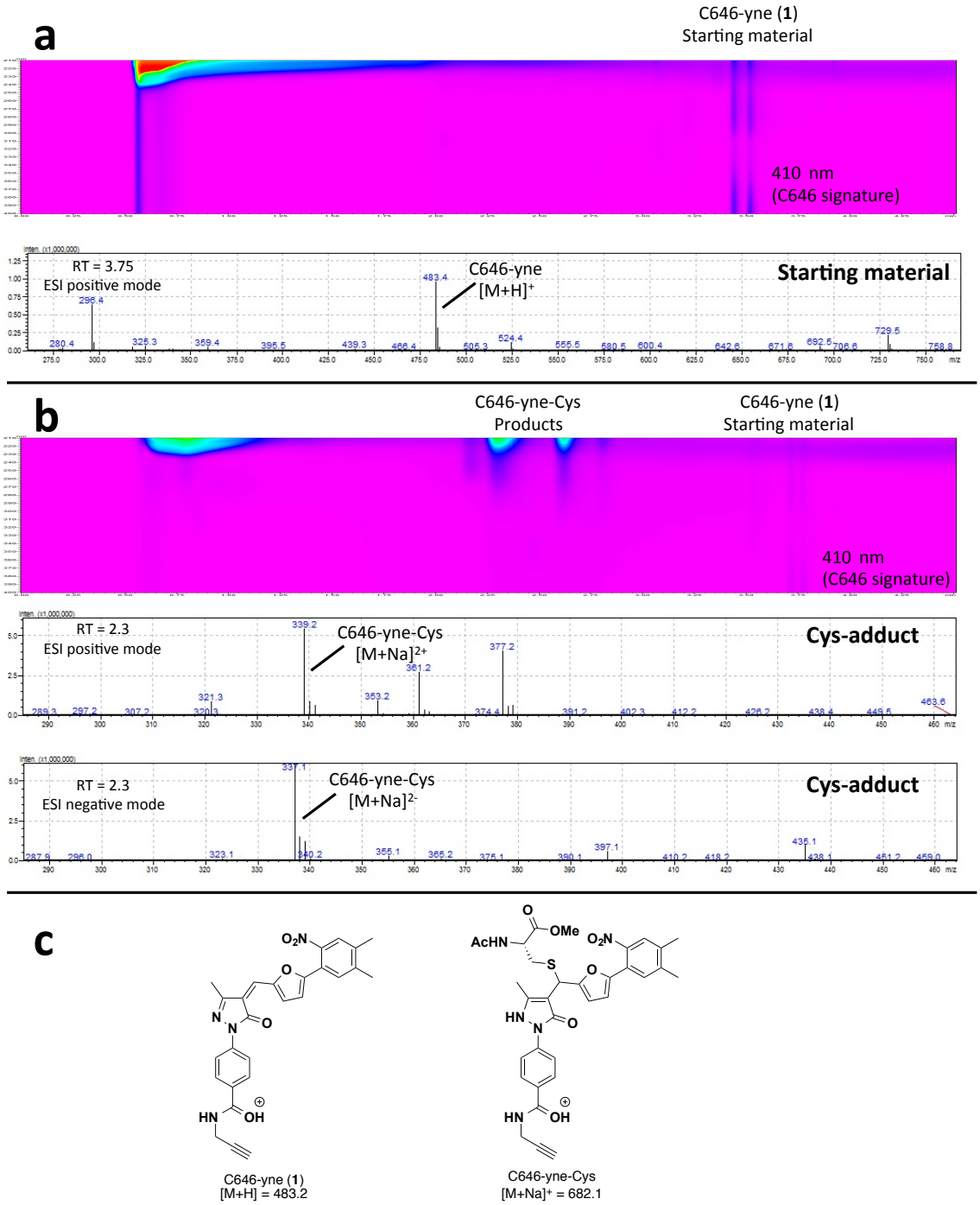


Figure S2: LC-MS analysis suggests that C646-yne forms a covalent adduct with cysteine. (a) LC-MS of C646-yne starting material. Top: UV trace. Bottom: MS. (b) LC-MS of C646-yne after incubation with Ac-Cys-OMe (5 mM) for 16 hrs. C646 is consumed and two new UV-absorbing peaks with masses corresponding to doubly charged Cys adducts are observed at lower retention time. Top: UV trace. Bottom: MS. (c) Structures of C646-yne starting material and putative C646-yne-Cys adduct.

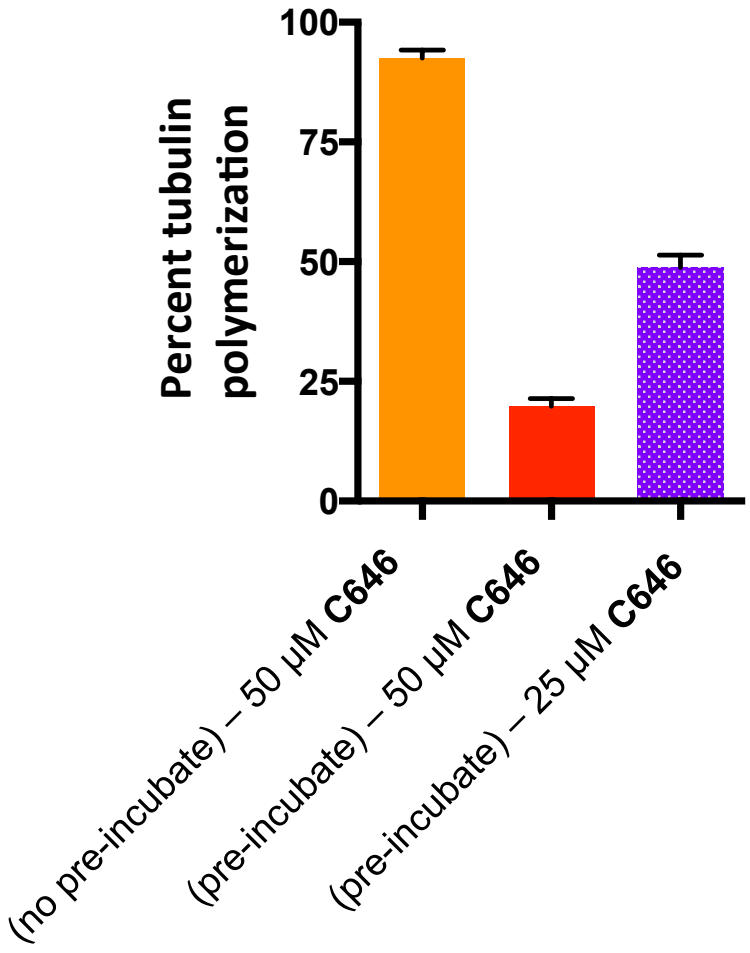


Figure S3: Inhibition of tubulin polymerization by C646 requires pre-incubation. Percent tubulin polymerization refers to signal for tubulin polymerization observed at 30 minutes in C646 treated samples relative to a DMSO-treated control (set to 100%). Pre-incubate = 1 hr pre-incubation with tubulin prior to assay.

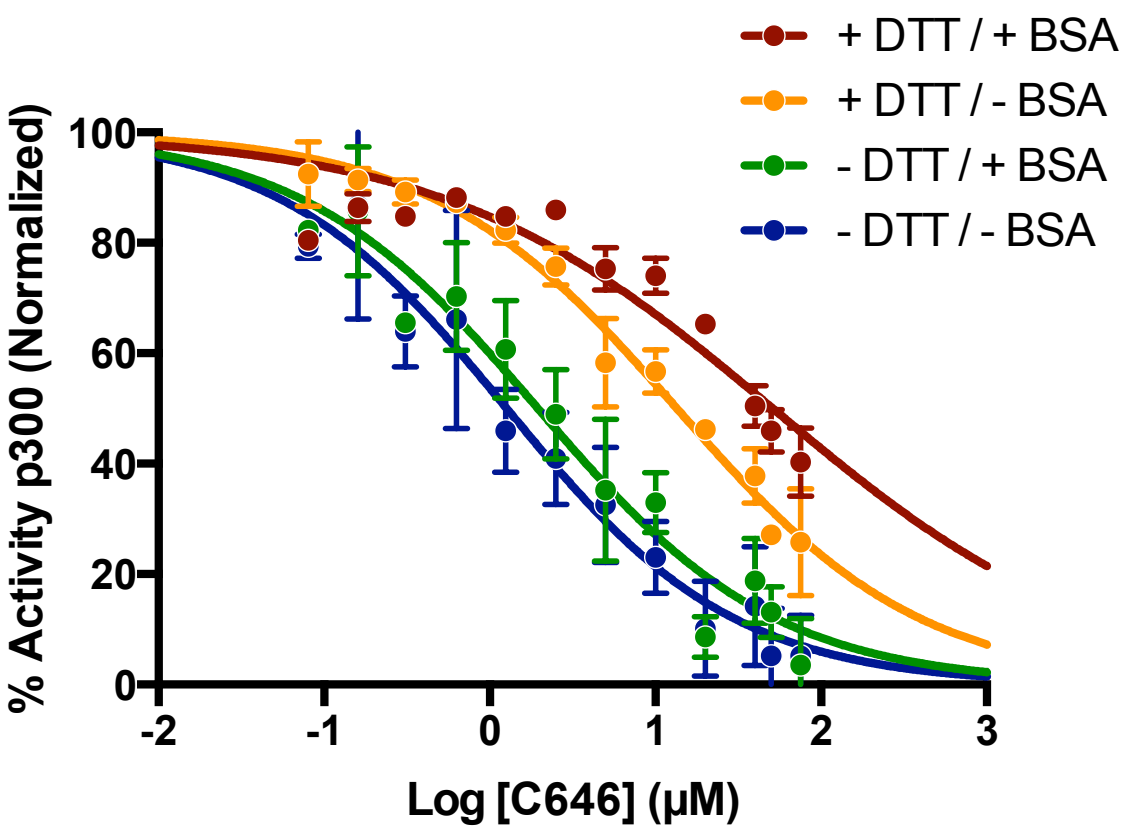
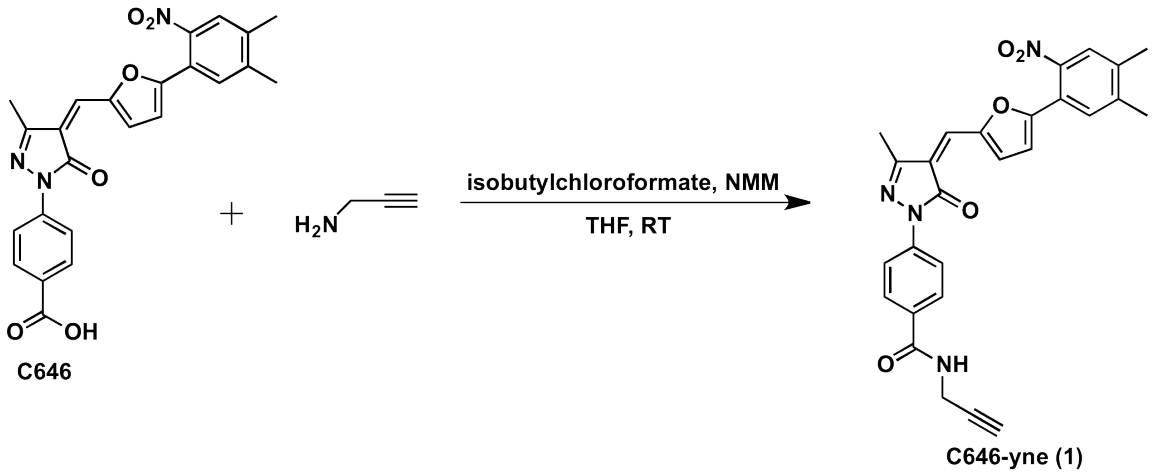


Figure S4: C646's p300 inhibitory activity is modulated by the presence of DTT and BSA.

Protein	gene	C646-yne (**1**)	no probe	ratio
Tubulin alpha-1B chain	TUBA1B	101	4	25
Tubulin alpha-1A chain	TUBA1A	99	4	25
Tubulin alpha-1C chain	TUBA1C	98	4	25
Tubulin beta chain	TUBB	71	2	36
Tubulin alpha-8 chain	TUBA8	64	1	64
Elongation factor 1-alpha 1	EEF1A1	60	5	12
Tubulin beta-2A chain	TUBB2A	49	2	25
60 kDa heat shock protein, mitochondrial	HSPD1	48	2	24
Tubulin beta-4A chain	TUBB4A	37	2	19
D-3-phosphoglycerate dehydrogenase	PHGDH	29		-
Alpha-enolase OS=Homo sapiens	ENO1	27	4	7
Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	19		-
Eukaryotic initiation factor 4A-I	EIF4A1	17	1	17
Creatine kinase B-type	CKB	17	2	9
Pyruvate kinase PKM	PKM	16	1	16
Heat shock protein HSP 90-beta	HSP90AB1	16	1	16
Actin, cytoplasmic 1	ACTB	16	4	4
Heat shock 70 kDa protein 1A/1B	HSPA1A	15	2	8
Delta-1-pyrroline-5-carboxylate synthase	ALDH18A1	13	0	-
Heat shock protein HSP 90-alpha	HSP90AA1	13	2	7
Exportin-1	XPO1	12	0	-
Heterogeneous nuclear ribonucleoprotein F	HNRNPF	12	0	-
Elongation factor Tu, mitochondrial	TUFM	12	0	-
Tubulin beta-6 chain	TUBB6	12	0	-
Aldose reductase	AKR1B1	11	0	-
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	11	0	-
Bifunctional glutamate/proline--tRNA ligase	EPRS	11	0	-
Heat shock 70 kDa protein 1-like	HSPA1L	11	0	-
Heterogeneous nuclear ribonucleoprotein L	HNRNPL	10	0	-
Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	10	0	-
Interleukin enhancer-binding factor 2	ILF2	9	0	-
3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	9	0	-
T-complex protein 1 subunit eta	CCT7	9	0	-
Vimentin	VIM	9	0	-
Heterogeneous nuclear ribonucleoprotein K	K	9	0	-
L-lactate dehydrogenase B chain	LDHB	9	1	9
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B	9	1	9
Elongation factor 2	EEF2	9	1	9
Heat shock cognate 71 kDa protein	HSPA8	9	1	9
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	9	2	5

Table S1. Proteins detected by LC-MS/MS following treatment of HEK-293 lysates with probe **1** (20 μM) or DMSO control, followed by click chemistry with biotin azide, streptavidin enrichment, wash, and tryptic digest. Listed are enriched proteins with spectral counts ≥ 9 and an enrichment ratio of ≥ 4 (**1**/control).



Scheme S1: Scheme for synthesis of C646-yne **1**.

General Synthetic Procedures

Chemicals were purchased from commercial sources and used without further purification unless otherwise noted. C646 was obtained from Cayman Chemical. Anhydrous solvents were prepared by passage over activated alumina. Purification of C646-yne (**1**) was carried out on a Waters 2545 Binary Gradient Module equipped with a Waters® 2767 Sample Manager fraction collector and a Luna 10 µm C18 110 Å (75 x 30 mm) column obtained from Phenomenex, Inc. Analytical LC/MS was performed using a Shimadzu LCMS-2020 Single Quadrupole utilizing a Kinetex 2.6 µm C18 100 Å (2.1 x 50 mm) column obtained from Phenomenex Inc. Analytical runs employed a gradient of 0→90% MeCN/0.1% aqueous formic acid over 4 minutes at a flow rate of 0.2 mL/min.

Synthesis of C646-yne (1)

To a stirring solution of C646 (3.7 mg, 8 µmol, 1.0 eq) in anhydrous, room temperature THF (360 µL) were added sequentially N-methylmorpholine (NMM, 2.2 µL, 20 µmol, 2.5 eq) and isobutylchloroformate (4.7 µL, 36 µmol, 4.5 eq). Upon addition of isobutylchloroformate the solution turned red and cloudy. The reaction was stirred at room temperature for 45 minutes, followed by addition of propargylamine (4.7 µL, 73 µmol, 9 eq). The solution clarified and consumption of C646 was monitored by TLC (2% MeOH in CH_2Cl_2). After 2 hours, all C646 was deemed consumed, solvent was removed under reduced pressure, and the crude reaction mixture was purified by preparative HPLC. Product was eluted using an isocratic gradient of 10% solvent B (CH_3CN/0.1% TFA) from 0-2 minutes, followed by a linear gradient of 10% - 95% solvent B over 6 minutes. Lyophilization of product-containing fractions afforded **1** as an orange solid (0.5 mg, 1.1 µmol, 12% yield). ESI $[M+H]^+$ calcd for $C_{27}H_{23}N_4O_5$ 483.2, observed 483.4.

Modeling parameters

The crystal structure of the KAT catalytic domain of human P300 (PDB code: 3BIY) was retrieved from the Protein Data Bank for use in the docking calculations.[1] The Protein Preparation Wizard of the Schrödinger suite was used to prepare the binding site of p300. The protein was processed by assigning bond orders, adding hydrogens, removing co-crystallized water molecules and creating disulfide bonds. Finally, a restrained minimization with a root mean square deviation (RMSD) value of 0.30 was applied using the OPLS 2005 force field to optimize the hydrogen bond network. The structures of C646 and C646-yne were sketched in the Maestro panel and geometrically optimized using the LigPrep module. Docking studies were performed using Glide (Glide, version 5.8; Schrödinger, LLC). The prepared p300 structure was employed to generate the receptor energy grid centered on the co-crystallized ligand (bisubstrate analog Lys-CoA). The extra-precision (XP) docking protocol was used to dock the prepared ligands.

Biological materials and methods
Recombinant p300 (1195-1662) was purchased from Enzo. Labchip EZ-Reader 12-sipper chip (#760404) and ProfilerPro Separation Buffer (#760367) were purchased from Perkin-Elmer. Tubulin Polymerization Assay Kit was purchased from Cytoskeleton, Inc. (#BK011P). C646-yne stock solutions were prepared by DMSO, and concentration was determined by comparing absorbance to a solution of C646 of known molarity.

Biochemical KAT activity assay
Acetyltransferase activity of p300 was assessed using a separation-based assay as reported previously.[2-3] This method detects acetylation of a FITC-labeled p300 substrate peptide (histone H4 3-14; FITC-Ahx-RGKGGKGLGKGG) based on its altered electrophoretic mobility relative to non-acetylated peptide. Briefly, KAT assays consisting of reaction buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.05% Triton-X-100) with p300 [50 nM] and FITC-H4 [2 μM] were plated in 384-well plates and allowed to equilibrate at room temperature for 10 min in the presence or absence of inhibitor. C646-yne concentrations used were calibrated by comparing UV absorbance at 410 nm to C646. Reactions were initiated by addition of acetyl-CoA (final concentration = 5 μM), bringing the final assay volume to 30 μL and quenched while the reaction was still in the linear phase (<15% product accumulation) by addition of 5 μL of 0.5 M neutral hydroxylamine. Reactions were then transferred to a Perkin-Elmer Lab-Chip EZ-Reader instrument for analysis by microfluidic electrophoresis. Optimized separation conditions were: downstream voltage of –500 V, upstream voltage of –2500 V, and a pressure of –1.5 psi. Percent conversion is calculated by ratiometric measurement of substrate/product peak heights. Percent activity p300 represents the relative acetylation observed in inhibitor treated reactions relative to untreated control reactions, with each corrected for nonenzymatic background acetylation. Dose-response analyses were performed in triplicate and analyzed by nonlinear least-squares regression fit to $Y = 100/(1 + 10\text{^}(\text{Log } IC_{50} - X)*H)$, where H = Hill slope (variable). IC_{50} values represent the concentration that inhibits 50% of KAT activity. All calculations were performed using Prism 6 (GraphPad) software.

Analysis of C646-yne targets by SDS-PAGE
HEK-293 cell proteomes (20 μg in pH 7.2 in PBS) were incubated with C646-yne **1** (0-50 μM; 2 mM stock in DMSO) for 16 hours. Proteins labeled by C646-yne were detected by Cu(I)-catalyzed [3 + 2] cycloaddition ("click chemistry").[4-5] Click reactions were initiated by sequential addition of TAMRA-azide (100 μM; 5 mM stock solution in DMSO), pre-mixed $CuSO_4$:THPTA (1 mM and 5 mM respectively; made from 50 mM stocks in H_2O), and sodium ascorbate (5 mM; 250 mM stock in H_2O). Samples were vortexed and incubated at room temperature for 1 hour. Cycloaddition reactions were quenched by addition of 4x SDS-loading buffer (strongly reducing) and subjected to SDS-PAGE (20 μL per well). Excess probe fluorescence was removed by destaining in a solution of 50% MeOH/40% H_2O/10% AcOH overnight. Gels were then washed with water and fluorescently visualized using an ImageQuant Las4010 (GE Healthcare) with green LED excitation (λ_{max} 520–550 nm) and a 575DF20 filter. For iodoacetamide competition experiments, proteomes were pre-treated with 10 mM iodoacetamide for 1 hour prior to C646-yne addition.

Enrichment and LC-MS/MS Identification of C646-yne targets

HEK-293 cell proteomes (1 mg/mL in PBS, 1.5 mL) were treated with C646-yne **1** (20 µM) for 16 hours. Labeled samples were subjected to Cu(I)-catalyzed [3 + 2] cycloaddition with TAMRA biotin-azide as previously described.[5-6] Final concentrations for click reaction components were: HEK-293 proteome (1.5 mg in PBS), probe **1** (50 µM), TAMRA biotin-azide (125 µM), TCEP (1.25 mM), TBTA (127.5 µM), *tert*-butanol (4.8%), and $CuSO_4$ (1.25 mM). After $CuSO_4$ addition samples were vortexed and incubated at room temperature for 1 hour over which period the solution became cloudy. Reactions were centrifuged to collect precipitated protein and the supernatant discarded. Samples were redissolved in 750 µL of MeOH (dry-ice chilled) with sonication, and again centrifuged (4300g *x* 10 min, 4 °C). The MeOH supernatant was discarded, and resulting protein pellet dried under a gentle stream of air. Proteins were redissolved in a solution of SDS (2.5% w/v SDS in PBS; 650 µL) with sonication, followed by heating at 60 °C to ensure complete resuspension. Samples were allowed to cool to room temperature and PBS was added to a final volume of 6 mL. Streptavidin beads (Pierce # 20347, 100 µL of 50% aqueous slurry per enrichment) were washed 3x with PBS and added to clicked proteomes derived from DMSO and C646-yne treated samples. Samples were diluted to 8 mL and rotated for 1 hour at room temperature. Beads were then pelleted by centrifugation (1400g x 2 min) and supernatant discarded. Beads were then sequentially washed with 0.2% SDS in PBS (8 mL x 3) and PBS (8 mL x 3) for a total of 6 washes (5 minutes each). Following centrifugation (1400g x 2 min) and removal of the last wash, samples were resuspended in 100 µL of 8M urea in Tris buffer (pH 8.0, 50 mM). Reductive alkylation was accomplished by sequential addition of TCEP (14 µL of fresh 100 mM stock) and iodoacetamide (3.5 µL of 500 mM stock). Samples were protected from light rotated for 25 minutes at room temperature. Beads were pelleted by centrifugation (1400g x 2 min), supernatant discarded, and subjected to tryptic digest by adding 400 µL of 50 mM Tris-HCl (pH 8.0), 0.4 µL of 1M $CaCl_2$ and 4 µL of Trypsin Gold (Promega, 0.25 µg/µL in 1% acetic acid). Samples were left shaking overnight at 37 °C, pelleted by centrifugation (1400g x 5 min), and supernatant transferred to eppendorf tubes. Addition of 25 µL of formic acid (LC-MS grade) was used to quench trypsin. Desalting and LC-MS/MS analysis was performed as described previously.[2]

Tubulin polymerization assay

Tubulin polymerization assays were performed according the manufacturer's instructions (Cytoskeleton # BK011P). Briefly, 10x stock solutions of paclitaxel (enhancer, 100 µM), $CaCl_2$ (inhibitor, 10 mM) and C646 (500-100 µM) were prepared at room temperature. Buffer 1 (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM Ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, pH 6.9, 10 µM fluorescent reporter), GTP (100 mM) and tubulin buffer (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, 60% v/v glycerol, pH 6.9) and tubulin (88 µl of 10 mg/mL were thawed on ice. *NOTE: tubulin left at room temperature will begin to polymerize at 10 mg/ml. It is extremely important to defrost the tubulin rapidly and immediately transfer to ice.* A tubulin polymerization solution consisting of buffer 1 (243 µL), tubulin buffer (112 µL), GTP stock (4.4 µL), and tubulin (85 µL) was mixed and placed on ice. To 180 µL of this solution was added 20 µL of C646 (10-50 µM, from 100-500 µM stock) and the solution was vortexed and incubated on ice. Following this same method, reactions were prepared for paclitaxel, $CaCl_2$ and DMSO (as a negative control). After 1 hour, 50 uL from each reaction was transferred to a 96-well plate (pre-warmed at 37 °C in a Biotek Synergy plate reader) and reading initiated immediately (γ_{ex} = 360 nm, γ_{em} = 460 nm, 37 °C, 1 read per minute over one hour. Percent polymerization refers to the fluorescence signal observed for inhibitor or activator treated reactions, relative to uninhibited DMSO control.

Cell-based histone acetylation assays

HepG2 cells were cultured in RPMI supplemented with 10% FBS and L-glutamine (2 mM). For histone acetylation analyses, HepG2 cells were plated in 6-well dishes (4 x 10^5 cells/well), and allowed to adhere for 24 hours. For samples receiving BSA, RPMI was supplemented with BSA used during plating. For dosing, C646 (9 µL, 10 mM in DMSO) was dissolved in RPMI (241 µL) and added directly to wells (final volume per well 2.25 mL, 0.4% DMSO). An analogous protocol was used for 20 µM C646 treatments. Cells were incubated for 24 hours prior to histone extraction.[7] Following removal of media, cells were washed once with PBS and incubated with 300 µL of Triton Extraction Buffer (TEB: PBS with 0.5% Triton-X 100, 2 mM PMSF, and 0.02% NaN_3), harvested by gentle lifting, and transferred to microcentrifuge tubes. Samples were incubated in TEB for 30 min on ice and pelleted by centrifugation (6500g x 10 min, 4 °C). Supernatant was removed and saved for cytosolic protein analysis. Nuclei were resuspended, washed again with TEB (150 µL), and pelleted by centrifugation (6500g x 10 min, 4 °C). For acid extraction of histones, each pellet was treated with 0.4N H_2SO_4 (75 µL) and rotated overnight at 4 °C. Samples were centrifuged (11000g x 10 min, 4 °C), and histones were precipitated from the supernatant by addition of 20% TCA (750 µL). After at least 1 hour, samples were centrifuged (16000g x 10 min, 4 °C), and the pellets were washed with acetone/ 0.1% HCl (750 µL) and neat acetone (750 µL), with centrifugations (16000g x 10 min at 4 °C following each wash. Samples were air-dried at room temperature, dissolved in ddH_2O, and quantified by Qubit protein assay kit (Life Technologies). Samples were prepared for western blot analysis using Bis-Tris NuPAGE gels (12%) and MES running buffer in Xcell SureLock MiniCells (Invitrogen) according to the manufacturer's instructions. Following primary antibody incubation (H3K27Ac antibody, Cell Signaling, # 4353), washing, secondary antibody incubation and washing according to the manufacturer's instructions (Cell Signaling Technology), Lumiglo (CST ##7003) was added and the chemiluminescent signal was visualized using an ImageQuant Las4010 Digitial Imaging System (GE Healthcare). Immunoblot signals were quantified using ImageQuant TL software (GE Healthcare).

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