Bio-orthogonal Affinity Purification of Kinase Substrates

Supporting Information

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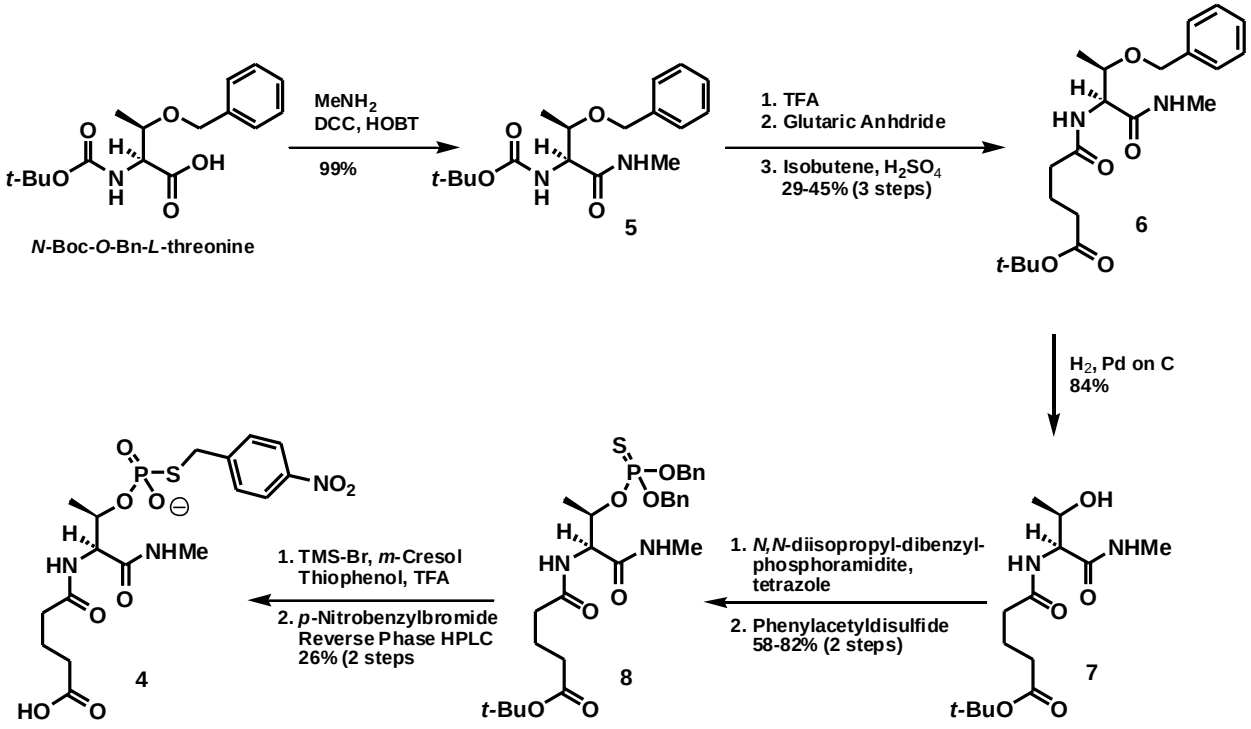
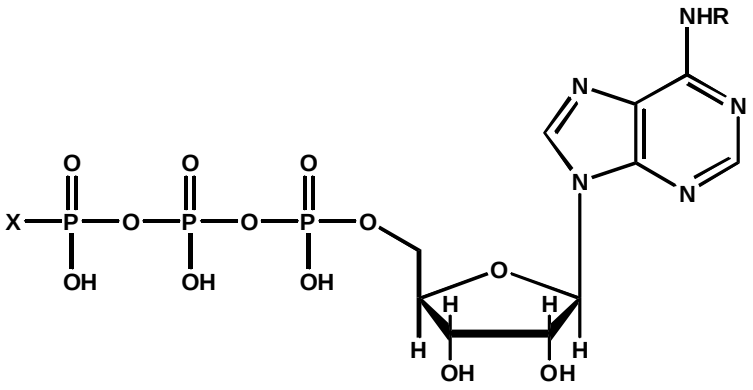
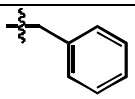
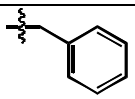
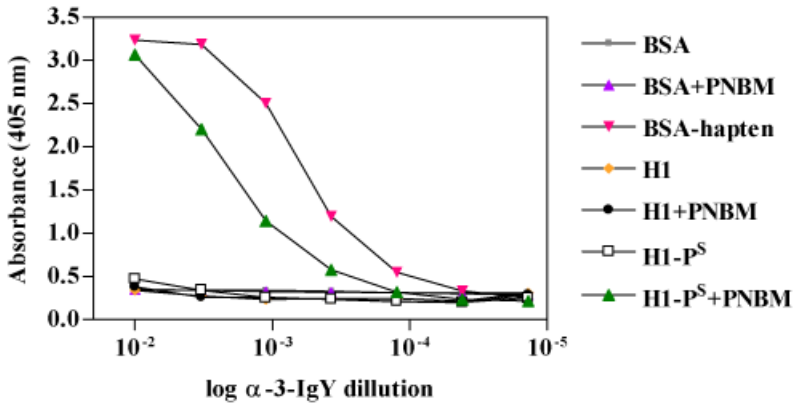
Supplemental Scheme S1. Synthesis of Hapten **4**



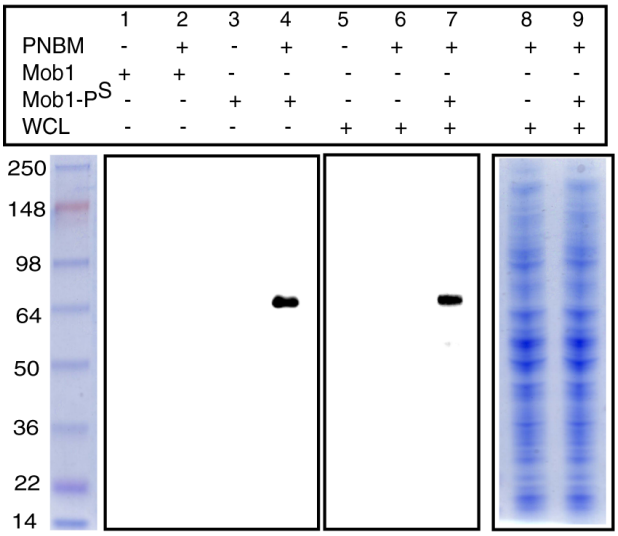


Supplemental Figure S1. Definitions of A*TP and A*TP?S

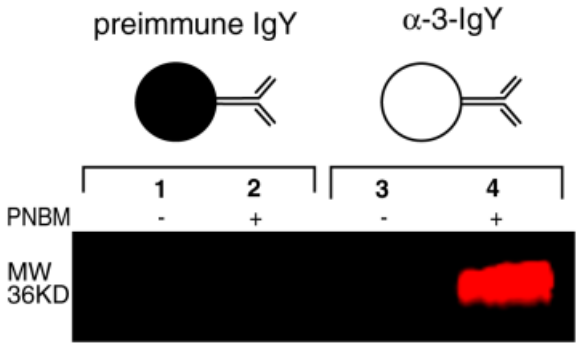
Compound	X	R
ATP	**OH**	**H**
N^6-benzyl ATP[1,2] (A*TP)	**OH**	
ATP?S	**SH**	**H**
N^6-benzyl ATP?S[3] (A*TP?S)	**SH**	



Supplemental Figure S2. ELISA Demonstrating a-**3**-IgY Recognition Determinants. Each protein antigen was coated in a 96 well plate and assayed by indirect ELISA, with colorimetric determination at 405 nm, reactions were done in triplicate.



Supplemental Figure S3. Western Blot analysis of Mob1 and WCL. 25 ng of Mob1 or Mob1-P^S was treated with DMSO (lanes 1 and 3) or 2.5 mM PNBM in DMSO (lanes 2 and 4). 15 µg WCL was treated with DMSO (lane 5), 2.5 mM PNBM in DMSO (lane 6), and 25 ng Mob1-P^S plus 2.5 mM PNBM in DMSO (lane 7). Lanes 8 and 9 show coomassie staining of samples identical to 6 and 7 respectively.



Supplemental Figure S4. Immunoprecipitation of Rh-H1-P^S, Requirement for PNBM and Immune Antibodies. 1 µg of Rh-H1-P^S treated with either 2.5 mM PNBM in DMSO or DMSO and then immunoprecipitations were attempted with preimmune or a-**3**-IgY sepharose beads. After gel electrophoresis, immoprecipitated proteins were visualized by in-gel fluorescence scanning (excitation at 523 nm, emission collected with a 580 nm band pass filter).

Supplemental Table S5. Kinases that have been shown to thiophosphorylate their substrates. The kinases listed below have been shown to thiophosphorylate defined substrates in vitro. Several other examples exist where proteins have been thiophosphorylated in crude preparations.[4,5]

Kinase	**Reference**
MAP kinase (p38α)	[6]
cyclin dependant kinase 1 (Cdk1)	[3] and this study
cyclin dependant kinase 2 (Cdk2)	[3]
phosphoinositide dependant kinase 1 (PDK1)	[7]
casein kinase 1 (CK1)	[8]
myosin light chain kinase (MLCK)	[8]
protein kinase A (PKA)	[9-11]
protein kinase B (Akt, PKB)	[12]
protein kinase C (PKC)	[13]
integrin linked kinase (ILK)	[14]
calcium/calmodulin dependent protein kinase II (CAMIIK)	[15]
C-terminal Src kinase (Csk)	[16]
cyclic nucleotide dependant protein kinase (PKG Iα)	[17]
Rho associated kinase (ROC)	[18]
MEK2	[19]
phosphorylase kinase	[11]

General Methods

Unless noted, chemical reagents and solvents were used unpurified from commercial sources. Reaction mixtures were magnetically stirred. Thin layer chromatography was performed on Merck precoated silica gel F-254 plates (0.25 mm). Concentration *in vacuo* was generally performed using a Büchi rotary evaporator. Flash column chromatography was performed on Baker 230-400 mesh silica gel.

Nuclear magnetic resonance spectra were recorded on a Varian 400 MHz instrument. Proton NMR spectra were recorded in ppm using the residual solvent signal as an internal standard: $CDCl_3$ (7.26 ppm), C_6D_6 (7.15 ppm) or d_6-DMSO (2.49 ppm). Carbon NMR were recorded in ppm relative to solvent signal: $CDCl_3$ (77.07 ppm), C_6D_6 (128.0 ppm) or d_6-DMSO (39.5 ppm). High resolution electron impact mass spectra were recorded on a MicroMass VG70E spectrometer by Sun Yuequan at the University of California-San Francisco Center for Mass Spectrometry.

Protected Threonine 5. A solution of N-Boc-O-Benzyl-(L)-threonine (3.00 g, 9.70 mmol) in THF (24 mL) was treated with hydroxybenzotriazole (HOBT) (1.378 g, 10.2 mmol) and methylamine (2.0 M in THF, 6.00 mL, 10.7 mmol) and cooled to 0 °C. Dicyclohexycarbodiimide (DCC) (2.105 g, 10.2 mmol) was added and the solution was allowed to warm to ambient temperature. After stirring for 16 h, ether (100 mL) was added and a white precipitate was removed by filtration through celite. The precipitate was washed with ether, and the filtrate was concentrated. Flash chromatography (hexanes-EtOAc-MeOH, 40:60:0.5) afforded 3.10 g (99%) of desired **4** as a white powder: R_f = 0.29 (hexane-EtOAc, 33:66). Analytical data was consistent with the literature.[20]

***t*-Butyl Ester 6.** A solution of protected threonine **4** (3.10 g, 9.62 mmol) in CH_2Cl_2 (27 mL) was cooled to 0 °C and treated with trifluoroacetic acid (3.0 mL, 38.5 mmol). The solution was allowed to warm to ambient temperature and stirred for 48 h. Toluene (10 mL) was added and the reaction mixture was concentrated to a pink oil. An additional 10 mL of toluene was added and the solution was concentrated again. The crude deprotected amine was carried on without further purification: R_f = 0.13 (EtOAc-MeOH, 90:10).

Crude amine in MeOH (20 mL) was treated with glutaric anhydride (2.75 g, 24.1 mmol) and 2,6-lutidine (4.5 mL, 38.5 mmol). The solution was stirred at ambient temperature for approximately 4 days. 1M HCl was added and the mixture was extracted with EtOAc three times and with CH_2Cl_2 once. The combined organic layers were dried over Na_2SO_4 (anhyd), filtered and concentrated. The crude acid was carried on without further purification: R_f = 0.29 (EtOAc-MeOH, 90:10).

Crude acid in CH_2Cl_2 (20 mL) was treated with concentrated sulfuric acid (0.050 mL, 0.96 mmol), cooled to 0 °C and sealed with a rubber septum. The solution was charged with isobutylene by bubbling through the stirring solution via a needle (with a second needle as an outlet). After approximately 1 hour the isobutylene bubbling was stopped, the two needles were removed and the reaction mixture stirred while allowing to warm to ambient temperature. After 16 hours, additional concentrated sulfuric acid (0.100 mL, 1.92 mmol) was added and the solution was recharged with isobutylene at 0 °C for 1 h. The reaction mixture was sealed with a rubber septum and stirred at ambient temperature for approximately 7 days. 10% sat. $NaHCO_3$

was added and the mixture was extracted 3 times with EtOAc. The combined organic layers were washed once with $NaHCO_3$ (sat aq), dried over Na_2SO_4, filtered and concentrated. Flash chromatography (CH_2Cl_2-EtOAc, 50:50 to 30:70 to 10:90) afforded 1.09 g (29%, 3 steps) *t*-butyl ester **5** as a glassy solid: R*f* = 0.27 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (m, 5H), 6.59 (m, 1H), 6.51 (br d, *J* = 6.6 Hz 1H), 4.64 (d, *J* = 11.5 Hz, 1H), 4.60 (d, *J* = 11.5 Hz, 1H), 4.53 (dd, *J* = 6.6, 3.0 Hz, 1H), 4.16 (dq, *J* = 6.3, 2.9 Hz, 1H), 2.81 (d, *J* = 4.9 Hz, 3H), 2.28 (m, 4H), 1.93 (m, 2H), 1.43 (s, 9H), 1.11 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 172.5, 170.0, 138.0, 128.5, 127.93, 127.85, 80.5, 74.2, 71,7, 56.1, 35.4, 34.6, 28.1, 26.3, 20.9, 15.5; HRMS calcd for $[C_{21}H_{32}N_2O_5 - O\text{-}t\text{Bu}]^+$: 319.165782, found: 319.166378.

Alcohol 7. 10% palladium on carbon (approx 0.1 g) was placed in a round bottom flask under argon. *t*-Butyl ester **5** (0.97 g) in MeOH (25 mL) was added. The mixture was evacuated and placed under hydrogen with a balloon. The mixture was stirred under hydrogen, refilling the balloon with hydrogen every 24 h. After 72 h the reaction mixture was carefully evacuated and purged with argon several times and filtered through celite. The filter cake was washed several times with CH_2Cl_2 and the filtrate was concentrated to afford 0.630 g (84%) of alcohol **6** as a white powder: R*f* = 0.13 (EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.93 (br s, 1H), 6.66 (br s, 1H), 4.34 (m, 2H), 4.01 (br s, 1H), 2.79 (d, *J* = 4.4 Hz, 3H), 2.32 (m, 2H), 2.26 (t, *J* = 7.1 Hz, 2H), 1.92 (m, 2H), 1.43 (s, 9H), 1.14 (d, *J* = 6.1 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.6, 172.5, 171.9, 80.6, 66.4, 56.6, 35.3, 34.5, 28.1, 26.2, 21.0, 18.2; HRMS calcd for $[C_{10}H_{17}N_2O_5]^+$ (M-O*t*-Bu): 229.118832, found: 229.118403.

Dibenzyloxyphosphorthioate 8. **A**lcohol **6** (0.200 g, 0.661 mmol) and dibenzyl-diisopropylphosphoramidite (0.280 mL, 0.827 mmol) were azeotropically dried by the evaporation of 2 mL dioxane, and redissolved in dioxane (6.5 mL). Tetrazole (0.070 g, 0.992 mmol) was added and the reaction mixture was stirred at 23 °C. After 2 h TLC showed complete coupling and phenylacetyl disulfide (0.459 g, 1.65 mmol) was added. The reaction mixture was at 23 °C for an addition 2 h. The mixture was concentrated and partitioned between ethyl acetate and water. The organic layer was separated and the aqueous layer was extracted again with ethyl acetate. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered and

concentrated. Flash chromatography (EtOAc-hexanes, 50:50 to 60:40 to 75:25) afforded 0.314 (82%) of desired dibenzylphosphorthioate **7** as a white powder: R_f = 0.47 (EtOAc-hexanes, 75:25); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (br s, 8H), 6.51 (m, 2H), 5.05 (m, 5 H), 4.54 (m, 1H), 2.72 (d, *J* = 4.4 Hz, 3H), 2.24 (m, 4H), 1.90 (m, 2H), 1.43 (s, 9H), 1.24 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.55, 172.50, 168.8, 135.72, 135.68, 135.6, 128.60, 128.59, 128.57, 128.2, 128.1, 80.5, 74.94, 74.88, 70.13, 70.08, 70.02, 56.85, 56.79, 35.2, 34.6, 28.1, 26.3, 20.8, 17.79, 17.77; 31P NMR (162 MHz) δ 68.6; ESIMS 601.93 (M+Na).

***p*-Nitrobenzylphosphorthioate 4 (hapten 4).** A mixture of dibenzyloxyphosphorothioate **7** (0.027 g, 0.0467 mmol), *m*-cresol (0.097 mL, 0.93 mmol), and thiophenol (0.095 mL, 0.93 mmol) was cooled to 0 °C and treated with trifluoroacetic acid (0.60 mL) and bromotrimethylsilane (0.123 mL, 0.93 mmol). The reaction mixture was stirred at 0 °C for 1.5 h, diluted with toluene and concentrated in vacuo. Additional toluene was added and the mixture was concentrated again. The resulting oil was dissolved in $NaHCO_3$ (sat aq, 5 mL) and washed once with pentane (5 mL). The aqueous layer (containing deprotected phosphorothioate) was treated with a solution of *p*-nitrobenzyl bromide (0.020 g, 0.093 mmol) in ethanol (1 mL). The reaction mixture was stirred at ambient temperature for 2 hours. After washing with a small amount of pentane, the aqueous mixture was purified by reverse-phase preparative HPLC (^{18}C prep column, 30 min gradient, H_2O-CH_3CN 90:10 to H_2O-CH_3CN 40:60, 254 nm). A peak at 22.8 min was collected and concentrated to afford 0.0135 g (61%, 2 steps) of desired phorphorthioate **3** as a white powder: ^{1}H NMR (400 MHz, DMSO) δ 8.17 (d, *J* = 8.5 Hz, 2H), 8.02 (br d, *J* = 8.3 Hz, 1H), 7.91 (m, 1H), 7.65 (d, *J* = 8.5 Hz, 2H), 4.60 (m, 1H), 4.30 (m, 1H), 4.04 (m, 2H), 2.57 (d, *J* = 4.2 Hz, 3H), 2.22 (q, *J* = 7.3 Hz, 4H), 1.71 (quint, *J* = 7.3 Hz, 2H), 1.14 (d, *J* = 6.1 Hz, 3H); ^{31}P NMR (162 MHz) δ 20.2; ESIMS (negative mode) 476.028 (M-H); Extinction coefficient = 6300 M^{-1} cm^{-1}.

***Para*-nitrobenzylmesylate (PNBM).** *Para*-nitrobenzyl alcohol (1.00 g, 6.53 mmol) in THF (10 mL) was treated with triethylamine (1.83 mL, 13.1 mmol), cooled to –10 °C and treated dropwise with methanesulfonyl chloride (0.610 mL, 7.84 mmol). After stirring for 30 min, 10% HCl (25 mL) was added. An additional 10 ml of water was added and the mixture was extracted

twice with ethyl acetate. The combined organic layers were dried over Na_2SO_4 (anhyd), filtered and concentrated to afford an off-white powder. Analytical data was consistent with the literature.[21]

Preparation of Hapten-KLH Conjugate. Hapten **4** (10 mg, 0.021 mmol) in DMF (0.5 mL) was treated with dicyclohexylcarbodiimide (4.3 mg, 0.021 mmol) and *N*-hydroxysuccinimide (2.4 mg, 0.021 mM) and stirred 5 h. The cloudy mixture was centrifuged and the clear supernatant was added to a solution of keyhole limpet hemocyanin (KLH, Pierce, 10 mg) in 0.1 M borate buffer (pH = 8). After stirring for several hours, NaOH (30 µL, 1.0 M aqueous) was added to maintain neutral pH, and the mixture was stirred an additional 16 hours. The reaction mixture was purified over a prepacked Sephadex G-25 gel filtration column (PD-10). Fractions containing hapten-KLH conjuagate **8** were determined by Bradford assay, and the coupling efficiency was estimated by determining the absorption at 280 nm. Three fractions containing between 1 and 2.5 mg/mL of protein were collected, and coupling efficiency was determined to be 120 haptens/KLH. BSA-Hapten **4** was prepared in the same manner, coupling efficiency was 11 haptens/BSA.

Purification of polyclonal sera. An affinity purification column was prepared as follows: Diaminodipropyl amine gel (2 mL, 16-20 uMole/mL, Pierce) was placed in a small fritted column. Buffer was allowed to drain off and the remaining solid was washed with PBS (6 mL). Hapten **3** (7.2 mg, 0.021 mmol) in conjugation buffer (2 mL, 0.1 M MES, 0.9% NaCl, pH= 7.4) was added, followed by EDC (120 mg) in conjugation buffer (0.75 mL). The ends of the column were capped and the column was placed on a rotator for 16 hours. The column was drained and washed with 1 M NaCl. Coupling efficiency was estimated by measuring the absorbance of the wash fractions at 280 nm. Approximately 5.2 mg of hapten was recovered, for a coupling efficiency of 26%. The affinity column was washed with PBS (10 mL), and 12 mL of total immune IgY (Aves Labs) was added. The column was drained until the anti-sera had fully entered the beads, and the column was capped and incubated at ambient temperature for 1.5 h. Unbound antibodies were washed from the column with 150 mL PBS and bound antibodies were eluted with acidic elution buffer (0.1 M glycine, pH=2.8), followed by basic elution buffer (0.1 M triethylamine pH 11). Individual fractions (1 mL) were immediately neutralized with 50 µL

1M TRIS, pH= 9.5 or 50 µL 1M TRIS, pH= 3.5. Fractions were pooled, concentrated, and exchanged into coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3) with an Amicon centrifugal 10,000 MWCO filter as per the manufacturers instructions to afford a-**3**-IgY at 5 mg/ml.

Preparation of Antibody Coated Sepharose Beads. Sepharose-CNBr (Amersham Biosciences), 1.0 ml hydrated volume, was washed extensively with ice cold 1.0 mM HCl, then a-**3**-IgY or preimmune IgY (500 µl, 5 mg/ml in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3)) was added and allowed to react for 3 hours with rotation at ambient temperature. Coupling efficiency was calculated to be 75% based on absorbance at 280 nm. Antibody binding supernatant was removed, the beads were washed with additional coupling buffer, and unreacted sites on the beads were blocked by overnight incubation at 4 °C with 1.0 ml 0.1 M Tris-HCl pH 8.0. The beads were then washed extensively with alternating 0.1 M acetate, 0.5 M NaCl, pH= 3.5 and 0.1 M Tris-HCl, 0.5 M NaCl, pH=8.0. After one final wash with PBS the beads were stored at 4 °C until further use.

Preparation of histone H1-nitrobenzylthiophosphate. Histone-H1 (Calbiochem, 500 µg) in kinase buffer (10 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 100 µM ATPγS pH **= 7.5**, 500 µL final volume) was treated with Cdk1(F80G)/CycB (5 µg) and incubated for 16 hours at 30°C . The kinase reaction was purified through Micro Bio-Spin P6 columns (Bio-Rad Laboratories) to afford Histone thiophosphate (H1-P^S). H1-P^S (240 µL, approx 240 µg) in TRIS buffer (10 mM TRIS-HCl, pH = 7.4) was treated with a 12 µL of 50 mM PNBM in DMSO. The reaction was incubated for 2 hours at ambient temperature to afford histone nitrobenzylthiophosphate (H1-P^S+PNBM).

Rhodamine labelling of H1-nitrobenzylthiophosphate. Rhodamine-NHS ester was prepared by treatment of 5(6)-carboxytetramethylrhodamine (2.5 mg, 5.8 µmol) with N-hydroxysuccinimide (3.6 mg, 31 µmol), and diisopropylcarbodiimine (7 µl, 47 µmol) in DMF (90 µl) for 18 hours at ambient temperature. The reaction mixture was then diluted three fold with DMSO (to afford an estimated 20 mM Rh-NHS). H1-P^S+PNBM, or H1-P^S was treated with Rh-NHS (0.5 µl of 20 mM Rh-NHS per 100 µl of 0.3 mg/ml H1-P^S+PNBM) for 10 minutes at

ambient temperature. The proteins were immediately purified into TRIS buffer (10 mM TRIS-HCl, pH = 7.4) with Micro Bio-Spin P6 columns. Protein concentration was estimated by comparison with know amounts of H1 on coomassie stained SDS-PAGE gels. The extent of rhodamine labeling was quantitated (0.2 rhodamine/H1) by fluorescence gel imaging of the prepared conjugates as compared to a monorhodamine labeled protein Histone H2A (120C).

Preparation of Mob1 and Swe1 nitrobenzylthiophosphate conjugates. Bacterially expressed Mob1 (maltose binding protein fusion) and Swe1 (glutathione transferase fusion) were kindly provided by Mart Loog, Morgan Lab. Solutions of each protein (30 μL, 0.5 mg/ml) were treated with Cdk1(F80G)/cyclinB (1 μg) in kinase buffer, the total reaction volume was 150 μL. Thiophosphorylation reactions proceeded overnight at 30°C to afford Mob1-P^S and Swe1-P^S. Without further purification and in the same buffer the proteins were alkylated with 7.5 μL of 50 mM PNBM in DMSO for 2 hours at ambient temperature to afford the protein nitrobenzylthiophosphate esters (Mob1-P^S+PNBM and Swe1-P^S+PNBM).

ELISA. 96-well plates (Nunc Immunosorb) were coated with 50 μL/well antigen (2.5 μg/mL in PBS) and incubated at 4 °C overnight. After washing three times with PBS, the wells were blocked with 200 μL BSA (10 mg/mL in PBS-T (PBS with 0.05% Tween-20)) for 2 h at ambient temperature. The plates were washed four times with PBS-T, then 50 μL serial dilutions of affinity purified polyclonal IgY (100 μg/mL diluted from 1:100 to 1: 100,000 in PBS-T containing 0.1% BSA) were added to the wells, and the plates were incubated at 4 °C overnight. After washing four times with PBS-T and twice with PBS, the plates were treated with 100 μL/well Anti-Chicken IgY (IgG)-Alkaline Phosphatase antibody (Sigma) and incubated at ambient temperature for 1 h. After washing three times with PBS-T and twice with PBS, the plates were developed by treatment with 100 μL/well of ABTS (Southern Biotechnology, Birmingham, AL) solution (0.3 mg/mL ABTS in 65 mM citrate, 90 mM Na_2HPO_4, pH=4.0 containing 0.1% hydrogen peroxide). After 10 minutes the absorbance at 405 nm was read with a microplate reader.

Immunoblot Analyis. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose paper. The blots were blocked with 5% BSA in TBS-T (Tris-buffered saline, pH=7, with 0.05%

Tween-20) for 1 h at ambient. The blots were next incubated with a solution of a-3-IgY (2000:1 dilution (100 µg/ml starting concentration) in TBS-T containing 5% BSA at 4°C overnight. After washing 4 times (10 min each) with TBS-T. The blots were treated with a solution anti-Chicken IgY (IgG)-Alkaline Phosphatase antibody (Sigma) (5000:1 dilution in TBS-T containing 5% BSA), and washed again 4 times (10 min each) with TBS-T. The blots were developed with Pico SuperSignal (Pierce), and chemiluminescence was imaged on an Alpha Innotech.

Immunoprecipitation of Rh-H1-P^S+PNBM. HeLa-S3 cells (NCCC) were lysed in RIPA buffer ((50 mM Tris-HCl, 2 mM EGTA, 150 mM NaCl, 1% SDS, and 1% NP-40, pH=8.0) with Roche complete mini protease tablet, and 1 µM PMSF) for 15 min at 4 °C, and then centrifuged at 14,000 rcf for 10 min at 4 °C. Soluble lysates were treated with 2.6 mM PNBM for 2 hours at ambient temperature, then purified through PD-10 Sephadex column. Alkylated lysates were precleared with preimmune IgY-sepharose beads (50 µl beads per 1.0 ml lysate) for 15 min at 4 °C, and protein concentration was measured using a Bradford assay. Sepharose beads coated with a-3-IgY or preimmune IgY were preblocked with BSA (200 µl of a 50% suspension, 200 µg BSA in 1.0 ml RIPA lysis buffer). For each experiment 1 µg of Rh-H1-P^S-PNBN, or Rh-H1-P^S, was mixed with either 200 µg of BSA, or increasing amounts of precleared HeLa lysate. Total volume was adjusted to 1.3 ml with RIPA buffer, and 50 ul of BSA blocked a-3-IgY or preimmune IgY Sepharose beads were added, immunoprecipitations were incubated with rotation at 4 °C overnight. Following removal of the binding supernatant the beads were washed 4X with 1.0 ml RIPA wash buffer (50 mM Tris-HCl, 2 mM EGTA, 150 mM NaCl, and 1% NP40, pH=7.5). Bound proteins were eluted with 2X SDS-PAGE sample buffer, electrophoresed, and Rhodamine imaged on a Typhoon (Amersham Biosciences), excitation at 523 nM, emission collected with a 580 nM band pass filter.

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