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Supporting Information

A Pronucleotide Probe for Live-Cell Imaging of Protein AMPylation

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Supplementary figures.

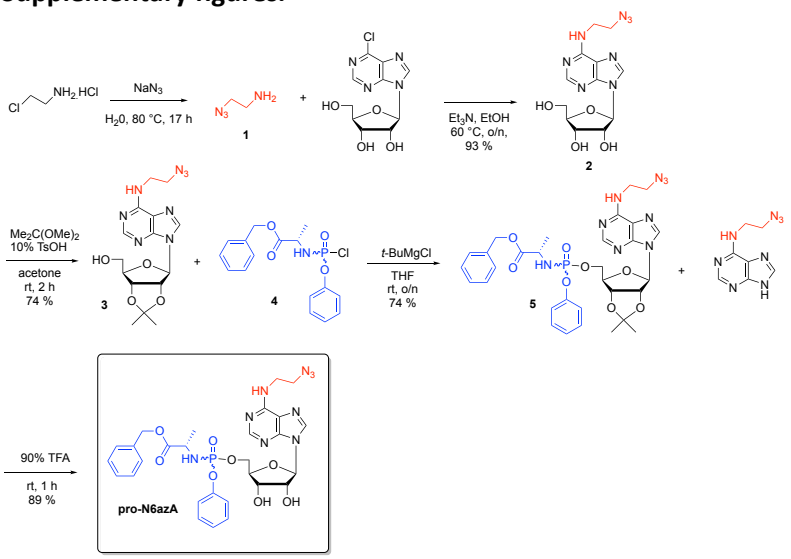


Figure S1. Synthesis of the pro-N6azA probe.

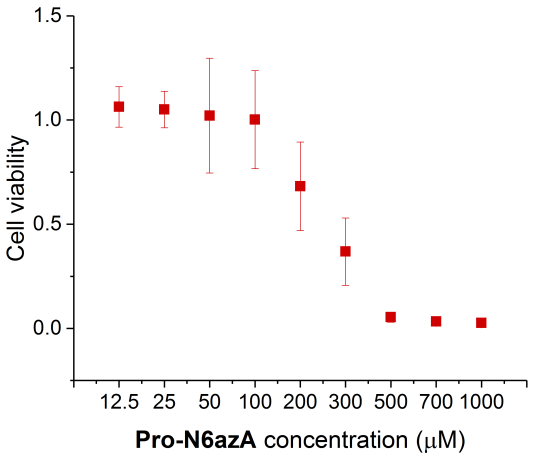


Figure S2. Cytotoxicity measurement of the pro-N6azA probe.

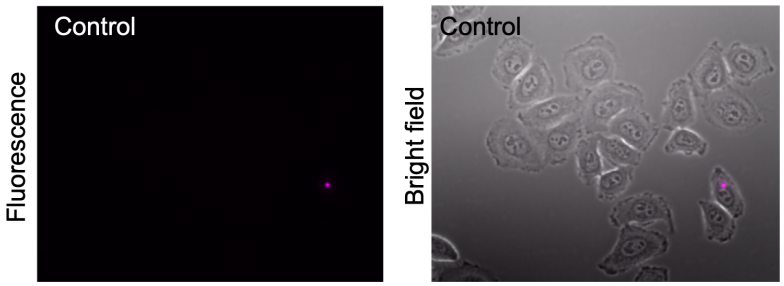


Figure S3. Fluorescence background control. HeLa cells were treated with 100µM DBCO-TAMRA in PBS and fluorescence was scanned after further 24 h incubation. (Compare with images of cells treated with **pro-N6azA** probe Fig. 4c). Pink colour represents the TAMRA fluorescence.

Synthesis.

General methods. Reagents and solvents were purchased from commercial suppliers (*Sigma-Aldrich Co. LLC*, *Thermo Fisher Scientific Inc.*, *Merck KGaA*, *TCI Europe GmbH*, *Fluorochem Ltd.* and *Alfa Aesar GmbH*) and used without further purification. HPLC-grade solvents or anhydrous solvents (max. 0.01 % water content, stored over molecular sieve under an argon atmosphere, *Sigma-Aldrich Co. LLC*) were used for all reactions. All experiments were monitored by analytical thin layer chromatography (TLC). TLC was performed on pre-coated silica gel plates (60 F-254, 0.25 mm, *Merck KGaA*) with detection by UV (λ = 254 and/or 366 nm) and/or by colouration using a potassium permanganate ($KMnO_4$) stain and subsequent heat treatment. Flash chromatography was performed on silica gel 60 (0.035 – 0.070 mm, mesh 60 Å, *Merck KGaA*) with the indicated eluent. ^{1}H, proton-decoupled ^{13}C and proton-decoupled ^{31}P NMR spectra were recorded on a *Bruker* Avance III HD 300 (300 MHz), a *Bruker* Avance I 360 (360 MHz) or a *Bruker* Avance III HD (500 MHz) at 298 K. Chemical shifts are reported in delta (δ) units in parts per million (ppm) relative to distinguished solvent signals. The following abbreviations are used for the assignment of the signals: s – singlet, d – doublet, t – triplet, q – quartet, m – multiplet. Coupling constants *J* are given in Hertz [Hz]. HR-MS spectra were recorded in the ESI mode on a Thermo Scientific LTQ-FT Ultra (FT-ICR-MS) coupled with an UltiMate 3000 HPLC system (*Thermo Fisher Scientific Inc.*), the separation was performed using a Waters XBridge C18 column (3.5 µm, 4.6 x 100 mm, flow rate = 1.1 ml/min) and LCQ-Fleet Ion Trap Mass Spectrometer with an ESI (electrospray ionisation) ion source and a Dionex HPLC system using a Waters Xbridge BEH13 C18 column (5 µm, 4.6 x 100 mm). Spectra were analysed using MestReNova.

2-azidoethan-1-amine (**1**)**.** 300 mg (25.9 mmol, 1 eq.) 2-chloroethylamine hydrochloride and 5,045 g (77.6 mmol, 3 eq) sodium azide were dissolved in 5 mL water and heated to 80 °C under stirring. After 17 h, the solution was basified to a pH of 13 by adding KOH pellets. The product was extracted with diethyl ether, followed by drying the combined organic phases over Na_2SO_4 and evaporation of the Et_2O yielded **1** as a colorless oil, quantitatively. HRMS (ESI): calculated 86.06 $[M]^+$, found 86.816 $[M]^+$. ^{1}H NMR (300 MHz, methanol-d4): δ [ppm]: 3.40 (t, *J* = 5.9 Hz, 2H), 2.79 (t, J = 5.7 Hz, 2H). ^{13}C NMR (75 MHz, methanol-d4): δ [ppm]: 54.66, 41.59.

6-(2-azidoethylamino)-9-(β-D-ribofuranosyl)-9*H*-purine (**2**)**.** 416 mg (4.83 mmol, 4 eq.) 2-azidoethan-1-amine were mixed with 2 mL EtOH and 700 µL (4.83 mmol, 4 eq.) triethylamine (Et_3N) and stirred for 5 min at room temperature. Afterwards, 347 mg (1.21 mmol, 1 eq.) 6-chloro-9-(β-D-ribofuranosyl)-9*H*-purine in 25 mL ethanol were added, the reaction mixture subsequently heated to 60 °C and stirred overnight. After cooling down to room temperature, the mixture was directly loaded onto silica. The crude product was then applied on a 50 mL silica column in DCM and purified using DCM with 10 % MeOH. The combined fractions were then concentrated *in vacuo* and yielded **2** (366 mg, 90%) as a yellow solid. TLC: R_F = 0.4 (MeOH:$CHCl_3$ 7:1). HRMS (ESI): calculated 337.138 $[M+H]^+$, found 337.1366 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d6): δ [ppm]: 8.38 (s, 1H), 8.24 (s, 1H), 8.07 (s, 1H), 5.89 (d, *J* = 6.2 Hz, 1H), 5.44 (d, *J* = 6.2 Hz, 1H), 5.36 (dd, *J* = 7.1, 4.6 Hz, 1H), 5.18 (d, *J* = 4.6 Hz, 1H), 4.61 (d, *J* = 6.7 Hz, 1H), 4.16 – 4.12 (m, 1H), 3.96 (q, *J* = 3.5 Hz, 1H), 3.74 – 3.64 (broad, 3H), 3.60-3.51 (broad, 3H). ^{13}C NMR (100 MHz, DMSO-d6): δ [ppm]: 152.25, 139.98, 87.86, 85.86, 73.48, 70.61, 61.62.

6-(2-azidoethylamino)-9-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)-9*H*-purine (**3**)**.** 350 mg (1 mmol, 1 eq.) of the unprotected nucleoside **2** were suspended in 10 mL acetone. Afterwards, 1.224 mL (10 mmol, 10 eq.) 2,2-dimethoxypropane and 951 mg (5 mmol, 5 eq.) *p*-toluene sulfonic acid monohydrate were added and the reaction mixture was stirred at room temperature for 2 h. The reaction was stopped by adding $NaHCO_3$ until a pH of 10 was reached, followed by an extraction with EtOAc. The combined organic phases were dried over Na_2SO_4 and concentrated. The crude product was dissolved in DCM and added on a 100 mL silica column in DCM. After washing the column with 50 mL 2% MeOH in DCM, the product was eluted with 5 % MeOH in DCM. The product containing fractions were combined, concentrated at reduced pressure and finally dried *in vacuo* to give **3** (278 mg, 74%) as an yellowish solid. TLC: R_F = 0.42 (MeOH:$CHCl_3$ 7:1). HRMS (ESI): calculated 377.1680 $[M+H]^+$, found 377.1680 $[M+H]^+$. ^{1}H NMR (300 MHz, DMSO-d6): δ [ppm]: 8.38 (s, 1H), 8.26 (s, 1H), 8.07 (s, 1H), 6.14 (d, *J* = 3.1 Hz, 1H), 5.35 (dd, *J* = 6.2, 3.1 Hz, 1H), 5.25 – 5.17 (m, 1H), 4.97 (dd, *J* = 6.2, 2.6 Hz, 1H), 4.22 (td, *J* = 4.9, 2.5 Hz, 1H), 3.69 (s, 2H), 3.54 (broad, 5H), 1.54 (s, 3H), 1.32 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d6): δ [ppm]: 152.49, 139.78, 113.03, 89.61, 86.43, 83.28, 81.35, 61.55, 27.06, 25.18.

Benzyl (chloro(phenoxy)phosphoryl)alaninate (**4**)**.** 267 mg (3.4 mmol, 1 eq.) of benzyl alaninate 4-methylbenzenesulfonate were dissolved in 7.2 mL anhydrous DCM at -78 °C. After adding 0.11 mL (3.4 mmol, 1 eq.) of phenyl phosphorodichloridate, 0.21 mL (6.8 mmol, 2 eq.) of anhydrous triethylamine in DCM were added dropwise. The solution was stirred at -78 °C for 5 min and subsequently at room temperature for 1 h. After evaporating the DCM, 8.6 mL anhydrous tetrahydrofuran were added, the suspension filtrated and tetrahydrofuran finally removed *in vacuo* to give a colorless oil, quantitative.

6-(2-azidoethylamino)-9-(2,3-*O*-isopropylidene-β-D-ribofuranosyl)-9*H*-purine 5'-*O*-[phenyl(benzyloxy-L-alaninyl)]phosphate (**5**)**.** 200 mg (0.6 mmol, 1 eq.) of the protected nucleoside **3**

were dissolved in 8 mL anhydrous tetrahydrofuran and 1.3 mL (1.2 mmol, 2 eq.) of *t*-BuMgCl were added dropwise. After stirring at room temperature for 15 min, 849 mg (2.4 mmol, 4 eq.) benzyl (chloro(phenoxy)phosphoryl)alaninate (**4**) in 8 mL anhydrous tetrahydrofuran were added and the mixture stirred at room temperature overnight. Afterwards, the reaction was quenched with NH_4Cl and the product was extracted with EtOAc. The combined organic phases were dried over Na_2SO_4 and the product was concentrated *in vacuo* to yield **5** (308 mg, 74%) as a light-yellow gel. HRMS (ESI): calculated 694.248 $[M+H]^+$, found 694.2493 $[M+H]^+$. The crude product was used in following reaction.

6-(2-azidoethylamino)-9-(β-D-ribofuranosyl)-9*H*-purine 5'-*O*-[phenyl(benzyloxy-L-alaninyl)]phosphate (**6**)**.** 276 mg (0.4 mmol) of the protected pronucleotide **5** were dissolved in 5 mL 90% trifluoracetic acid and stirred at room temperature for 1 h. The reaction was quenched with MeOH which was co-evaporated afterwards with TFA. The crude product was dissolved in DCM and purified on a 100 mL column in DCM using a gradient from 5 % MeOH in DCM to 10 % MeOH in DCM. The product containing fractions were finally concentrated under reduced pressure and dried *in vacuo* to yield **6** (232 mg, 89%) as a colorless gel. TLC: R_F = 0.6 and 0.68 (MeOH:$CHCl_3$ 7:1). HRMS (ESI): calculated 654.218 $[M+H]^+$, found 654.218 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ [ppm]: 8.03 (s, 2H), 8.00 (s, 1H), 7.32 (s, 5H), 7.17 (dd, *J* = 8.4, 6.6 Hz, 4H), 7.07 (s, 5H), 5.94 (d, *J* = 5.3 Hz, 1H), 5.09 (s, 3H), 4.38 (s, 10H), 4.21 (s, 2H), 3.94 (s, 10H), 3.62 (s, 7H), 1.36 – 1.30 (m, 6H), 0.07 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm]: 135.12, 129.68, 128.64, 128.53, 128.1, 125.17, 120.05, 119.88, 90.03, 71.29, 67.37, 20.76. ^{31}P NMR (162 MHz, $CDCl_3$): δ [ppm]: 2.83, 2.54.

Chemical proteomics.

Cell culture. Human epitheloid cervix carcinoma cells (HeLa, CCL-2) were cultivated in high glucose Dulbeccos´s Modified Eagle´s Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine. Cells were grown under a humidified atmosphere at 37 °C and 5% CO_2. Cells were seeded into 6 cm diameter dishes and grown to 80-90% confluency. Human neuroblastoma cells SH-SY5Y (CRL-226) were cultivated in DMEM/F12 1:1 media supplemented with 10% (v/v) FBS. Fibroblast cells BJ (ATCC® CRL-2522™) were cultured in DMEM supplemented with 10% (v/v) FBS.

Protein concentration. Protein concentrations were determined by bicinchoniic acid assay (BCA, *Carl Roth GmbH + Co.*).

Cytotoxicity measurement. HeLa cells were seeded at a density of 5,000 cells per well (100 µL of a solution of 50,000 cells/mL) in a transparent, flat-bottom 96-well plate. Cells were grown over night in a humidified atmosphere at 37 °C and 5% CO_2. The next day the medium was removed and replaced by fresh medium supplemented with 1000, 700, 500, 300, 200, 100, 50, 25 and 12.5 µM pro-N6azA or 1% (v/v) DMSO as a control. The cells were incubated for 24 h. To determine metabolic activity of the cells, 20 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide solution (MTT, 5

mg/mL in PBS) were added to each well. The cells were incubated for 4 h. The medium was completely removed and the formazan crystals were resuspended in 200 µL DMSO and absorbance at 570 nm and at a reference wavelength of 630 nm was determined in an infinite F200 pro plate reader (*Tecan*). All data points were measured in triplicate. The data was normalized with respect to the DMSO control.

Probe treatment, click chemistry and enrichment. Cells (6-cm dishes) were treated with the probes at 80-90% confluency. Culture medium was removed and the cells were labelled in fresh media containing 100 µM pro-N6azA (100mM in DMSO stock solution) for 16 h at 37 °C in cells incubator. Subsequent cell lysis was done in 1 mL ice cold lysis buffer (1% (v/v) NP40, 1% (w/v) sodium deoxycholate and 1 tablet protease inhibitor (cOmplete™, Mini, EDTA-free protease inhibitor cocktail, *Roche*) in 10 mL PBS) while mechanically detached by scraping and subsequently transferred into an eppendorf tube or by ultrasonication at 40 % intensity for 10 s. Lysis was done for 30 min at 4 °C while rotating. Insoluble fraction was pelletized (10 min, 14,000 g, 4 °C) and protein concentration was determined by BCA. To 500 µg (HeLa cells) of a protein lysate in total volume of 970 µL 0.2% (w/v) SDS in PBS 10 µL 5-rhodmamine-azide (10 mM in DMSO, *Jena Bioscience*; in-gel analysis) or 10 µL azide-PEG_3-biotin (10 mM in DMSO, *Jena Bioscience*; for MS samples), 10 µL Tris(2-carboxyethyl)phosphine (TCEP) (53 mM in ddH_2O) and 1.2 µL tris(benzyltriazolylmethyl)amine (TBTA) (83.5 mM in DMSO) were added. Samples were gently vortexed and the click reaction was initiated by the addition of 20 µL $CuSO_4$ solution (50 mM in ddH_2O). The mixture was incubated at 25 °C for 1.5 h. Afterwards proteins were precipitated by addition of 4 mL acetone and incubation overnight at -20 °C. The protein pellet was harvested by centrifugation at 9,000 g for 15 min at 4 °C and washed twice with 1 mL of ice-cold methanol. Proteins were reconstituted in 500 µL 0.2% (w/v) SDS in PBS, the remaining not soluble particles were spun down at 9,000 g for 5 min and resulting supernatant was loaded onto the beads and incubated at 25 °C for 1.5 h while rotating. The 50 µL of avidin-agarose beads (*Sigma-Aldrich*) were washed in advance thrice with 1 mL 0.2% (w/v) SDS in PBS. Afterwards through the enrichment protocol, the beads were always spun at 400 g, 25 °C, for 2 min. Beads were subsequently washed thrice with 1 mL 0.2% (w/v) SDS in PBS, twice with 1 mL 6 M urea in ddH_2O and thrice with 1 mL PBS.

In-gel analysis. Rhodamine-tagged proteins were released from the beads with 50 µL of gel loading buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue in 125mM Tris-HCl, pH 6.8) and loaded on a 12.5% SDS-PAGE. After electrophoretic separation, fluorescently labelled proteins were visualized using a Fujifilm LAS-4000 equipped with a Fujifilm Fujinon VR43LMD3 lens and a 575DF20 filter (Fujifilm) operated in Cy3 fluorescence detection mode.

Proteomics. Enriched proteins were on beads digested in 200 µL digestion buffer (20 mM HEPES, pH 7.5, 7 M urea, 2 M thiourea). Proteins were reduced (0.2 µL 1 M DTT, 45 min, 25 °C) and alkylated (2 µL, 30 min, 25 °C, in the dark). The alkylation reaction was quenched by addition of 0.8 µL 1M DTT and

incubation for 30 min at 25 °C. Proteins were pre-digested with 1 µL LysC (*Wako*) at 25 °C for 4 h. 600 µL 50 mM TEAB buffer was added and the proteins were digested overnight with 1.5 µL sequencing grade trypsin (0.5 mg/mL, Promega) at 37 °C. The following day the beads were settled and the supernatant was acidified with 10 µL formic acid to a pH of 2–3. Peptides were desalted and on-column dimethyl labelled using 50 mg SepPak C18 cartridges (*Waters Corp.*) on a vacuum manifold. The cartridges were equilibrated with 1 mL acetonitrile, 1 mL 80% acetonitrile and 3 mL 0.5% formic acid. The samples were loaded on the cartridges and subsequently washed with 5 mL 0.5% formic acid. Cartridges were subsequently washed with 2 mL 0.5% formic acid. The peptides were eluted with two times 200 µL 80% acetonitrile, 0.5% formic acid. DMSO and probe-treated samples were combined and dried by lyophilization. Peptides were reconstituted in 30 µL 1% (v/v) formic acid, prepared for mass spectrometry by filtering through a membrane filter (Ultrafree-MC and –LC, Durapore PVDF-0.22 µm, *Merck Millipore*) and transferred into mass vials. Experiments were conducted in 8 replicates for first initial chemical proteomic enrichment experiment and 6 replicates for competition experiments.

Mass Spectrometry. MS analysis was either performed on an Q Exactive Plus instrument coupled to an Ultimate3000 Nano-HPLC via an electrospray easy source (all *Thermo Fisher Scientific*). Samples were loaded on a 2 cm PepMap RSLC C18 trap column (particles 3 µm, 100A, inner diameter 75 µm, *Thermo Fisher Scientific*) with 0.1% TFA and separated on a 50 cm PepMap RSLC C18 column (particles 2 µm, 100A, inner diameter 75 µm, *Thermo Fisher Scientific*) constantly heated at 50 °C. The gradient was run from 5-32% acetonitrile, 0.1% formic acid during a 152 min method (7 min 5%, 105 min to 22%, 10 min to 32%, 10 min to 90%, 10 min wash at 90%, 10 min equilibration at 5%) at a flow rate of 300 nL/min. The Q Exactive Plus instrument survey scans (m/z 300-1,500) were acquired in the orbitrap with a resolution of 70,000 at m/z 200 and the maximum injection time set to 80 ms (target value 3e6). Data dependent HCD fragmentation scans of the 12 most intense ions of the survey scans were acquired in the orbitrap at a resolution of 17,500, maximum injection time of 50 ms as well as minimum and maximum AGC targets of 5e3 and 5e4, respectively. The isolation window was set to 1.6 m/z. Unassigned and singly charged ions were excluded for measurement and the dynamic exclusion of peptides enabled for 60 s. The lock-mass ion 445.12002 from ambient air was used for real-time mass calibration on the Q Exactive Plus. Data were acquired using Xcalibur software version 3.1sp3 (*Thermo Fisher Scientific*).

Data analysis. Raw files were analyzed using MaxQuant software with the Andromeda search engine. Searches were performed against the Uniprot database for Homo sapiens (taxon identifier: 9606, 7th December 2018, including isoforms). At least two unique peptides were required for protein identification. False discovery rate determination was carried out using a decoy database and thresholds were set to 1 % FDR both at peptide-spectrum match and at protein levels. Statistical analysis of the MaxQuant result table proteinGroups.txt was done with Perseus 1.5.1.6. Putative

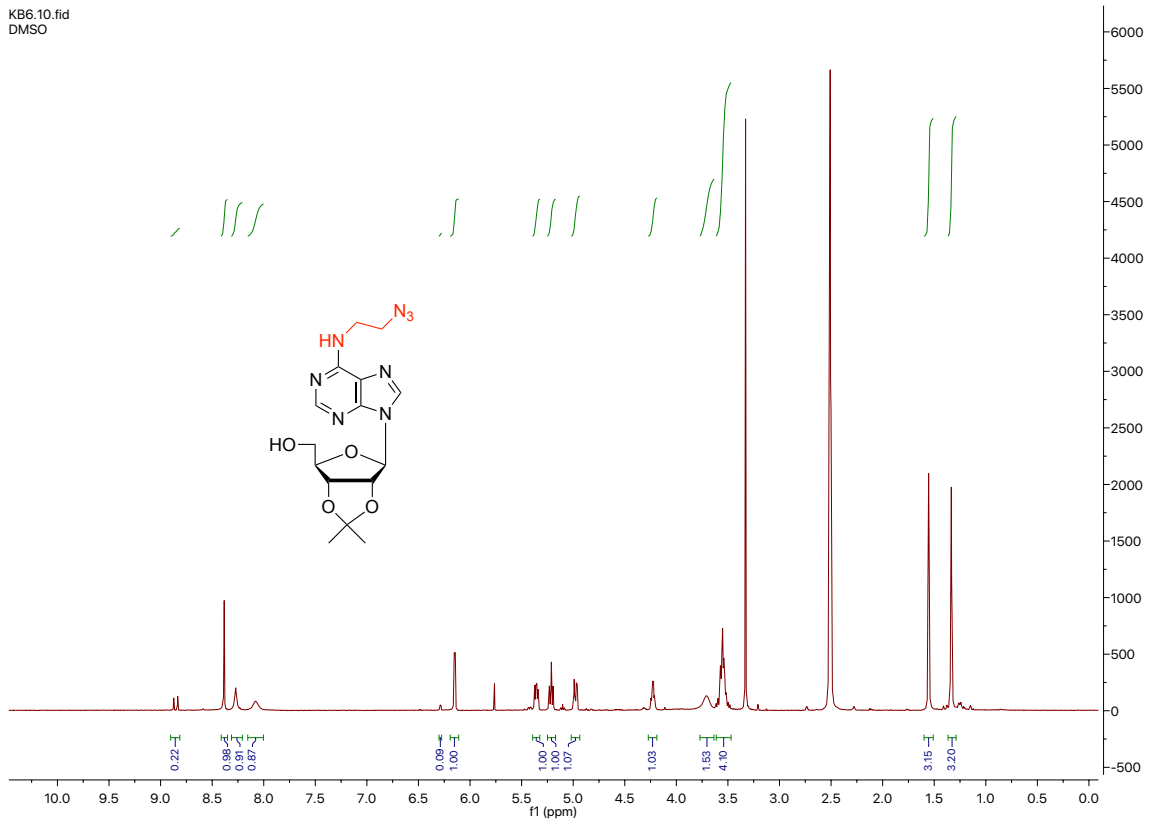
contaminants and reverse hits were removed. Normalized LFQ intensities were $\log_2$-transformed, hits with less than 3 valid values in each group were removed, missing values were imputed from the total matrix and $-\log_{10}$(*p*-values) were obtained by a two-sided two sample Student's *t*-test over replicates with the initial significance level of $p = 0.05$ adjustment by the multiple testing correction method of Benjamini and Hochberg (FDR = 0.05), the $-\log_{10}$ of *p*-values were plotted against the $\log_2$ of geometric mean of LFQ ratios.

Data availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD016011.

Probe staining of fixed cells. Cells were cultured with medium containing 100 µM pro-N6azA or 1:1,000 dilution of DMSO for 16 h prior to fixation. PFA-fixed cells on coverslips were permeabilized using 0.1 % Triton X100 in PBS for 5 min. Then they were incubated with click-chemistry staining mix (10 µM rhodamine-alkyne, 1 mM $CuSO_4$, 10 mM freshly prepared sodium ascorbate in PBS) at rt for 2 h in the dark, followed by several washes with PBS. For nuclear counterstaining, cells were incubated with 0.1 µg/mL DAPI in PBS for 15 min.

Fluorescence live imaging. For timelapse imaging of AMPylation in living cells, cells were cultured on 3 cm glass bottom dishes and were treated with 100 µM pro-N6azA in culture medium for 16h at 37 °C, 5% CO_2 and ambient oxygen levels. For live staining, the medium was removed and cells were washed three times with PBS prior to incubation in the SPACC mix containing 100 µM DBCO-TAMRA (1:1,000 from 100 mM stock) in culture medium for 1h at normal culture conditions. Afterwards, cells were washed again three times with PBS. Then, imaging was performed at a *Leica* SP8 confocal laser scanning microscope at 5% CO_2 and pre-warmed to 37 °C by taking images of selected positions every 20 seconds over 25 minutes, using the 546 laser and 2x zoom in 25x magnification. To check for the stability of both live staining and AMPylation, cells were imaged again 24h after SPACC, taking pictures in the 546 and brightfield channels.

NMR spectra.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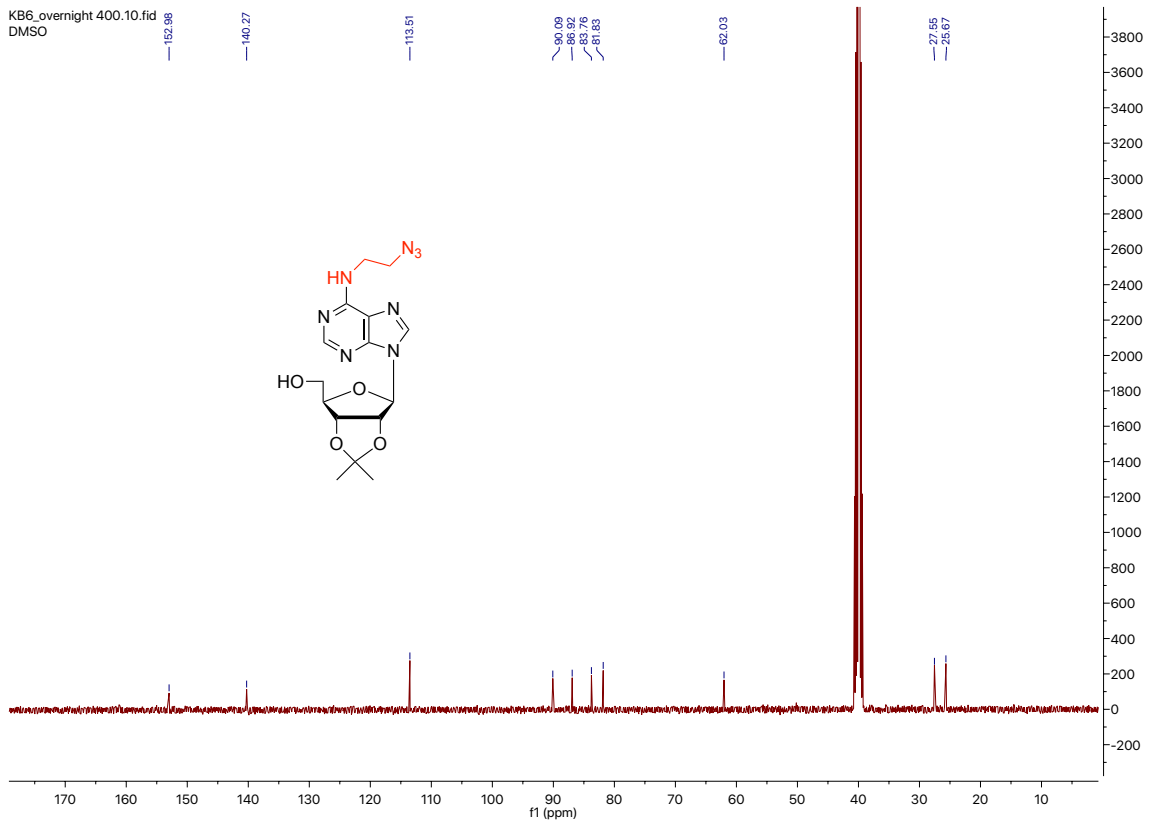
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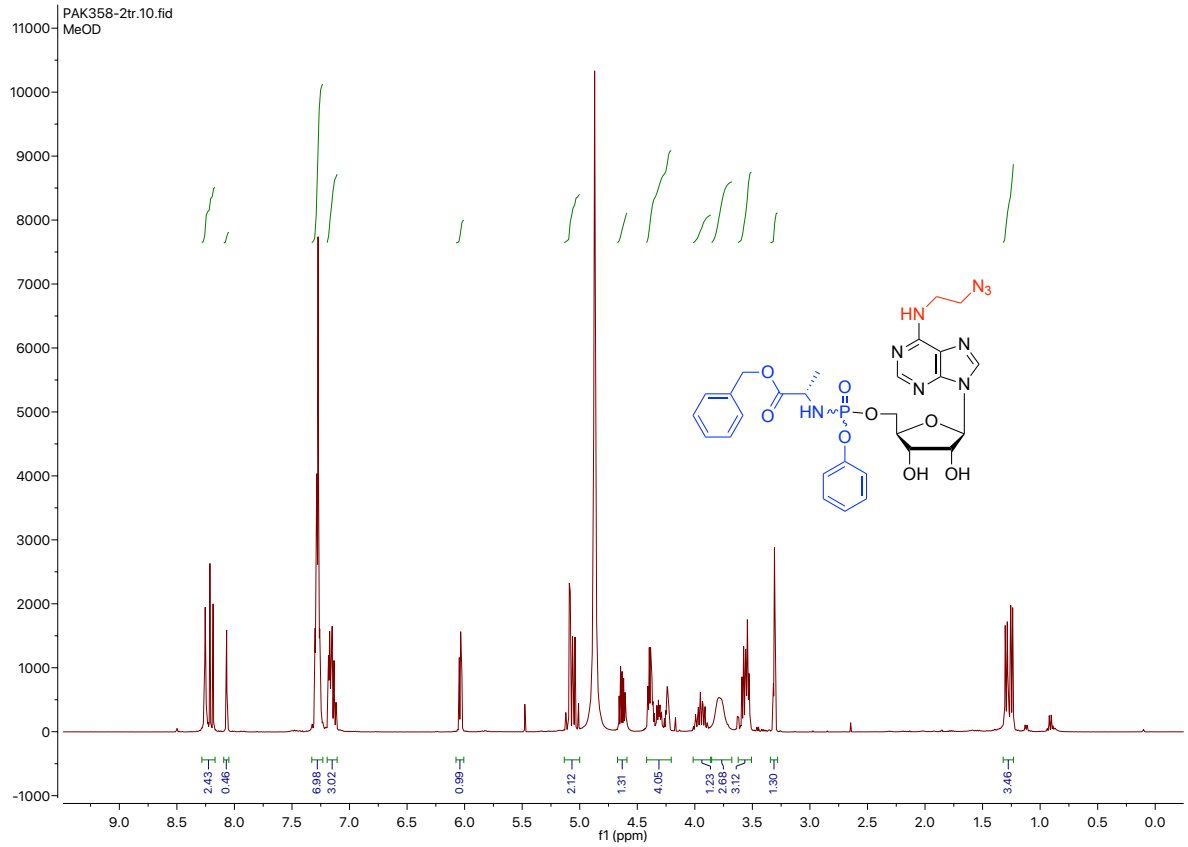
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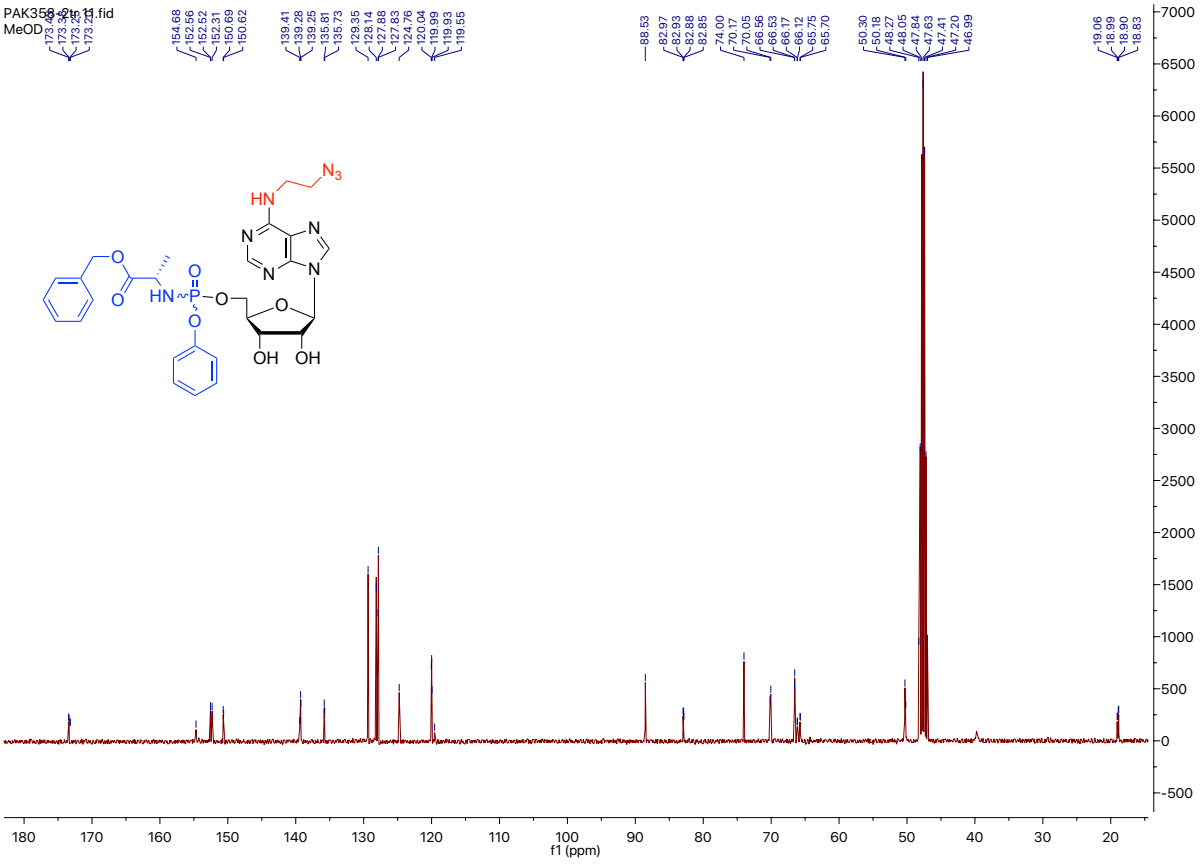
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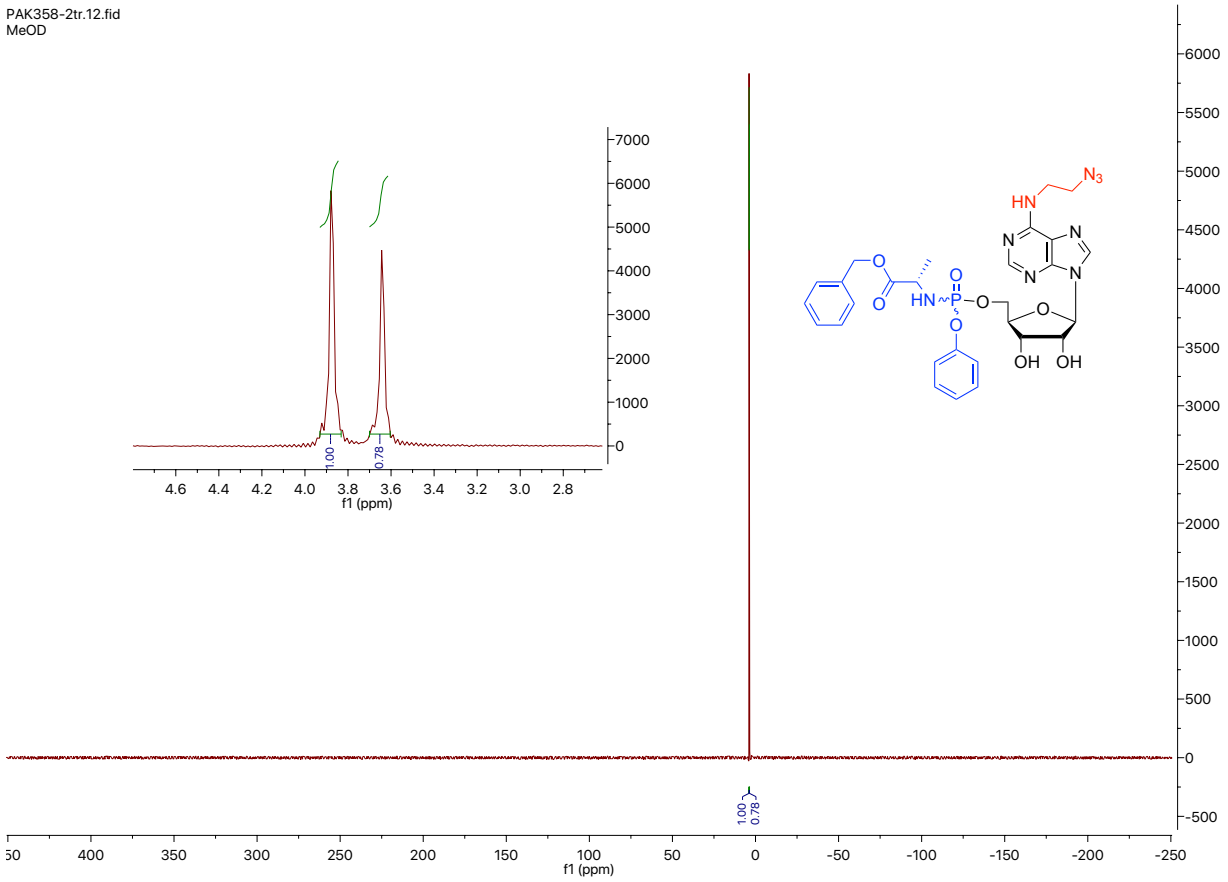
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