

Method for the Synthesis of Mono-ADP-ribose Conjugated Peptides

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Supporting Information

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B. Materials and Methods

Materials. Boc-(*t*-butoxycarbonyl) and Fmoc (fluorenylmethoxycarbonyl)-L-amino acids, *N*^α-Fmoc-*N*^β-(*N*-Boc-amino oxyacetyl) diaminopropionic acid (Fmoc-Dpr(Boc-Aoa)-OH), biotin *p*-nitrophenyl ester (Biotin-ONp), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), *p*-methylbenzhydrylamine (MBHA) resin, and rink amide (MBHA or aminomethyl) resins were purchased from Novabiochem (Läufelfingen, Switzerland). D-Biotin was from Anaspec (Fremont, CA, USA). 2-(*N*-Boc)amino-4-[*O*-[*N*-(2-Cl-Z)-*N*-methyl]amino]hydroxybutanoic acid was synthesized as previously described.¹ Rink amide ChemMatrix resin was from Matrix Innovation (Montreal, Canada). HPLC grade MeCN, dichloromethane (DCM), dimethylformamide (DMF), MeOH, and *N*-methylpyrrolidinone (NMP) were purchased from Fischer Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid (TFA) was purchased from Halocarbon (River Edge, NJ, USA). Biotech grade piperidine was from Sigma-Aldrich (St Louis, MO, USA). *N,N*-Diisopropylethylamine (DIPEA) was from Applied Biosystems (Foster City, CA, USA). Anhydrous hydrogen fluoride (HF) was purchased from Matheson Tri-Gas (Irving, TX, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) at the highest available purity.

Nicotinamide[2,5',8-³H]adenine dinucleotide ([³H]-NAD; 0.25 mCi/mL; 30 Ci/mmol), Amplify fluorographic reagent, glutathione sepharose 4 fast flow, and streptavidin-horseradish peroxidase (HRP) conjugate (RPN1231) were from GE Health Life Sciences (Piscataway, NJ, USA). 6-Biotin-17-nicotinamide-adenine-dinucleotide² (biotinylated NAD; 250 μM), 10× activated DNA, and the poly(ADP-ribose) glycohydrolase (PARG) catalytic domain (461-976) (1 μg/mL) were purchased from

Trevigen (Gaithersburg, MD, USA). Snake venom phosphodiesterase I (*Crotalus adamanteus*; ≥ 20 U/mg) was purchased from Worthington Biochemical (Lakewood, NJ, USA). Calf intestinal alkaline phosphatase (CIP; 10 U/ μ L), restriction endonucleases, and T4 DNA ligase were from New England Biolabs (Ipswich, MA, USA). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA). Nickel-nitriloacetic acid (Ni-NTA) resin, KOD DNA polymerase, pET-3a, and pET-15b vectors were from Novagen (Gibbstown, NJ, USA). The pFastBac HT B vector was from Invitrogen (Carlsbad, CA, USA). The QuikChange XL II site directed mutagenesis kit was from Agilent (La Jolla, CA, USA). DNA purification kits (QIAprep spin minikit, QIAquick gel extraction kit, QIAquick PCR purification kit) were from Qiagen (Valencia, CA, USA). High capacity streptavidin-agarose resin was from Pierce (Rockford, IL, USA). Mouse α -His Tag (clone HIS.H8) monoclonal (mAb) (05-949), and rabbit α -hH2B polyclonal (07-371) antibodies were from Millipore (Billerica, MA, USA). Rabbit α -PARP-9 polyclonal (ab53796) was from Abcam (Cambridge, MA, USA). Goat α -mouse IgG (H+L)-HRP, and goat α -rabbit IgG (H+L)-HRP conjugates were from Biorad (Hercules, CA, USA). Mouse α -Flag M2 monoclonal antibody (F1804) was from Sigma-Aldrich. Oligonucleotides were purchased from Integrated DNA Technologies (IDT) (Coralville, IA, USA)

Equipment. An all Kel-F apparatus (Peptide Institute, Osaka, Japan) was used for HF cleavage. Deionized double distilled H₂O (ddH₂O) was used throughout and was prepared by a Millipore Synergy UV ultrapure water system. Electrospray ionization-mass spectrometry (ESI-MS) was performed on a Perkin Elmer-Sciex API100 single quadrupole instrument using LC2Tune 1.4/Biomultiview 1.3b2 (PE-Sciex, Toronto, Canada) software.

Analytical high-performance liquid chromatography (HPLC) was performed using Hewlett Packard (HP, Santa Clara, CA, USA) 1100 series instrumentation (Chemstation for LC 3D Rev A.10.02[1757], quaternary pump G1311A, column heater G1316A, diode array detector G1328A, auto-sampler G1313A). Preparative HPLC was performed using a Waters (Milford, MA, USA) Delta Prep 4000

system with a Waters Prep LC system controller, and a Waters 486 tunable absorbance detector. Separations were performed in gradient mode over 30 min for analytical separations, or over 60 min for other separations. Solvent A consisted of 0.1% (v/v) TFA in H₂O and solvent B consisted of 90 % (v/v) MeCN/0.1 % (v/v) TFA in H₂O. Analysis was run at 1 mL/min with detection at 214 nm and 280 nm. ADP-ribose conjugates and ligation reactions were additionally monitored at 259 nm to detect adenine absorbance. Separations were achieved on C18 stationary phases using a Vydac 218TP5415 column (5 μm; 4.6 × 150 mm) for analytical separations, a Vydac 218TP1010 column (7 μm; 10 × 250 mm) for semi-preparative separations, a Vydac 218TP1022 column (10 μm; 22 × 250 mm) for preparative separations, and a Vydac 218TP152050 column (15-20 μm; 50 × 250 mm) for process separations. A flow-rate of 1, 5, 18, or 30 mL/min was used for analytical, semi-preparative, preparative, and process scale separations, respectively. Peptides were quantified by amino acid analysis (AAA) at the W.M. Keck Foundation Biotechnology Resource Laboratory (Yale University, CT, USA) using a Hitachi L-8900 amino acid analyzer.

Photocrosslinking was performed using an Oriel 50-200 W Hg Arc Lamp (Newport 66477) powered by an Oriel 50-200 W universal arc lamp power supply (Newport 69907). A 365 nm bandpass filter (Oriel 56430; 25.4 mm ø) was used. A Newport 840-C optical power meter with a Newport 818-UV photodiode detector was used to measure lamp power output.

Peptide Synthesis. Peptides **1**, **2**, **3**, and **9** were synthesized by manual solid-phase peptide synthesis (SPPS) on MBHA resin using HBTU/DIPEA *in situ* neutralization³ and Boc-chemistry. Peptides **4** and **5** were synthesized on rink amide (aminomethyl)polystyrene resin by Fmoc-chemistry on a CEM liberty microwave-assisted peptide synthesizer (CEM; Matthews, NC, USA) using standard instrument protocols. Peptide **13** (Phe-Dpr(Aoa)-NH₂) was synthesized by Fmoc-manual SPPS on rink amide MBHA resin for NMR studies. Peptides **6–8** were synthesized on rink amide ChemMatrix resin by a combination of manual Fmoc-synthesis and automated synthesis using the CEM liberty. For peptides

synthesized on MBHA resin, the resin hydrochloride salt was neutralized by 3 × 15 min treatments with 5 % (v/v) DIPEA-DMF (10 mL) prior to coupling the first amino acid. For peptides synthesized on rink amide resins, an Fmoc-deprotection step was performed prior to coupling the first amino acid.

For manual peptide synthesis, Boc- and Fmoc-deprotections were achieved by 2 × 1 min treatments with neat TFA or 3 × 5 min treatments with 20 % (v/v) piperidine-DMF respectively. A 1 min DMF flow wash was subsequently performed, followed by 15-60 min couplings with 4 eq of preactivated amino-acid. Amino acid activation was achieved by dissolving amino acids (4 eq) in 0.5 M HBTU-DMF solution (3.92 eq) to which DIPEA (5.68 eq) was added. Amino acids were preactivated for 1 min prior to their addition to the resin. Coupling yields were determined using the quantitative ninhydrin test.⁴ Where necessary, couplings were repeated to give coupling yields greater than 99.7%. Boc-amino acids with the following side-chain protection were used: Glu(OcHx), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl). Fmoc-amino acids with the following side-chain protection were used: Glu(OtBu), Lys(Boc), Ser(tBu), Thr(tBu). Upon completion of peptide synthesis, resins were washed thoroughly with DMF × 3, DCM × 3, and MeOH × 3. The resins were subsequently dried under vacuum in a desiccator overnight prior to cleavage and deprotection with anhydrous HF for Boc-peptides, or TFA for Fmoc-peptides.

For Boc-synthesized peptides **1**, **2**, **3**, and **9**, the *N*^ε-biotinyl-lysine residue was incorporated into the sequence by coupling Boc-Lys(Fmoc)-OH to the resin, followed by Fmoc-deprotection. Biotin-ONp (1.35 eq) in DMF was subsequently added to the resin and left to couple overnight. The resin was subsequently washed with DMF, followed by synthesis of the remaining peptide chain.

For Fmoc-synthesized peptides **7** and **8**, the *N*^ε-biotinyl-lysine residue was incorporated into the peptide by coupling Fmoc-Lys(Alloc)-OH to the resin. The Alloc group was removed following synthesis of the H2B(3-19) sequence. The resin was thoroughly washed with anhydrous DCM, followed

by the addition of anhydrous DCM (~1 mL/0.25 g resin), phenylsilane (24 eq), and Pd(PPh₃)₄ (0.25 eq).⁵ The reaction vessel was filled with Ar(g) and left to agitate for 30 min. The resin was then washed with anhydrous DCM, and the deprotection repeated for 30 min. The resin was then washed with DCM (5 × 10 mL), 0.5 % (v/v) DIPEA-DMF (3 × 10 mL), 0.5 % (w/v) sodium diethylthiocarbamate-DMF (5 × 10 mL), 1:1 DCM-DMF (5 × 10 mL), and 0.5 % (w/v) 1-hydroxybenzotriazole (HOBt)-DMF (3 × 10 mL). D-Biotin (10 eq) was activated with 0.5 M HBTU (9.8 eq) in 1:1 DMF-NMP and DIPEA (12 eq). After the mixture became transparent, it was added to the resin and left to couple for 3 h. The resin was subsequently thoroughly washed with 1:1 NMP-DMF, and DMF, followed by synthesis of the remaining peptide chain.

Coupling of Fmoc-Dpr(Boc-Aoa)-OH (2 eq) was achieved using PyBOP (1.98 eq) and DIPEA (4 eq) for 1 h. Subsequent amino acid couplings were performed for 30 min with HBTU (1.98 eq), 2 eq of amino acid, and 3.5 eq DIPEA to reduce the risk of acylation of the mono-Boc-protected aminoxy group. This included the coupling of the Bpa photocrosslinker in peptides **7** and **8**, which was coupled for 1 h.

HF Cleavage. HF cleavage (10mL HF/g resin) was performed for 1 h at 0-4 °C with 5 % (v/v) *p*-cresol as a scavenger. Following HF cleavage, the HF was removed under reduced pressure, peptides were precipitated in ice-cold ether, filtered, dissolved in 40% (v/v) aqueous MeCN containing 0.1% (v/v) TFA, and lyophilized. Peptides were purified by HPLC as described under peptide purification.

TFA Cleavage. TFA cleavage was performed for 1 h at RT with 95:2.5:2.5 TFA-triisopropylsilane-H₂O (10-20 mL/g resin). The TFA was subsequently evaporated with a nitrogen stream. The peptides were precipitated with ice-cold ether, filtered, dissolved in 40% (v/v) aqueous MeCN containing 0.1% (v/v) TFA, and lyophilized. Peptides were purified by HPLC as described under peptide purification.

Ligation of ADP-ribose to Peptides. Ligation reactions (1-25 mg of peptide) were performed in 0.5 M sodium acetate at pH 4.0 for peptide **3**, and pH 4.5 for peptides **2**, **6**, **9**, and **13**. Ligation to peptide **8** was conducted in 20 % (v/v) MeCN, 0.5 M sodium acetate pH 4.5 to aid solubility. The use of lower concentrations of sodium acetate (e.g. 0.1 M) was found to result in slower reaction rates and reduced product yields for peptide **3**. Ligation to peptide **3** was performed at 30 °C for 3 days with the peptide at 8.9 mM, and ADP-ribose at 89 mM concentration. At this point 40 % of peptide **3** had reacted with ADP-ribose. Peptide concentrations of approximately 10 mM have been favored in the literature for fast ligation rates to *N*-methyl aminoxy amino acid **11**.⁶ The use of > 30 eq of ADPR, when ligating to **3**, resulted in the formation of non-specific ligation products. Ligations to peptides **2**, **6**, **8**, **9**, and **13** were performed at RT to 30 °C for 3 h with 3.3-14.2 mM peptide and 43.9-104.2 mM ADP-ribose. Peptides were subsequently purified by HPLC. Representative HPLC and ESI-MS data for the ligation of ADP-ribose to **2** and **3** is reported in Figure 1.

Peptide Purification. Peptides were purified by reversed-phase HPLC (conditions described under equipment) with detection at 214 nm. Peptides **2a**, and **9a** were purified on a Vydac C18 semi-preparative scale column. Peptides **1**, **3**, **3a**, **6**, **6a**, **7**, **8a**, **9**, and **13a** were purified on a Vydac C18 preparative column. Peptides **2**, **4**, and **5** were purified on a Vydac C18 process column. Peptides **8** and **13** were not purified prior to ligation.

Peptides were separated using the following linear gradients: Peptides **1-4**, 7-20 % solvent B; Peptide **6**, 0-30 % solvent B; Peptide **7**, 10-40 % solvent B; Peptides **5** and **9**, 0-20 % solvent B. ADP-ribose conjugates were purified using the following linear gradients: Peptide **2a**, 7-20 % solvent B; Peptide **3a**, 10-15 % solvent B; Peptide **6a**, 0-30 % solvent B; Peptide **8a**, 15-30 % solvent B; Peptide **9a**, 0-20 % solvent B; Peptide **13a**, 0-15 % solvent B. After purification, pure fractions were combined, lyophilized, and characterized by analytical reversed-phase HPLC, and ESI-MS.

Peptide Analytical Data. Analytical RP-HPLC and ESI-MS data for peptides is reported in Figure S11 and Figure S12.

Peptide 1. Yield 62 %; HPLC: $t_R = 16.2$ min (7-20 % solvent B); Purity: 95.7 %; AAA: Ala 4.1 (4), Glx 1.1 (1), Gly 1.0 (1), Lys 6.2 (6), Pro 4.1 (4), Ser 1.6 (2), Thr 0.9 (1), Val 1.0 (1); ESI-MS: m/z $[M+2H]^{2+}$ 1123.9 (calcd. 1123.8), $[M+3H]^{3+}$ 749.5 (calcd. 749.6), $[M+4H]^{4+}$ 562.5 (calcd. 562.4); MW 2245.69.

Peptide 2. Yield: 43 %; HPLC: $t_R = 17.4$ min (7-20 % solvent B); Purity: 97.0 %; ESI-MS: m/z $[M+2H]^{2+}$ 1138.6 (calcd. 1138.6), $[M+3H]^{3+}$ 759.4 (calcd. 759.4), $[M+4H]^{4+}$ 569.4 (calcd. 569.8); MW 2275.72 g/mol.

Peptide 2a. Yield: 72 %; HPLC: $t_R = 16.9$ min (7-20 % solvent B); Purity: 97.0 %; AAA: Ala 4.0 (4), Gly 1.7* (1), Lys 6 (6), Pro 4.2 (4), Ser 1.8 (2), Thr 0.8 (1), Val 1.0 (1); *Note that acid hydrolysis of the adenine ring from ADP-ribose produces a species in 40-60 % molar yield which co-elutes with glycine during AAA;⁷ ESI-MS: m/z $[M+2H]^{2+}$ 1409.9 (calcd. 1409.5), $[M-AMP+2H]^{2+}$ 1235.9 (calcd. 1236.4), $[M+3H]^{3+}$ 940.0 (calcd. 940.0), $[M-AMP+3H]^{3+}$ 824.0 (calcd. 924.6); MW 2817.02 g/mol.

Peptide 3. Yield: 65 %; HPLC: $t_R = 15.4$ min (7-20 % solvent B); Purity: 98.0 %; ESI-MS: m/z $[M+2H]^{2+}$ 1124.3 (calcd. 1124.4), $[M+3H]^{3+}$ 749.8 (calcd. 749.9), $[M+4H]^{4+}$ 562.7 (calcd. 562.7); MW 2246.7 g/mol.

Peptide 3a. Yield: 10.8 %; HPLC: $t_R = 15.4, 15.6$ min (7-20 % solvent B); Purity: 93.4 %; AAA: Ala 4.1 (4), Gly 1.6* (1), Lys 5.8 (6), Pro 4.0 (4), Ser 2.1 (2), Thr 0.9 (1), Val 1.0 (1); *Note that acid hydrolysis of the adenine ring from ADP-ribose produces a species in 40-60 % molar yield which coelutes with glycine during AAA;⁷ ESI-MS: m/z $[M+2H]^{2+}$ 1394.8 (calcd. 1395.0), $[M-AMP+2H]^{2+}$ 1220.8 (calcd. 1221.9), $[M+3H]^{3+}$ 929.9 (calcd. 930.3), $[M-AMP+3H]^{3+}$ 814.9 (calcd. 814.9); MW 2788.02 g/mol.

Peptide 4. Yield: 40 %; HPLC: $t_R = 8.0$ min (7-20 % solvent B); Purity: > 99.5 %; ESI-MS: m/z $[M+2H]^{2+}$ 1010.1 (calcd. 1010.7), $[M+3H]^{3+}$ 674.0 (calcd. 674.1), $[M+4H]^{4+}$ 505.8 (calcd. 505.8); MW 2019.39 g/mol.

Peptide 5. Yield: 23 %; HPLC: $t_R = 12.4$ min (0-30 % solvent B); Purity: > 99.5 %; ESI-MS: m/z $[M+2H]^{2+}$ 982.8 (calcd. 981.7), $[M+3H]^{3+}$ 655.5 (calcd. 654.8); MW 1961.37 g/mol.

Peptide 6. Yield: 25 %; HPLC: $t_R = 12.5$ min (0-30 % solvent B); Purity: > 99.5 %; ESI-MS: m/z $[M+H]^+$ 2049.7 (calcd. 2050.4), $[M+2H]^{2+}$ 1025.9 (calcd. 1025.7), $[M+3H]^{3+}$ 684.0 (calcd. 684.1); MW 2049.42 g/mol.

Peptide 6a. Yield: 74 %; HPLC: $t_R = 13.2$ min (0-30 % solvent B); Purity: > 99.5 %; ESI-MS: m/z $[M+2H]^{2+}$ 1295.8 (calcd. 1296.4), $[M-AMP+2H]^{2+}$ 1122.3 (calcd. 1123.8), $[M+3H]^{3+}$ 864.2 (calcd. 864.6), $[M-AMP+3H]^{3+}$ 748.7 (calcd. 749.5); MW 2590.72 g/mol.

Peptide 7. Yield 16.9 %; HPLC: $t_R = 16.5$ min (0-50 % solvent B, 50 °C); Purity: > 98.0 %; ESI-MS: m/z $[M+2H]^{2+}$ 1248.9 (calcd. 1249.5), $[M+3H]^{3+}$ 833.0 (calcd. 833.3), $[M+4H]^{4+}$ 625.0 (calcd. 625.2); MW 2496.9 g/mol.

Peptide 8. HPLC: $t_R = 18.5$ min (0-50 % solvent B); Peptide was not purified; ESI-MS: m/z $[M+2H]^{2+}$ 1264.9 (calcd. 1264.5), $[M+3H]^{3+}$ 843.7 (calcd. 843.3); MW 2527.0 g/mol.

Peptide 8a. Yield: 62 %; HPLC: $t_R = 16.2$ min (0-50 % solvent B); Purity: > 99.5 %; ESI-MS: m/z $[M+2H]^{2+}$ 1535.8 (calcd. 1535.2), $[M-AMP+2H]^{2+}$ 1361.8 (calcd. 1362.5), $[M+3H]^{3+}$ 1023.9 (calcd. 1027.8), $[M-AMP+3H]^{3+}$ 907.9 (calcd. 908.7); MW 3068.3 g/mol.

Peptide 9. Yield 54.4 %; HPLC: $t_R = 12.7$ min (0-30 % solvent B); Purity: 95.4 %; ESI-MS: m/z $[M+H]^+$ 702.5 (calcd. 702.8); MW 701.8 g/mol.

Peptide 9a. Yield: 54 %; HPLC: $t_R = 12.1$ min (0-30 % solvent B); Purity: 97.4 %; AAA: Gly 3.4* (3), Lys 1 (1); *Note that acid hydrolysis of the adenine ring from ADP-ribose produces a species in 40-60 % molar yield which coelutes with glycine during AAA;⁷ ESI-MS: m/z $[M+H]^+$ 1243.2 (calcd. 1243.4), $[M-AMP+H]^+$ 896.1 (calcd. 897.3), $[M+2H]^{2+}$ 622.3 (calcd. 622.2); MW 1242.4 g/mol.

Peptide 13. HPLC: $t_R = 7.3$ min (0-15 % solvent B); Peptide was not purified; ESI-MS: m/z $[2M+H]^+$ 649.0 (calcd. 647.3), $[M+H]^+$ 324.0 (calcd. 324.2); MW 323.4 g/mol.

Peptide 13a. Yield: 49 %; HPLC: $t_R = 10.8$ min (0-15 % solvent B); Purity: 98.7 %; ESI-MS: m/z $[M+H]^+$ 865.0 (calcd. 865.2), $[M+H-AMP]^+$ 518.0 (calcd. 519.4); MW 864.7 g/mol.

Inverse PCR to Generate mH2A1.1 Coding DNA. cDNA for mH2A1.2 (BC013331; IMAGE: 4077577) was obtained from Open Biosystems (Huntsville, AL, USA) in pDNR-LIB. The region encoding mH2A1.2 (198-229) differs from the mH2A1.1 sequence. Two 5'-phosphorylated HPLC purified primers (Table S1) were used to substitute the mH2A1.1 sequence for this region using inverse PCR. Inverse PCR was performed using a QuikChange XL II kit. The reaction was assembled (with 15 pmol of each primer), and cycled as described in the product information. The PCR reaction was then digested with DpnI (20 U) at 37 °C for 2 h to digest the parental vector, and gel purified using the QIAQuik gel extraction kit. The DNA (5 μ L) was cyclized with T4 DNA ligase (800 U) in 50 μ L total volume at 16 °C overnight. The ligation mixture was transformed into DH5 α *E. coli* cells. The plasmid was purified by standard protocols and sequenced.

PCR-Based Gene Synthesis. The H2A.2 gene was synthesized using PCR-based gene synthesis.⁸ The DNA sequence was codon optimized for *E. coli* using the OPTIMIZER (<http://genomes.urv.es/OPTIMIZER/>).⁹ The optimized sequence (Table S2) was used to generate overlapping oligonucleotides (≤ 60 bp length; Table S3) using assembly PCR oligomaker (http://publish.yorku.ca/~pjohanson/Assembly_PCRoligomaker.html),¹⁰ and ordered with standard desalting. Oligonucleotides were dissolved in water to 100 μ M. The internal oligonucleotides were combined to generate a stock solution containing 5 μ M of each oligonucleotide in water. The PCR reaction was assembled using 10 \times KOD polymerase buffer #1 (5 μ L), 2 mM dNTPs (5 μ L), 25 mM MgCl₂ (3 μ L), 5 μ M internal oligonucleotide stock (0.2 μ L; 20 nM of each oligonucleotide), 100 μ M external oligonucleotides (0.2 μ L; 0.4 μ M of each oligonucleotide), and water to 50 μ L. KOD DNA

polymerase (1 U) was added. The reaction was denatured at 98 °C (15 sec), and then subjected to 25 cycles of 98 °C (15 sec), 50 °C (2 sec), and 72 °C (20 sec). The mixture was then put through a QIAquick PCR purification kit, eluted with water, digested with NdeI and BamHI, and gel purified prior to ligation into digested pET-3a (see molecular cloning section).

Molecular Cloning to Generate Histone, *Macro* Domain, and PARP Expression Vectors.

Restriction/ligation cloning was used to generate pET-3a expression vectors for histones; pET-15b expression vectors for *macro* domains and PARP1; and a pFastBac HT B vector for PARP10. DNA inserts were generated by PCR off cDNA templates, or synthetic DNA for H2A.2 (Table S2), using primers with 5'-overhangs (Table S4), incorporating appropriate restriction sites. For mH2A1.1, the inverse-PCR generated plasmid was used as a template for PCR. PARP1 (BC037545; IMAGE: 5193735), PARP10 (BC136542; IMAGE 9020532) and human histone H2B.1 (BC051872; IMAGE: 6526835) cDNA was from Open Biosystems. PARP10 cDNA was initially subcloned into pcDNA3.1(+) using the KpnI-Flag-PARP10 (FOR) and XbaI-PARP10 (REV) primers, and then cloned into pFastBac HT B using the BamHI-Flag-PARP10 (FOR) and Xba-PARP10 (REV) primers. The inserts and expression vectors were digested with appropriate restriction enzymes, and gel purified. Inserts were ligated into digested vectors using T4 DNA ligase at 16 °C overnight. The ligation mixtures were transformed into DH5 α *E. coli* cells. The plasmids were purified by standard protocols and sequenced.

Site-Directed Mutagenesis. pET-15b expression vectors encoding for the mH2A1.1 (G224E) *macro* domain mutant¹¹ or the PARP1 (D214A) mutant¹² were generated using the Quikchange II XL site-directed mutagenesis kit according to the product manual. The primer sequences used are reported in Table S1. The mH2A1.1 *macro* domain and PARP1 pET-15b plasmids were used as templates.

Cell Culture. HeLa, and HeLa S3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose (Invitrogen) containing 10 % (v/v) fetal bovine serum (FBS). Farage cells were grown in RPMI-1640 media (Invitrogen) containing 10 % (v/v) FBS. Cells were maintained at 37 °C in a humidified incubator with 5 % CO₂. *Sf9* cells were grown in Grace's supplemented insect media (TNM-FH; Invitrogen) containing 10 % (v/v) FBS, 0.1 % (v/v) pluronic F-68, and pen-strep (100 U/mL penicillin; 100 µg/mL streptomycin) at 27 °C. When grown in suspension, *Sf9* cells were shaken at 150 RPM. *E. coli* were grown in lysogeny broth (LB; Miller's formulation) at 37 °C with shaking at 250 RPM, or on LB-agar plates. Ampicillin was used at 100 µg/mL for plasmid selection and maintenance in *E. coli*.

Expression and Reconstitution of Histone Dimers. Histones were expressed, purified, and reconstituted into dimers using the protocol of Luger, *et al.*¹³ with modifications. Briefly, histones were expressed in BL21(DE3)pLysS, and purified from inclusion bodies by denaturing size exclusion chromatography (SEC) on a sephacryl S200 HR column (~1 L column volume) in histone purification buffer (20 mM Tris-Cl pH 7.5, 7 M guanidine-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol) followed by HPLC purification (40-60 % solvent B) on a C18 process scale column. Recombinant histones were reconstituted into dimers by mixing equimolar amounts of each histone in unfolding buffer (20 mM Tris-Cl pH 7.5, 6 M guanidine-HCl, 5 mM DTT), and dialyzing into refolding buffer (10 mM Tris-Cl pH 7.5, 2 M NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol). The dimers were then purified by SEC on a superdex 200 10/300 GL column in refolding buffer, diluted to 50 % glycerol, and stored at -20 °C.

Expression and Purification of His₆-PARP1(D214A). The PARP1(D214A) mutant was selected for expression in *E. coli*. This mutation produces a caspase-3 uncleavable PARP1, which is expressed several fold more than native PARP1 in *E. coli*, and has a similar specific activity to the native protein.¹² The protein was expressed in C43(DE3) cells.¹⁴ Cultures were induced with 1 mM IPTG at 0.5 OD₆₀₀,

with expression for 4 h at 37 °C. The cells were then pelleted (3 k ×g, 15 min, 4 °C), resuspended in PBS-complete protease inhibitors, lysed using an Emulsiflex-C5 (Avestin, Canada), and pelleted (15 k ×g, 15 min, 4 °C). Ammonium sulfate (0.226 g/mL) was added to the supernatant to 40 % saturation (0.226 g/mL), and the precipitant was subsequently pelleted (18 k ×g, 30 min, 4 °C). To the supernatant was added ammonium sulfate (0.187 g/mL) to bring the supernatant to 70 % saturation. The precipitate was pelleted, and the supernatant decanted. The pellet was resuspended in 1 mM DTT-PBS, dialyzed against this buffer for 4 h, and then loaded onto a 1 mL HisTrap HP column (GE Health) equilibrated with binding buffer A (50 mM sodium phosphate pH 8, 300 mM NaCl, 5 mM imidazole, 1 mM DTT, 0.1 % (v/v) triton x-100, 20 % (v/v) glycerol, and complete protease inhibitors). The column was thoroughly washed with binding buffer A; 1 M NaCl in binding buffer A, and then eluted with a linear gradient of 5-150 mM imidazole in binding buffer A over 30 column volumes. The eluents were then purified by SEC on a superdex 200 10/300 GL column in 2× PARP1 buffer (40 mM Tris-Cl pH 8, 400 mM NaCl, 2 mM DTT), made to 50 % glycerol, 0.1 mg/mL BSA, and stored at -20 or -80 °C until use.

Expression and Purification of His₆-Flag-PARP10. His₆-Flag-PARP10 was expressed using the bac-to-bac system (Invitrogen) according to the product instructions with modifications. Briefly, the pFastBac HT B-His₆-Flag-PARP10 vector was transformed into DH10Bac competent cells to generate recombinant bacmids. Bacmid DNA was extracted by alkaline lysis, followed by ethanol precipitation. Isolated bacmid DNA was verified for the presence of the PARP10 gene by PCR. Bacmid DNA was transfected into a monolayer of *Sf9* cells using Cellfectin reagent (Invitrogen). After three days at 27 °C the virus containing supernatant was collected. Three rounds of virus amplification were performed in *Sf9* cell monolayers to increase the viral titer. *Sf9* cells in suspension were then infected with an optimized amount of recombinant virus, and mixed at 150 RPM at 27 °C on an orbital shaker for 48 h. The cells were then pelleted (400 ×g, 20 min, 4 °C), and lysed by sonication (6 × 10 sec) in ice-cold lysis buffer (50 mM sodium phosphate pH 8, 300 mM NaCl, 10 mM imidazole, 10 % (v/v) glycerol, complete protease inhibitors, 1 mM MgCl₂). To this mixture was added DNase I (20 µg/mL) and RNase

(420 U/mL). The mixture was incubated on ice for 30 min, and then pelleted (10 k ×g, 15 min, 4 °C). The expressed protein was purified from the supernatant using Ni-NTA beads. After binding for 1 h at 4°C the beads were thoroughly washed with lysis buffer containing 1 M NaCl. The column was eluted with an imidazole step gradient (10, 20, 50, 100, and 500 mM imidazole) in lysis buffer. The eluents were then purified by SEC on a superdex 200 10/300 GL column in 2× PARP10 buffer (100 mM Tris-Cl pH 8, 8 mM MgCl₂, 0.4 mM DTT), made to 50 % glycerol, and stored at -20 or -80 °C until use.

Expression and Purification of GST-PARP10(818-1025). The GW-pGST-PARP10(818-1025) plasmid¹⁵ was obtained from Henning Kleine and Bernhard Lüscher (RWTH Aachen University, Germany). The plasmid was transformed into BL21(DE3) *E. coli*, which were grown to OD 0.6 at 37 °C, 250 RPM in LB broth containing 100 µg/mL ampicillin. Expression was induced with 1 mM IPTG, and the cells were grown for a further 3 h at 37 °C. The cells were subsequently pelleted (3 k ×g, 15 min, 4 °C), and lysed in TNE buffer (20 mM Tris-Cl pH 8, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, complete protease inhibitors) using an Emulsiflex-C5, and pelleted (15 k ×g, 15 min, 4 °C). The cleared supernatant was incubated with glutathione sepharose 4 fast flow (GE health) for 1 h at 4 °C. The supernatant was then drained, and the beads thoroughly washed with GST wash buffer (100 mM Tris pH 8, 120 mM NaCl). The protein was eluted in GST wash buffer containing 40 mM reduced glutathione, followed by analysis of purity and quantification against BSA standards by SDS-PAGE with coomassie R-250 staining.

Expression of Macro Domains. *Macro* domains were expressed according to the protocol described by Kustatscher, *et al.*¹¹ with modifications. Briefly, *macro* domains were expressed in BL21(DE3) at 37 °C for 3 h with induction by 1 mM IPTG. The cells were lysed in binding buffer B (50 mM sodium phosphate pH 7.4, 500 mM NaCl, 1 mM MgCl₂, 5 mM β-mercaptoethanol, 10 % glycerol, complete protease inhibitors) using an Emulsiflex C-5 (Avestin, Canada), followed by sonication (3 × 20 sec).

The *macro* domains were purified from the supernatant using Ni-NTA beads. After binding for 1 h at 4°C the beads were thoroughly washed with binding buffer B; 1 M NaCl, 40 mM imidazole in binding buffer B, and then eluted with 500 mM imidazole in binding buffer B. The eluents were then purified by SEC on a superdex 75 10/300 GL column in 1 mM DTT-PBS, made to 20 % glycerol, and stored at -80 °C until use. *Macro* domains were assessed for their capacity to bind ADP-ribose using the pull-down assay described by Karras, *et al*¹⁶ (Figure S13).

Protein Analytical Data. Protein SDS-PAGE data is reported in Figure S14. Western blots for His₆-PARP1(D214A) and His₆-Flag-PARP10 are reported in Figure S14. HPLC and ESI-MS data for expressed proteins is reported in Figure S15 and Figure S16 respectively.

H2B.1-Flag. HPLC: $t_R = 11.2$ min (40-60 % solvent B); Purity: 99.3 %; ESI-MS: m/z 14,881.16 \pm 2.65 (reconstructed); MW 14,882.0.

H2B.1(E2A)-Flag. HPLC: $t_R = 11.4$ min (40-60 % solvent B); Purity: 99.2 %; ESI-MS: m/z 14,827.07 \pm 5.54 (reconstructed); MW 14,824.0.

H2A.2. HPLC: $t_R = 13.0$ min (40-60 % solvent B); Purity: > 99.5 %; ESI-MS: m/z 13,966.6 \pm 2.1 (reconstructed); MW 13,964.3.

His₆-mH2A1.1 *macro* domain. HPLC: $t_R = 22.9$ min (0-100 % solvent B); Purity: > 96 %; ESI-MS: m/z 24,025.3 \pm 5.6 (reconstructed); MW 24,026.2.

His₆-mH2A1.1(G224E) *macro* domain. HPLC: $t_R = 22.9$ min (0-100 % solvent B); Purity: > 98 %; ESI-MS: m/z 24,102.5 \pm 4.2 (reconstructed); MW 24,098.3.

His₆-mH2A1.2 *macro* domain. HPLC: $t_R = 23.5$ min (0-100 % solvent B); Purity: 96.2 %; ESI-MS: m/z 24,461.0 \pm 3.4 (reconstructed); MW 24,459.8.

***In vitro* ADP-Ribosylation of H2B-H2A Dimers Using Full-Length PARP10 or the PARP10 Catalytic Domain.** ADP-ribosylation assays were assembled using recombinant histone H2B-Flag/H2A

or H2B(E2A)-Flag/H2A dimers containing the equivalent of 0.5 μg H2B-Flag, 100 ng of His₆-Flag-PARP10 enzyme or 1 μg GST-PARP10(818-1025), and biotinylated NAD (5 μM) in 30 μL total volume. Dimers were incubated with biotinylated NAD to control for chemical versus enzymatic ADP-ribosylation. Benzamide was added to 10 mM concentration as a negative control to demonstrate that the observed auto-ADP-ribosylation was enzymatic. The reactions were conducted for 30 min in PARP10 reaction buffer (50 mM Tris-Cl pH 8, 4 mM MgCl₂, 0.2 mM DTT) at 30 °C. The reaction was stopped by the addition of reducing SDS-PAGE loading buffer (10 μL ; without heating), and immediately separated by electrophoresis on 12 % bis-tris midi gels (Biorad) using 1 \times NuPAGE MES SDS buffer (Invitrogen). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μm pore size) using NuPage transfer buffer (Invitrogen). The membrane was subsequently blocked with 5 % skim milk powder-phosphate buffered saline tween-20 (PBST; 0.1 % tween-20 in PBS), washed 3 \times 5 min with PBST, and incubated with 1:5000 streptavidin-HRP. After washing with PBST for 3 \times 5 min, the membrane was incubated with ECL reagent, and visualized following exposure to film (Figure S1). Immunoblotting with α -Flag M2 mAb (Sigma), followed by goat α -mouse IRDye 800CW (926-32210; Licor) secondary, with Scanning on an Odyssey infrared imaging system (Licor) was used to control for H2B loading. Coomassie R-250 staining was used as a loading control for His₆-Flag-PARP10 and GST-PARP10(818-1025).

In nucleo ADP-Ribosylation. HeLa cells were grown to 90 % confluence in a 15 cm cell culture dish, washed with PBS, scraped into ice-cold PBS, pelleted (700 $\times\text{g}$, 10 min), and washed with PBS two more times. The cells were taken up in 2 mL hypotonic lysis buffer (10 mM Tris-Cl pH 8, 1 mM KCl, 1.5 mM MgCl₂, complete protease inhibitors, sigma phosphatase inhibitor cocktail 1 (P2850; 1 $\mu\text{L}/\text{mL}$) and 2 (P5726; 1 $\mu\text{L}/\text{mL}$)) and Dounce homogenized with 20 up and down strokes of the tight pestle. The nuclei were pelleted at 600 $\times\text{g}$, and the supernatant discarded. The nuclei were resuspended in 2 mL hypotonic lysis buffer, pelleted at 600 $\times\text{g}$, and the supernatant removed. The nuclei were taken up in

0.45 mL incubation buffer (50 mM Tris-Cl pH 8, 10 mM MgCl₂, 0.1 mM EDTA) and left to warm to RT for 5 min. [³H]-NAD (12.5 μCi) was added to the nuclei, gently mixed with a pipette tip, and left for 30 min at RT with occasional mixing. The nuclei were subsequently pelleted (600 ×g, 10 min), and washed with 0.5 mL incubation buffer. The histones were extracted by adding 0.5 mL ice-cold 0.2 M H₂SO₄ to the nuclei pellet, vortexing to break up the pellet, and then mixing on a rotator for 1.5 h at 4 °C. The mixture was then pelleted (15 k ×g, 10 min, 4 °C), and the histones precipitated from the supernatant by the addition of 210 μL of 100 % (w/v) trichloroacetic acid. The sample was incubated on ice for 30 min, and then pelleted (15 k ×g, 20 min, 4 °C). The pellet was washed with 0.5 mL ice-cold acetone, and subsequently dissolved in 1× reducing SDS-PAGE buffer. The sample was immediately separated on a 15 % SDS-PAGE gel, or on a 15 % triton-acid urea gel¹⁷ followed by separation in the second dimension on a 12 % bis-tris gel run in 1× NuPAGE MOPS SDS buffer (Invitrogen). The gels were stained with coomassie R-250, impregnated with Amplify fluorographic reagent, dried, and visualized by fluorography (Figure S2).

ADP-Ribosylation of H2B Peptides 4, 5, and 6a By PARP1. To peptides **4**, **5**, and **6a** (0.65 nmol) in PARP1 buffer (50 mM Tris-Cl pH 8, 10 mM MgCl₂, 1.25 mM DTT) containing 1× activated DNA (Trevigen) was added biotinylated NAD (to 10 μM) and His₆-PARP1(D214A) (10 ng). Reactions were performed in 40 μL total volume at 25 °C for 1 h. Reactions were stopped with 10 μL 4× SDS-PAGE loading buffer, and separated on 12 % bis-tris gels using NuPAGE MES SDS running buffer. The peptides were transferred to PVDF using Towbin buffer-0.08 % (w/v) SDS-10 % (v/v) MeOH (25 mM Tris-Cl pH 8.3, 192 mM glycine, 0.08 % (w/v) SDS, 10 % (v/v) MeOH) at 100 V continuous for 30 min. The membrane was subsequently blocked with 5 % (w/v) skim milk power-PBST, washed 3 × 5 min with PBST, and incubated with 1:5000 streptavidin-HRP (GE Health) in PBST for 30 min. After washing with PBST for 3 × 5 min, the membrane was incubated with ECL reagent, and visualized following exposure to film (Figure S8). Groups without enzyme, but containing biotinylated NAD were

used to control for chemical ADP-ribosylation. Groups without enzyme or biotinylated NAD were used to ensure that streptavidin-HRP did not bind to the peptides. Peptide **4** was used to confirm whether the ADP-ribose in peptide **6a** was required for stimulating the transfer of biotinylated ADPR onto **6a** by PARP1, and to determine if PARP1 is able to initiate ADP-ribosylation at Glu-2 on this peptide. Peptide **5** was used as a control where Glu-2 was unavailable for ADP-ribosylation.

Confirming the Site-Specific Ligation of ADP-Ribose to Aminoxy Functionalized Peptides. The site-specific ligation of ADP-ribose to aminoxy-functionalized peptides was assessed by trypsinization of **2a** and **1**, followed by analytical HPLC (0-30 % solvent B). The **2a** digest chromatogram (Figure S4b) was examined for peaks featuring strong 259 nm absorbance associated with the ADP-ribose adenine. The **2a** digest chromatogram was also compared to the chromatogram obtained from the **1** tryptic digest (Figure S4a) for changes in tryptic peptide retention times, and the of presence peaks that absorb at 259 nm. Digests were performed in 50 mM ammonium bicarbonate buffer pH 8 with a 1:80 enzyme to substrate ratio, using sequencing grade modified trypsin (Promega) at 37 °C for 1 h. A peak eluting at 8.3 min for the **2a** digests was identified, which featured strong 259 nm absorbance, and corresponded to the mass of the amino-terminal peptide Pro-Dpr[Aoa(ADP-ribose)]-Pro-Ala-Lys ($[M+H]^+$ m/z 1112.0 (calcd. 1112.4)) (Figure S4). In addition, an attempt was made to ligate ADP-ribose to **1**, using the previously described ligation conditions. No ligation product was observed by HPLC over a 3 day period. In comparison, ligation of ADP-ribose under the same conditions to **2** was rapid (< 1 h), consistent with ligation occurring through the aminoxy group present in **2**.

Confirming that ADP-ribose Conjugation Occurs Through the Distal Ribose Residue. To **9a** (12 µg; 9.7 nmol) in 49 µL tris salts buffer (100 mM Tris-Cl pH 8, 100 mM NaCl, 15 mM MgCl₂) was added snake venom phosphodiesterase (0.1 U; Worthington Biochemical). The mixture was incubated at 25 °C for 1 h, and subsequently analyzed by analytical HPLC (0-30 % solvent B) and ESI-MS (Figure S5). The species generated no longer absorbed at 259 nm, suggesting the loss of adenine, and had a

mass corresponding to the conjugation of ribose-5-phosphate to **9** ($[M+H]^+$ m/z 914.5 (calcd. 914.4)). The reaction product was purified by analytical HPLC (0-30 % solvent B), and lyophilized. The lyophilized material was dissolved in 49 μ L 1 \times NEBuffer 3 (100 mM NaCl, 50 mM Tris-Cl pH 7.9, 10 mM MgCl₂, 1 mM DTT), to which calf intestinal alkaline phosphatase (10 U; New England Biolabs) was added. The mixture was incubated at 37 °C for 1 h, and subsequently analyzed by analytical HPLC (0-30 % solvent B) and ESI-MS (Figure S5). The species generated had a mass corresponding to the conjugation of ribose to **9** ($[M+H]^+$ m/z 834.4 (calcd. 834.4 g/mol)), demonstrating that ADP-ribose reacts with the aminoxy group in through the distal ribose residue.

¹H NMR of Peptide 13a. Peptide **13a** was dissolved in D₂O (Cambridge Isotope Laboratories; MA, USA) and lyophilized. This process was repeated twice, with **13a** subsequently dissolved in 0.4 mL D₂O. The pH of the mixture was \sim 7. 1D (¹H) (Figure S6) and 2D (COSY, TOCSY) spectra were acquired on a Bruker Avance 600 MHz (with cryoprobe) spectrometer (Bruker Biospin, Germany) at 298 °K. Chemical shifts are reported in parts per million (ppm) relative to the residual D₂O peak (δ 4.79 at 298 K). NMR spectra (Figure S6) were processed using Topspin 1.3 (Bruker Biospin, Germany). To aid in interpreting the acquired spectra, **13a** was digested with snake venom phosphodiesterase (as per **9a** above), purified by HPLC, and subjected to the same NMR experiments as **13a**. Oxime stereochemistry was assigned according to published data for D-ribose oximes.¹⁸ Adenine ring protons were assigned based upon published ¹H spectra for ADPR.¹⁹

Preparation of Nuclear Lysates. Nuclear lysates were prepared from HeLa S3 cells as described by Dignam, *et al*²⁰ and from Farage cells as described by Roeder.²¹ Lysates were precleared by incubation with high capacity streptavidin-agarose beads (Pierce) for 3 h at 4 °C, then aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

Stability of ADP-Ribose Conjugated Peptides. The stability of **6a** (14 μ g; 5.4 nmol) to 0.1 M NaOH, 1 mM HCl, 1 M hydroxylamine pH 7.0, 1 \times PBS, 0.1 M Tris-Cl pH 8, PARG (6 ng), and HeLa S3 cell lysates (108 μ g) was assessed (Figure S7) in 250 μ L total volume at 4 $^{\circ}$ C to mimic conditions used for the pull-down assays. For the PARG group a buffer consisting of 20 mM potassium phosphate pH 7.4, 50 mM KCl, 0.1 mg/mL BSA, 0.1 % (v/v) triton x-100 was used. Samples were prepared in triplicate. At 4 and 24 h, samples (100 μ L) were separated by analytical HPLC (7-20 % solvent B) and compared to a standard curve to determine the percentage **6a** remaining (Figure S7). ESI-MS was used to confirm that the remaining material corresponded to **6a**.

Pull-Down Assays. Peptides (24 μ M) and *macro* domains (0.8 μ M) were combined and incubated on a rotator at 4 $^{\circ}$ C for 3 h in binding buffer (to 300 μ L total volume; 1mM DTT, 0.02 % (v/v) NP40-PBS). Where appropriate, HeLa S3 lysates were added such that the *macro* domain concentration was 5 % (w/w) of total protein. To 50 μ L aliquots of a 50 % slurry of high capacity streptavidin-agarose beads (Pierce), prewashed in binding buffer, was added 250 μ L of the peptide-*macro* domain mixture. The remaining mixture was used for input samples. The mixture was placed on a rotator at 4 $^{\circ}$ C for 1 h to capture the formed complexes. The beads were subsequently washed with binding buffer 5 \times 1 mL, and the bound species eluted by heating at 95 $^{\circ}$ C for 10 min in 100 μ L of 1 \times reducing SDS-PAGE sample buffer. The mixture was spun through a bio-rad micro bio-spin column to separate beads from the supernatant. Samples were subsequently separated on 12 % bis-tris gels in 1 \times NuPAGE MES SDS buffer (Invitrogen) and transferred to PVDF using Towbin buffer-10 % (v/v) MeOH (25 mM Tris-Cl pH 8.3, 192 mM glycine, 10 % (v/v) MeOH) at 100 V continuous for 60 min. The membrane was subsequently blocked with 5 % (w/v) skim milk powder in tris-buffered saline tween-20 (TBST), washed 3 \times 5 min with TBST, and incubated with 1:5000 streptavidin-HRP (GE Health) in TBST for 30 min. The membrane was then washed 3 \times 5 min with TBST, incubated with 1:3000 mouse α -His Tag mAb in 5 % (w/v) skim milk powder-TBST for 1 h, washed 3 \times 5 min with TBST, and incubated with 1:5000

goat α -mouse IgG (H+L)-HRP for 1 h. After washing with TBST for 3×5 min, the membrane was incubated with ECL reagent, and visualized following exposure to film.

Photocrosslinking. Peptides **2a**, **7** or **8a** (24 μ M), His₆-mH2A1.1 *macro* domain (832 nM, or 167 nM for the crosslinking with pull-down experiment), and HeLa S3 lysates (to yield a 5 % (w/w) concentration of the His₆-mH2A1.1 *macro* domain, or 1 % in the crosslinking with pull-down experiment) were combined and incubated on a rotator at 4 °C for 1 h in crosslinking buffer (to 150 μ L total volume; 1mM DTT-PBS). The mixtures (125 μ L; 25 μ L used for input samples) were then irradiated at 365 nm at 4 °C for 15 min. At this stage samples were either separated by SDS-PAGE and blotted, when used as a probe for ADPR binding proteins, or used for pull-down experiments. For the pull-down experiment (Figure 3), the irradiated peptide-*macro* domain mixtures were incubated with 25 μ L aliquots of a 50 % slurry of high capacity streptavidin-agarose beads (Pierce), prewashed in crosslinking buffer, for 1 h at 4 °C. The beads were then washed with crosslinking buffer 5×1 mL, and the bound species eluted by incubating at RT for 15 min with 1 \times reducing SDS-PAGE sample buffer containing 5 mM D-Biotin, followed by heating at 95 °C for 15 min. The mixture was spun through a bio-rad micro bio-spin column to separate the beads from the supernatant. Samples (30 % of output) were separated on a 12 % bis-tris gel in 1 \times NuPAGE MES SDS buffer, transferred to PVDF, and blotted with streptavidin-HRP or α -His Tag clone HIS.H8 mAb (as described under pull-down assays), followed by incubation with ECL reagent, and visualization following exposure to film.

Enrichment of PARP9 from Farage nuclear lysates (Figure S10) was performed similar to the above experiment with the following changes. The lysates were dialyzed against 1 mM DTT in PBS for 3 hours prior to performing the experiment. Peptides **7** and **8a** were used at 7.2 μ M concentration, with 200 μ g of Farage nuclear extract, in 0.5 mL total volume of 1 mM DTT in PBS. The eluted proteins after separation by SDS-PAGE, and transfer to PVDF were blotted with α -PARP9 (for PARP9) and streptavidin-HRP (for peptides).

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C. Supplementary Figures S1-S16

Table S1: Site-directed mutagenesis primers.

Primer Name	Primer Sequence (5'→3')
mH2A1.1→.2 (inner)	pGAC AGC ATC ACT GTC GAT CGA GGC AAT GTC AGC CTG TAC AAC TTG CAG CTT CTG GCC AAG GAA G
mH2A1.1→.2 (outer)	pGTT CAC CCG ACA AAC ACT GAC TTC TAC ATC GGT GGT GAA GTA GGA AAC ACG CTG GAG AAG
mH2A1.1 domain (FOR)	<i>macro</i> (G224E) ACT GAC TTC TAC ATC GGT GAG GAA GTA GGA AAC ACG CTG G
mH2A1.1 domain (REV)	<i>macro</i> (G224E) CCA GCG TGT TTC CTA CTT CCT CAC CGA TGT AGA AGT CAG T
PARP1 (FOR)	(D214A) AAA GGC GAT GAG GTG GCT GGA GTG GAT GAA GTG
PARP1 (REV)	(D214A) CAC TTC ATC CAC TCC AGC CAC CTC ATC GCC TTT

Table S2: DNA sequence for NdeI-H2A.2-BamHI.

Primer Name	DNA Sequence (5'→3')
NdeI-H2A.2-BamHI	GGA ATT CCA TAT GTC TGG TCG TGG TAA ACA GGG TGG TAA AGC GCG TGC GAA AGC GAA ATC TCG TTC TTC TCG TGC GGG TCT GCA GTT CCC GGT TGG TCG TGT TCA CCG TCT GCT GCG TAA AGG TAA CTA CGC GGA ACG TGT TGG TGC GGG TGC GCC GGT TTA CAT GGC GGC GGT TCT GGA ATA CCT GAC CGC GGA AAT CCT GGA ACT GGC GGG TAA CGC GGC GCG TGA CAA CAA AAA AAC CCG TAT CAT CCC GCG TCA CCT GCA GCT GGC GAT CCG TAA CGA CGA AGA ACT GAA CAA ACT GCT GGG TAA AGT TAC CAT CGC GCA GGG TGG TGT TCT GCC GAA CAT CCA GGC GGT TCT GCT GCC GAA AAA AAC CGA ATC TCA CCA CAA AGC GAA AGG TAA ATA ATA AGG ATC CCG AT

Table S3: H2A.2 gene-synthesis PCR oligonucleotides.

Oligo Name	Oligo Sequence (5'→3')
H2A.2 F1 (external)	GGA ATT CCA TAT GTC TGG TCG TGG TAA ACA GGG TGG TAA AGC GCG TGC G
H2A.2 R1 (internal)	CGG GAA CTG CAG ACC CGC ACG AGA AGA ACG AGA TTT CGC TTT CGC ACG CGC TTT ACC
H2A.2 F2 (internal)	CGG GTC TGC AGT TCC CGG TTG GTC GTG TTC ACC GTC TGC TGC GTA AAG GTA ACT ACG CG
H2A.2 R2 (internal)	CGC CGC CAT GTA AAC CGG CGC ACC CGC ACC AAC ACG TTC CGC GTA GTT ACC TTT ACG C
H2A.2 F3 (internal)	GGT TTA CAT GGC GGC GGT TCT GGA ATA CCT GAC CGC GGA AAT CCT GGA ACT GGC GGG
H2A.2 R3 (internal)	CGC GGG ATG ATA CGG GTT TTT TTG TTG TCA CGC GCC GCG TTA CCC GCC AGT TCC AGG
H2A.2 F4 (internal)	CCC GTA TCA TCC CGC GTC ACC TGC AGC TGG CGA TCC GTA ACG ACG AAG AAC TGA ACA AAC
H2A.2 R4 (internal)	CAG AAC ACC ACC CTG CGC GAT GGT AAC TTT ACC CAG CAG TTT GTT CAG TTC TTC GTC GTT
H2A.2 F5 (internal)	CGC AGG GTG GTG TTC TGC CGA ACA TCC AGG CGG TTC TGC TGC CGA AAA AAA CCG AAT CTC
H2A.2 R5 (external)	ATC GGG ATC CTT ATT ATT TAC CTT TCG CTT TGT GGT GAG ATT CGG TTT TTT TCG GCA

Table S4: Primers for restriction/ligation cloning.

Primer Name	Primer Sequence (5'→3')
NdeI-H2B (FOR)	GGA ATT CCA TAT GCC GGA ACC AGC GAA GTC CGC
NdeI-H2B(E2A) (FOR)	GGA ATT CCA TAT GCC GGC ACC AGC GAA GTC CGC TCC CGC G
BamHI-H2B-Flag (REV)	CGG GAT CCT TAC TTA TCG TCG TCA TCC TTG TAA TCG GCT CCC TTA GCG CTG GTG TAC TTG GTG ACG G
NdeI-mH2A1 domain (FOR)	<i>macro</i> ATC TAC CAT ATG GGT GAA GTC AGT AAG GCA G
XhoI-mH2A1 domain (REV)	<i>macro</i> GAC TCT CGA GTT AGT TGG CGT CCA GCT TG
NdeI-PARP1 (FOR)	GGG AAT TCC ATA TGG CGG AGT CTT CGG ATA AGC
XhoI-PARP1 (REV)	TAT CTC GAG TTA CCA CAG GGA GGT C
KpnI-Flag-PARP10 (FOR)	AAG GTA CCG CCA TGG ACT ACA AAG ACG ATG ACG ACA AGG TTG CAA TGG CGG AGG CAG
XbaI-PARP10 (REV)	GCT CTA GAT CAA GTG TCT GGG GAG C
BamHI-Flag-PARP10 (FOR)	TGG GAT CCG ACT ACA AAG ACG ATG ACG

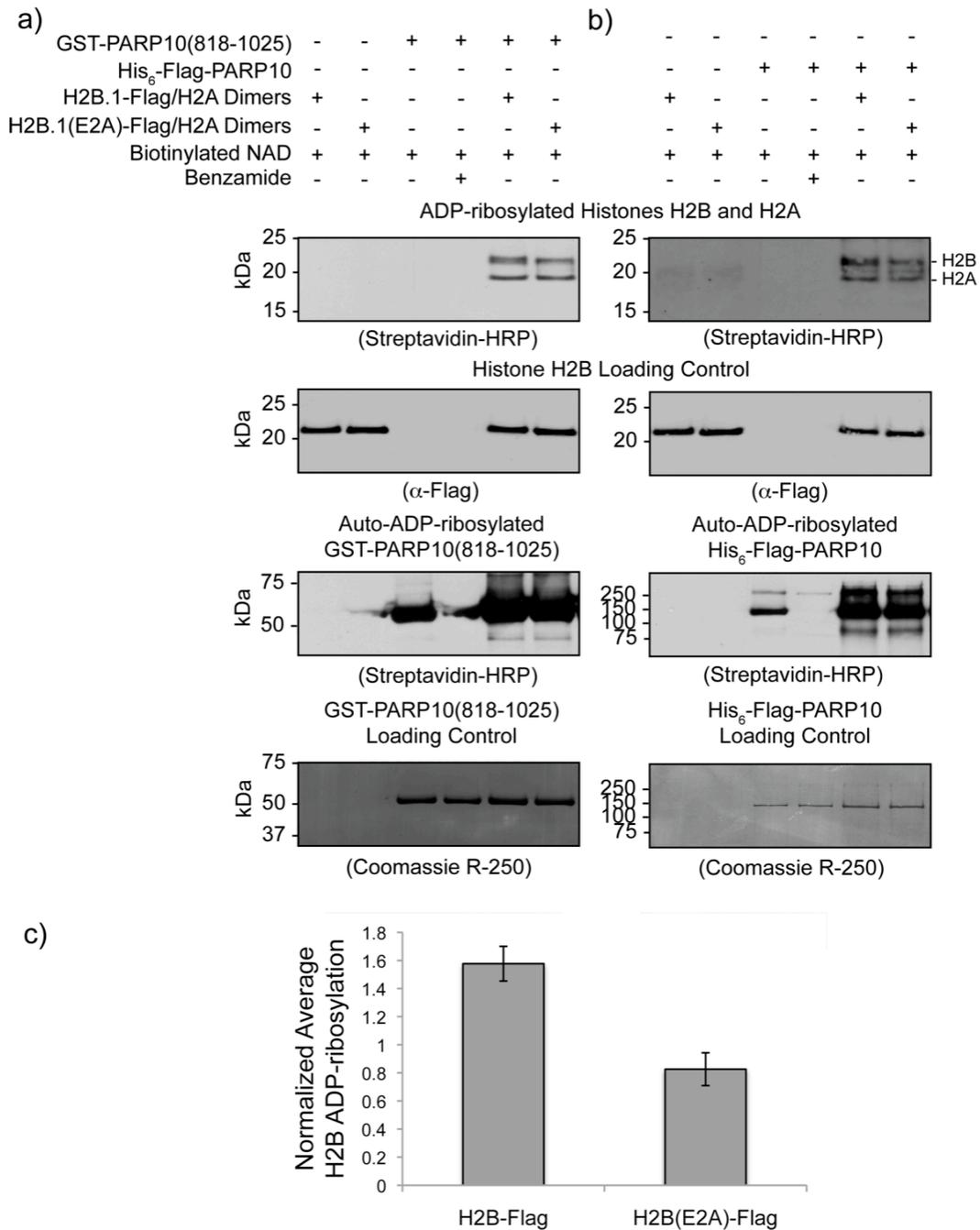


Figure S1. ADP-ribosylation of recombinant histone H2B-H2A dimers incorporating the wild-type H2B amino-terminal tail or an E2A mutation using recombinant (a) GST-PARP10(818-1025), or (b) His₆-Flag-PARP10, and biotinylated NAD (Trevigen). Mutating H2B glutamic acid-2 to an alanine prevents ADP-ribosylation at this position, and is observed to result in an ~ 50 % decrease in signal associated with ADP-ribosylation on histone H2B. Immunoblotting for the Flag-tag attached to the carboxyl-tail of the H2B histones was used as a loading control. Control groups where enzyme was not added did not exhibit ADP-ribose conjugation to the histones. The addition of benzamide, a PARP inhibitor, resulted in inhibition of auto-ADP-ribosylation of the enzyme. These data demonstrate that the observed ADP-ribosylation is enzymatic. (c) Histogram showing average ADP-ribosylated H2B band density normalized to H2A for H2B-Flag/H2A and H2B(E2A)-Flag/H2A dimers ($n = 3$). Error bars represent standard deviation.

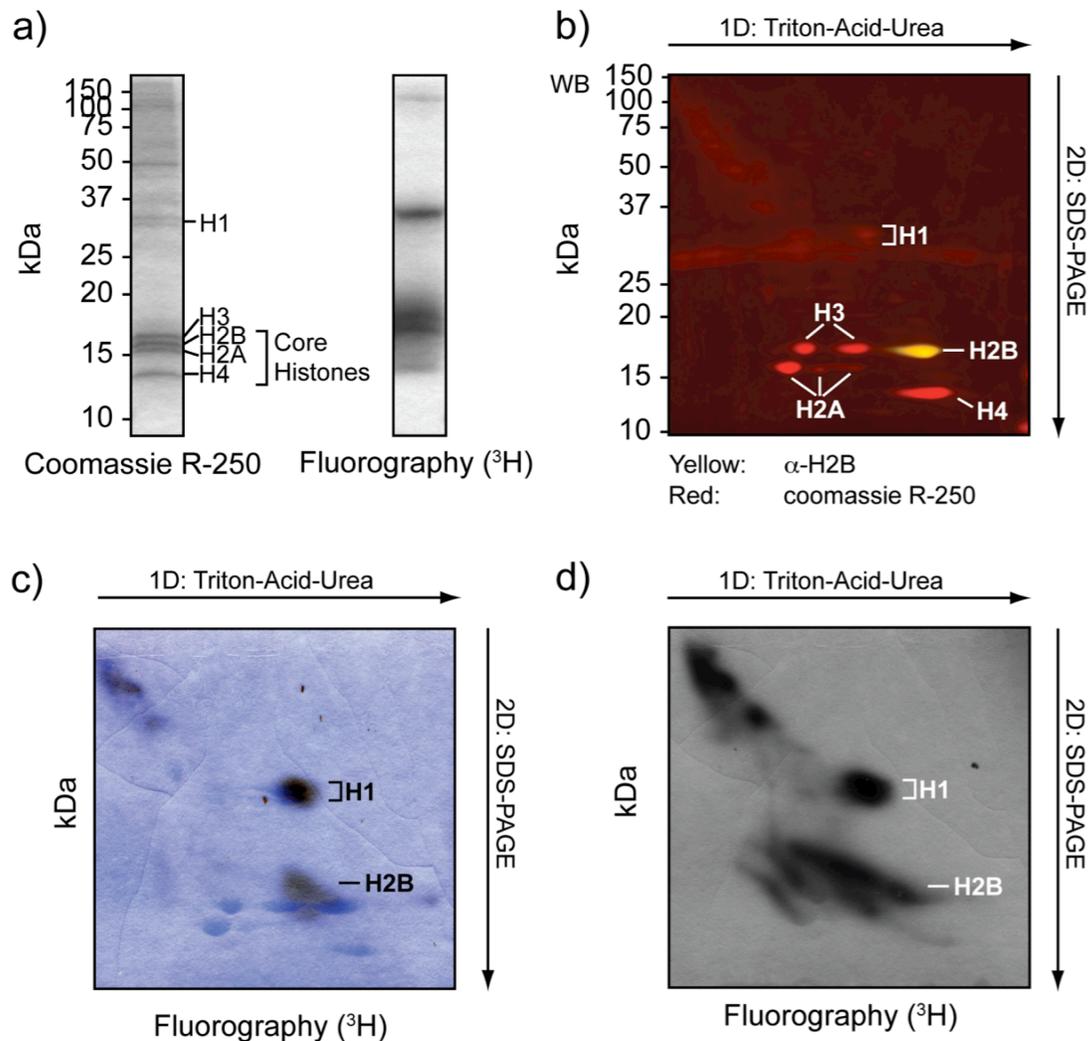


Figure S2. (a) 15 % SDS-PAGE of *in nucleo* [^3H]-ADP-ribosylated HeLa nuclear proteins after acid extraction and fluorographic detection. (b) Western blot of 2D 15 % triton-acid-urea/12 % bis-tris SDS-PAGE separated acid extracted, coomassie R-250 stained HeLa nuclear proteins. The red spots correspond to coomassie R-250 stained proteins. The yellow spot corresponds to H2B. Detection was achieved using a Li-COR Odyssey infrared imaging system. (c) and (d) Fluorographic detection of *in nucleo* [^3H]-ADP-ribosylated HeLa nuclear proteins after acid extraction separated on a 2D gel. Black spots in (c) correspond to fluorographic signal due to ^3H , with blue spots corresponding to coomassie R-250 stained proteins (for comparison to panel (b)). (d) Longer exposure time of the gel in (c). H1 and H2B were identified as the most modified histones.

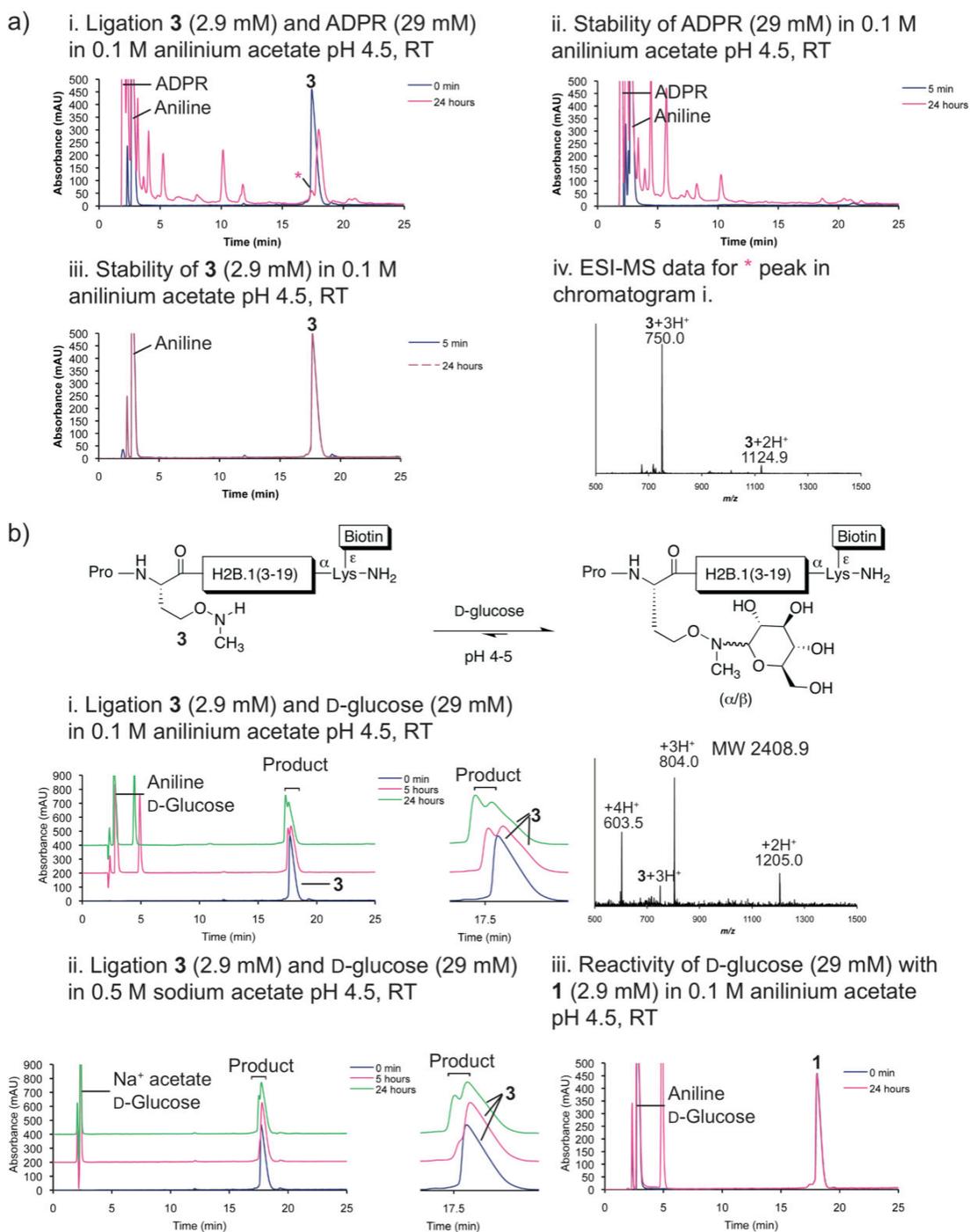


Figure S3. Aniline acceleration of (a) ADP-ribose and (b) D-glucose to peptide **3**. (a) (i.) Aniline acceleration with ADP-ribose resulted in the formation of multiple species. RP-HPLC of (ii.) ADP-ribose or (iii.) peptide **3** in anilinium acetate demonstrated that over time multiple ADP-ribose derived species formed, while **3** was stable in 0.1 M anilinium acetate pH 4.5. (iv.) ESI-MS of the * peak in (i.) did not reveal the presence of the ADP-ribose conjugate **3a**. (b) (i.) D-glucose in 0.1 M anilinium acetate pH 4.5 was found to ligate to **3** ~ 7-fold faster than (ii.) D-glucose in 0.5 M sodium acetate pH 4.5. (i.) The reaction went to ~ 80 % completion, with the expected product observed by ESI-MS (whole peak). (iii.) Non-specific reactions of D-glucose with **1**, which does not incorporate an *N*-methyl aminoxy group, were not observed in the presence of 0.1 M anilinium acetate pH 4.5.

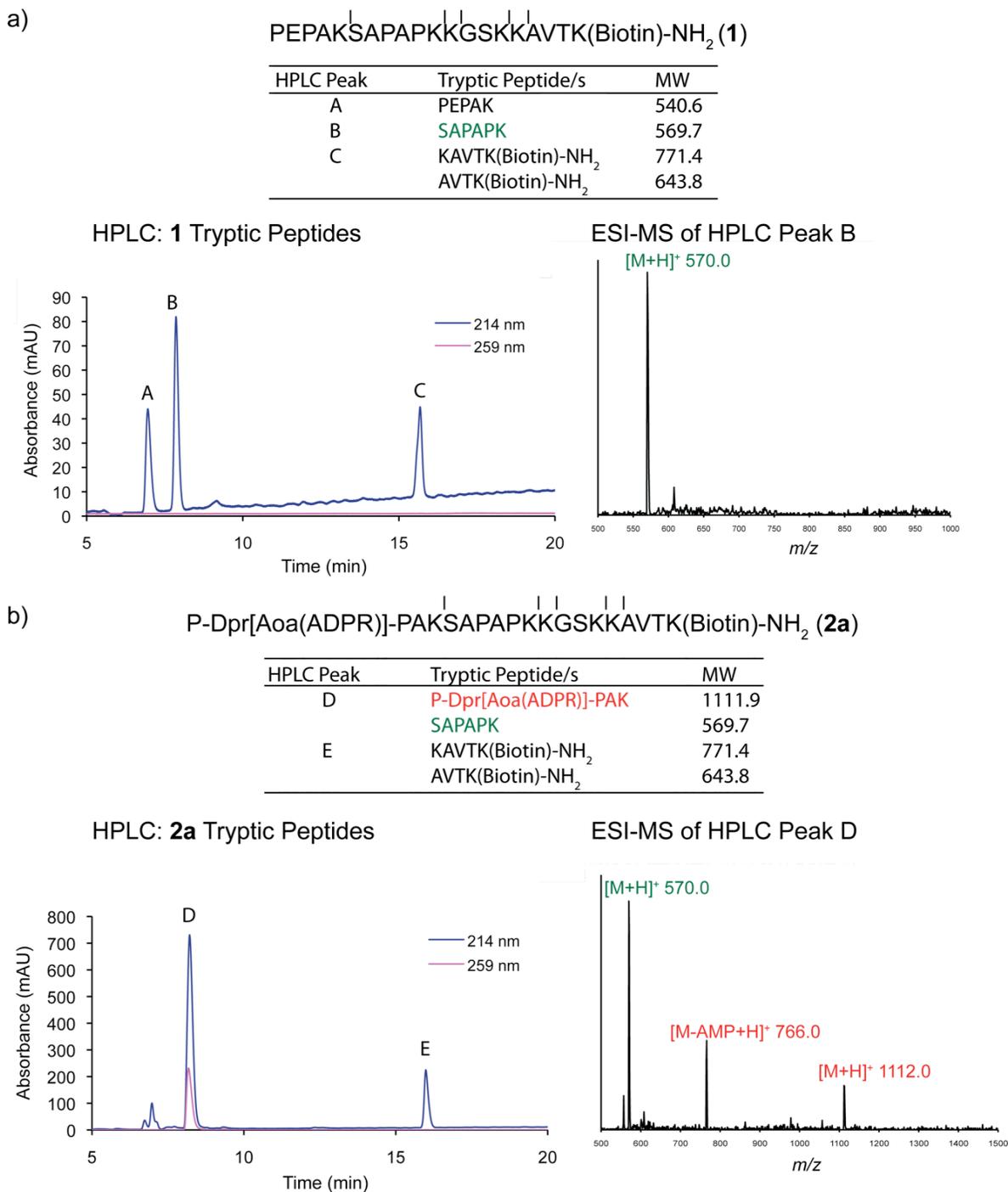


Figure S4. Test for the site-specific ligation of ADP-ribose. Tryptic digests of (a) peptide **1** and (b) peptide **2a** were separated by HPLC. Potential sites of proteolysis are marked by ticks above the peptide sequences. Peptides incorporating the adenine ring of ADP-ribose were identified by absorbance at 259 nm (HPLC peak D). Mass spectrometry revealed that peak D included the P-Dpr[Aoa(ADP-ribose)]-PAK ADP-ribose conjugated tryptic peptide, as well as the SAPAPK peptide. As the P-Dpr[Aoa(ADP-ribose)]-PAK peptide was the only species to have a mass corresponding to an ADP-ribose conjugated peptide, as well as a 259 nm absorbance, this experiment thereby demonstrated that ligation only occurred within the peptide region containing the aminoxy group.

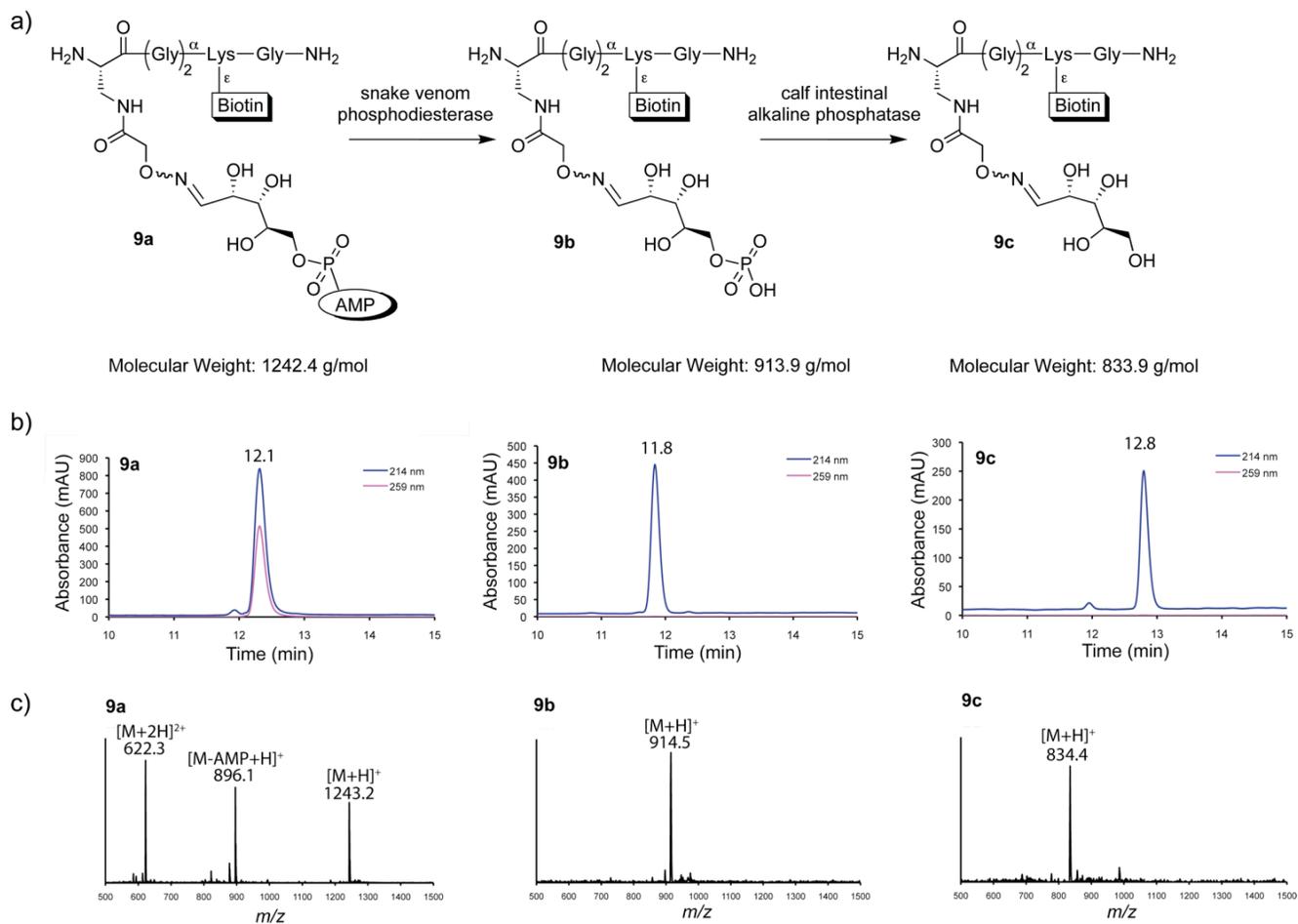


Figure S5. Testing if ADP-ribose is ligated to **9a** through the distal ribose residue. (a) The peptide was treated with snake venom phosphodiesterase, which cleaved AMP from the peptide, followed by calf intestinal phosphatase, which cleaved the phosphate from ribose-5-phosphate. (b) HPLC and (c) ESI-MS data for each step are shown. The 259 nm absorbance disappeared following treatment with snake venom phosphodiesterase, indicating the loss of the adenine ring.

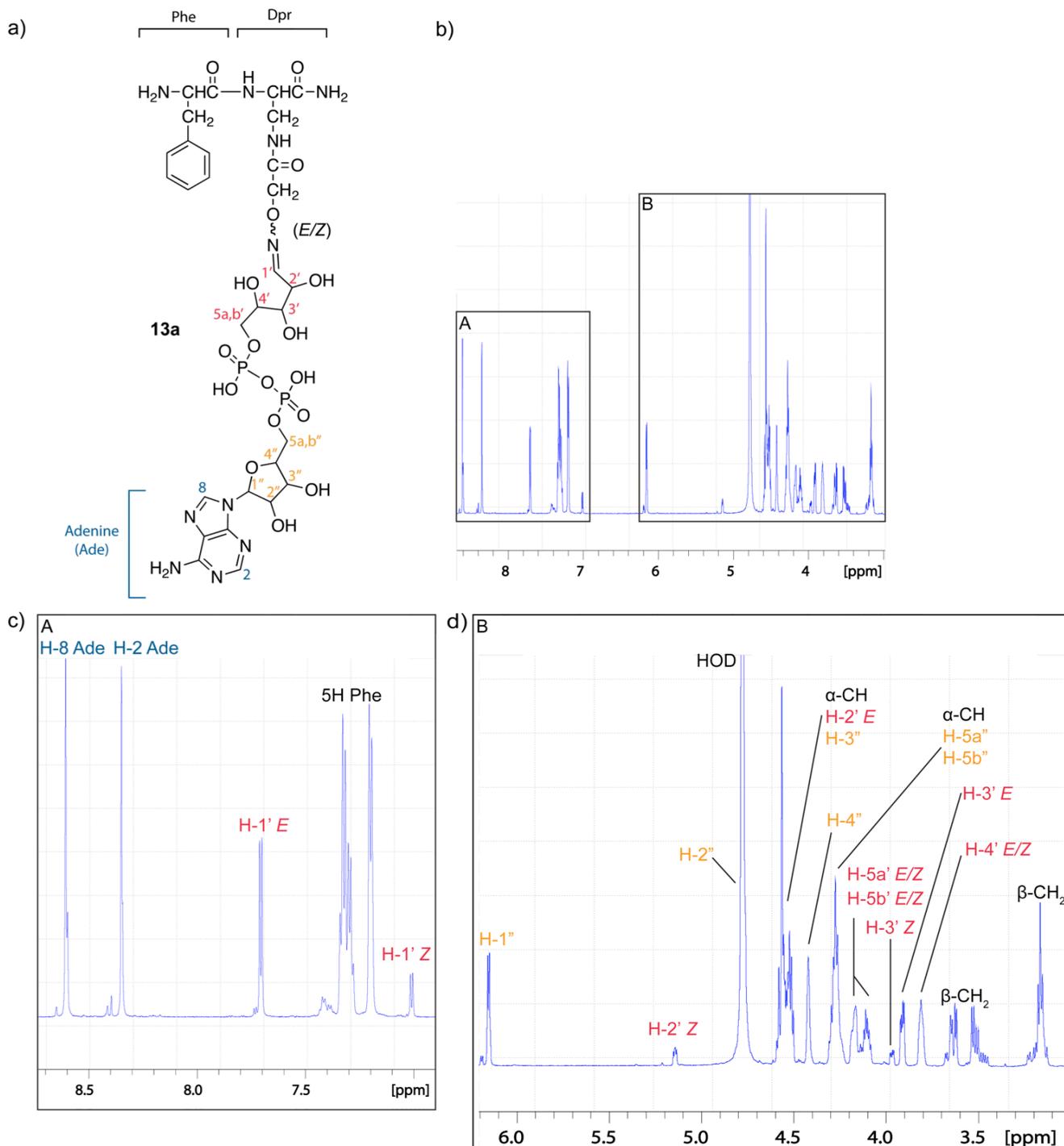


Figure S6. ^1H NMR (600 MHz, 298 K) of Phe-Dpr(Aoa(ADP-ribose))-NH₂ dipeptide **13a** recorded in D₂O at pH 7. (a) Structure of dipeptide **13a**. (b) ^1H NMR spectra with expanded regions (c) and (d) indicated. TOCSY was used to determine peaks corresponding to each of the individual ribose units. COSY was used to assign numbering to the ribose ring protons. Ribose units were assigned as proximal (orange numbering) or distal (red numbering) by cleaving AMP from **13a** using snake venom phosphodiesterase, and comparing the ^1H NMR spectra for this species, which contains only the distal ribose, with the ^1H spectra for **13a**. Oxime stereochemistry was assigned according to published data for D-ribose oximes.¹⁸ Adenine ring protons were assigned based upon published ^1H spectra for ADPR.¹⁹

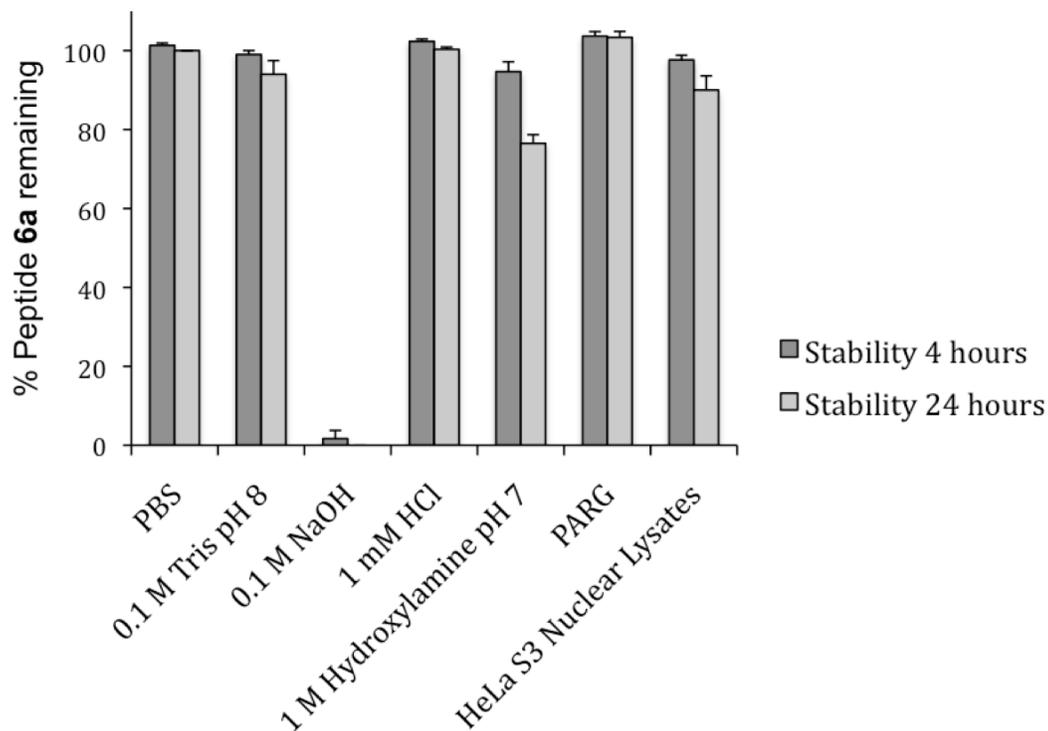


Figure S7. Chemical and enzymatic stability of ADP-ribosylated aminoxy peptide **6a**, at 4 °C, at 4 and 24 h as assessed by monitoring of the adenine 259 nm absorbance by HPLC ($n = 3$). The stability of **6a** was significantly greater than the native ADP-ribose-H2B ester linkage, which exhibits a half-life of < 1 h in 50 mM Tris-Cl pH 7.5, and 11 min in neutral hydroxylamine.²² In addition, **6a** was also stable to PARG, which has been suggested to cleave the ADP-ribose-H2B ester linkage,¹⁵ and to HeLa S3 salt-extracted nuclear lysates.

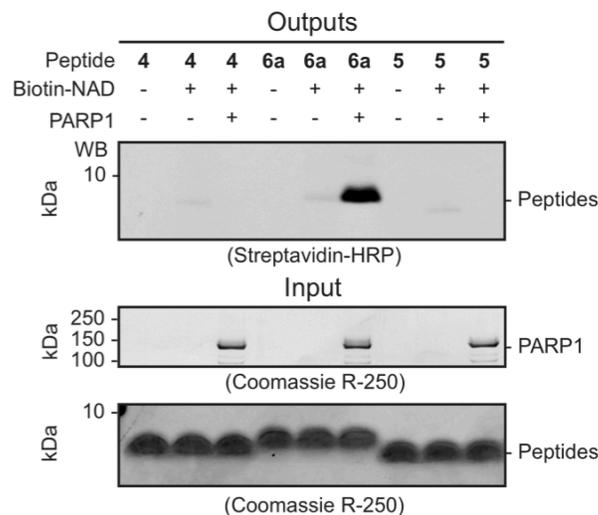


Figure S8. Transfer of ADP-ribose onto peptide **6a** using PARP1 and biotinylated NAD. Indicated reaction mixtures were resolved by SDS-PAGE, followed by western blotting against NAD associated biotin using streptavidin-HRP. Peptide only, and peptide plus biotinylated NAD groups were used to confirm that streptavidin-HRP does not interact with the peptides, and that biotinylated NAD did not chemically modify the peptides. Neither peptide **4**, which features the ADP-ribosylatable glutamic acid-2 residue, or peptide **5**, which is unable to be ADP-ribosylated at position 2 due to a glutamic acid to alanine mutation, were modified by PARP1. The lack of ADP-ribosylation of peptide **4** may be due to the absence of PARP1 ADP-ribosylation sites in this peptide, such as the recently described H2B lysine-30 residue.²³ In comparison, peptide **6a**, which contained a single ADP-ribose at position 2, was a substrate for PARP1, as demonstrated by the strong signal associated with the conjugation of biotinylated ADP-ribose onto this peptide. Thus, site-specific poly-ADP-ribose peptide conjugates can be accessed using this chemo-enzymatic strategy.

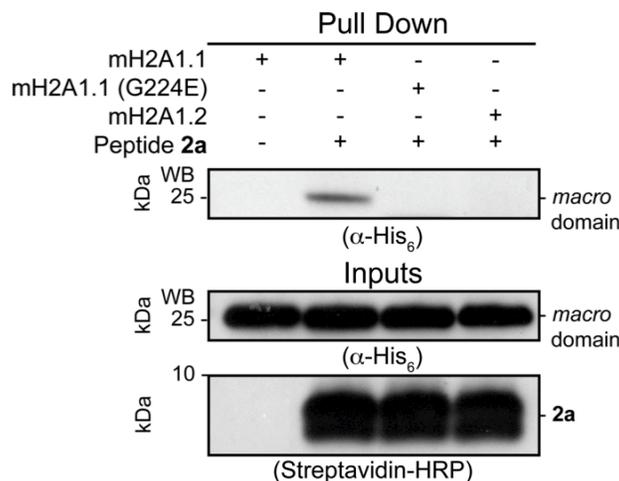


Figure S9. Pull-down assay assessing the ability of the mH2A1.1, mH2A1.1 (G224E) mutant, and mH2A1.2 *macro* domains to bind the ADP-ribose containing peptide **2a**. Complexes were captured using streptavidin-beads, and analyzed by SDS-PAGE, followed by western blotting with α -His₆ to detect the *macro* domains. Streptavidin-HRP blotting was used as a loading control for **2a**. Peptide **2a** was observed to bind the mH2A1.1 *macro* domain, but not the G224E mutant or the mH2A1.2 *macro* domain.

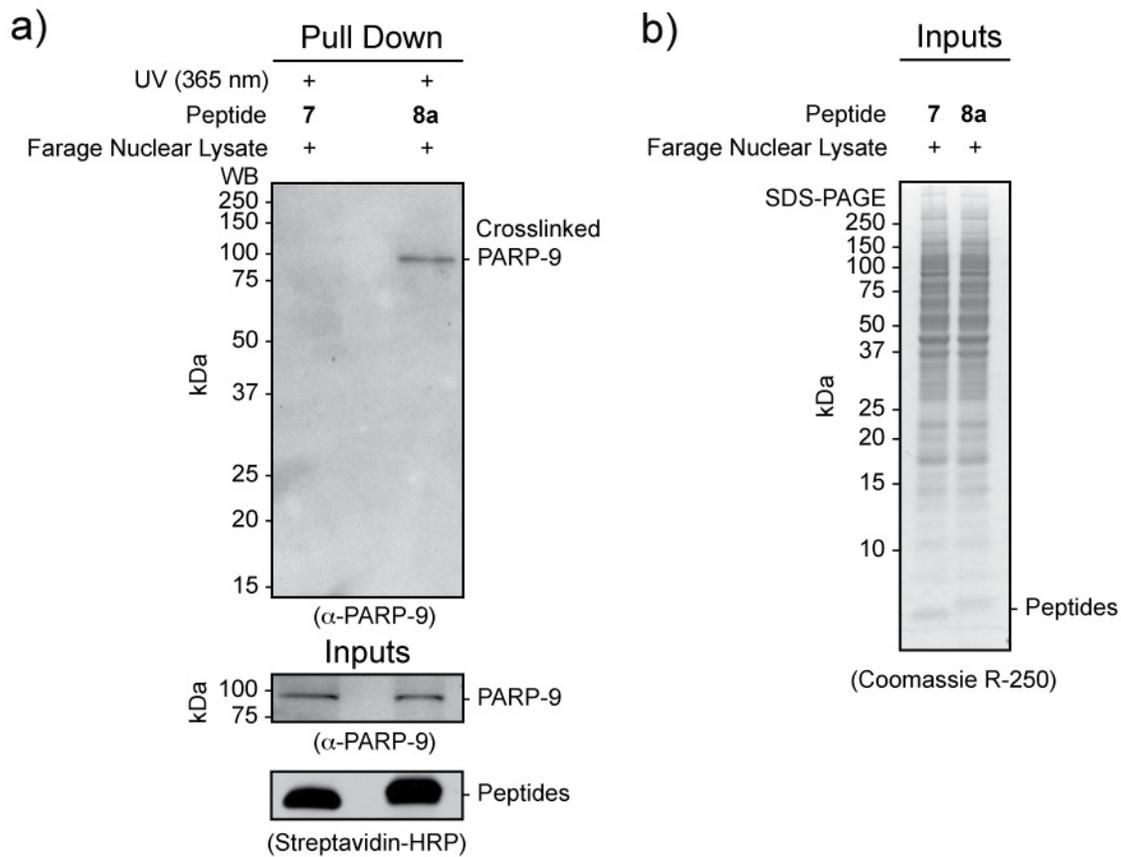


Figure S10. (a) The use of photocrosslinking peptide **8a** to enrich for the *macro* domain containing protein PARP-9. Peptides **7** or **8a** were doped into nuclear lysate from the Farage B lymphoma cell line. Following irradiation (365 nm), crosslinked species were immobilized on streptavidin-agarose beads, and thoroughly washed with 0.1 % SDS, 1 mM DTT in PBS. The immobilized proteins were eluted by boiling in reducing SDS-PAGE loading buffer containing 5 mM D-biotin, separated by SDS-PAGE and analyzed by western blotting against PARP9 (α -PARP-9). As a loading control, input samples were analyzed by western blotting for PARP9 (α -PARP-9) and peptides **7** and **8a** (streptavidin-HRP). A band (~96 kDa) corresponding to PARP9 was identified in the group containing the ADP-ribosylated peptide **8a** but not in the group containing peptide **7**. (b) Coomassie stained SDS-PAGE gel of the input protein and peptide mixture to demonstrate equal loading of nuclear lysates.

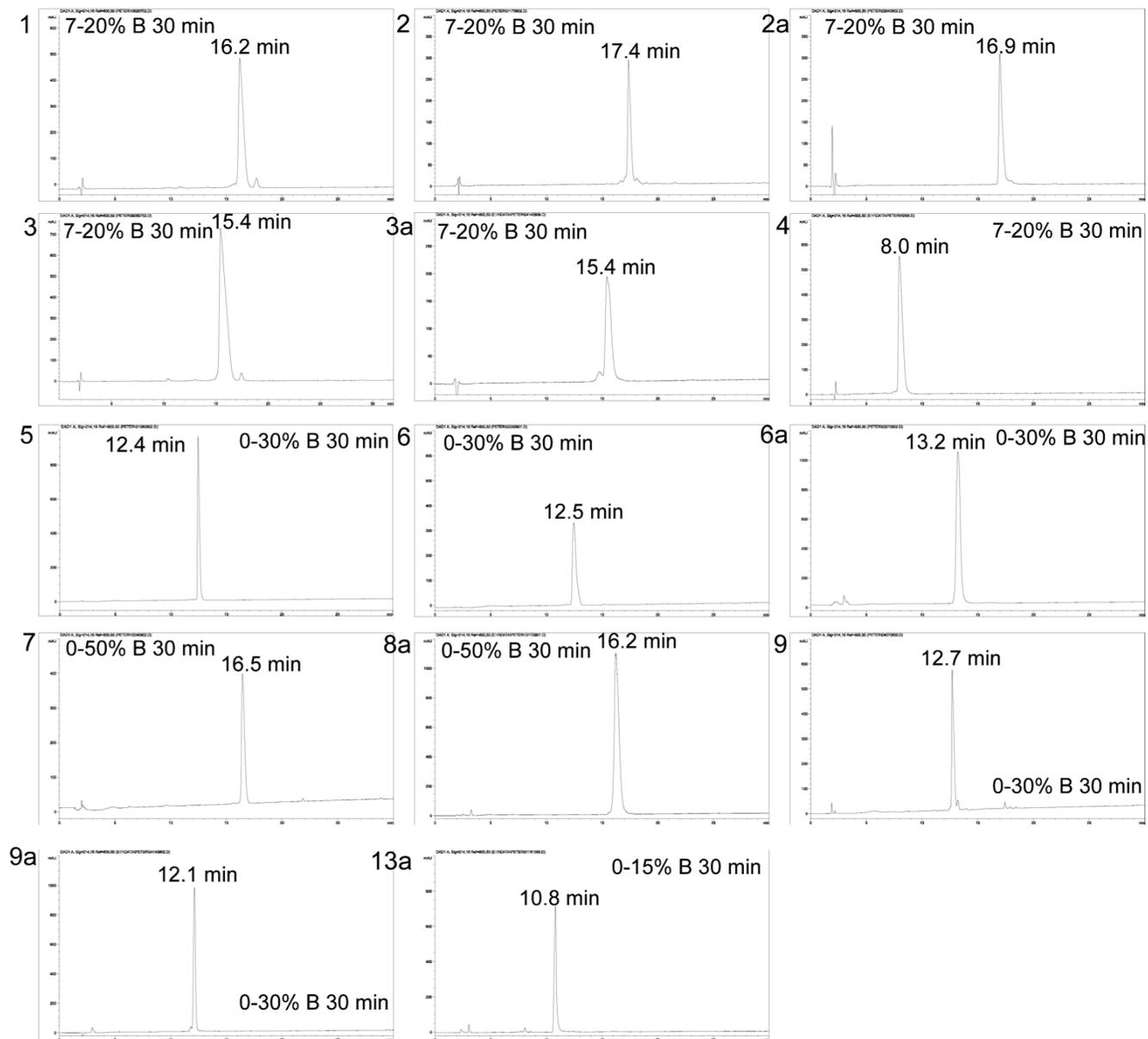


Figure S11. Analytical reverse-phase HPLC data for the purified synthetic peptides. Peptides **8** and **13** were not purified prior to ligation to ADP-ribose.

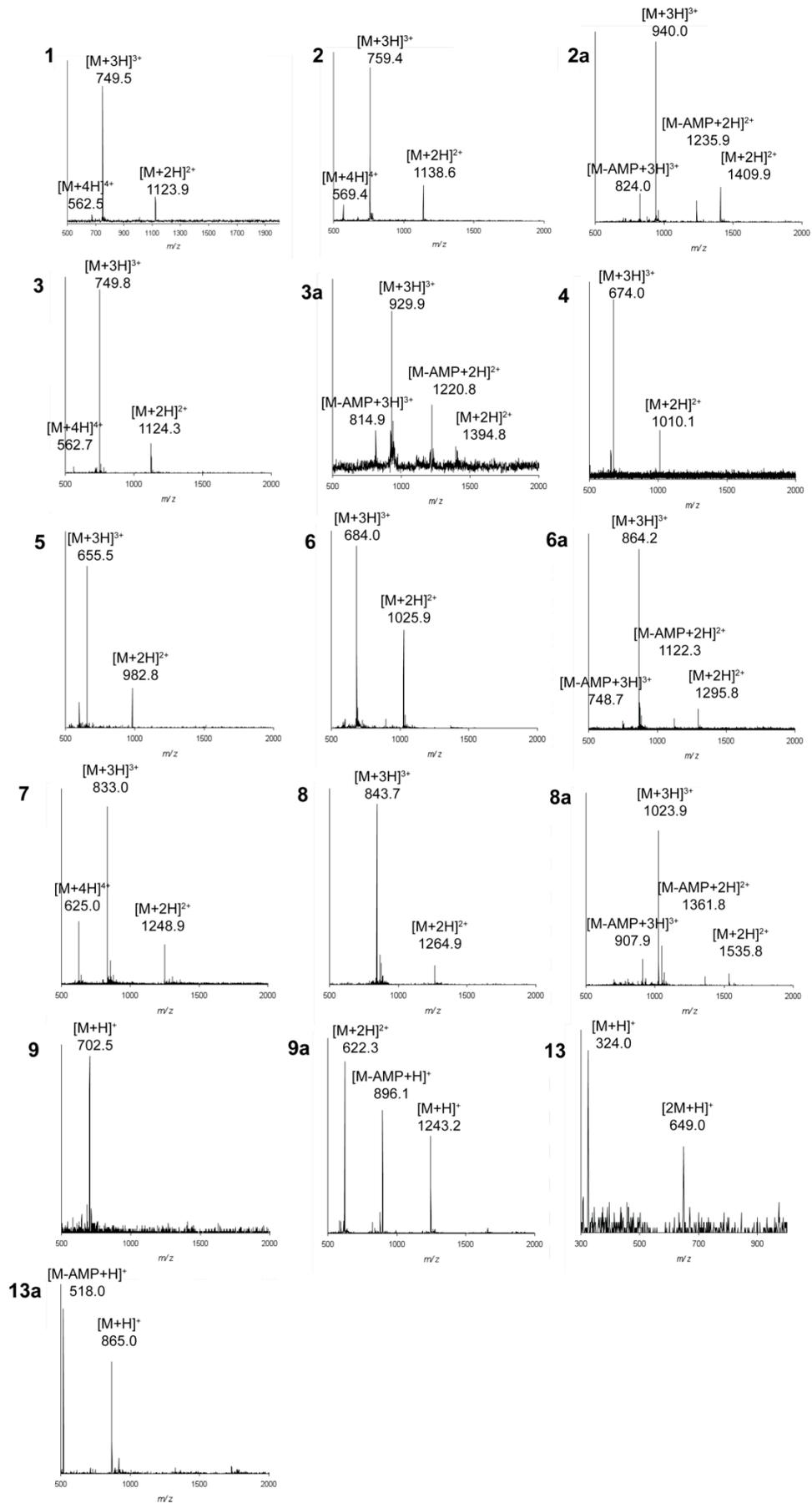


Figure S12. ESI-MS data for purified synthetic peptides.

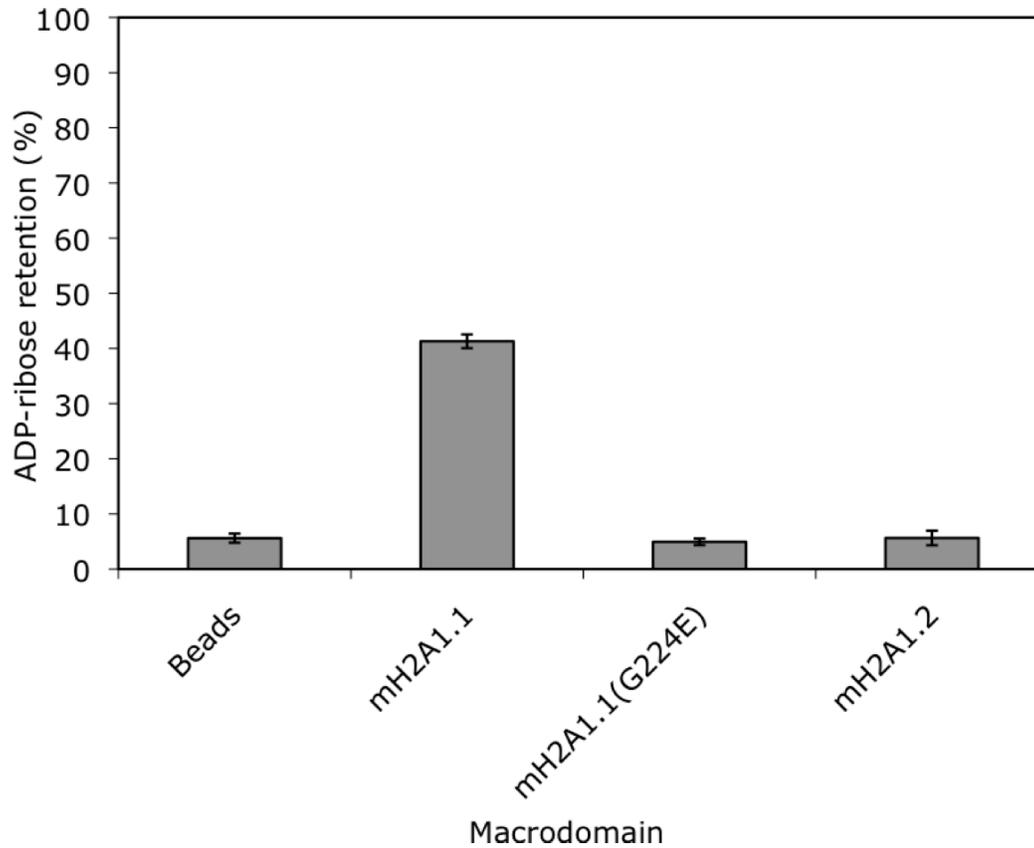


Figure S13. Pull-down assay to assess the percent binding of ADP-ribose to amino-terminal hexahistidine tagged *macro* domains immobilized on Ni-NTA beads. The mH2A1.1 *macro* domain is known to bind ADP-ribose, while mH2A1.2 does not bind ADP-ribose. Mutation of glycine-224 of mH2A1.1 to glutamic acid prevents ADP-ribose binding. Bars represent the average ADP-ribose retention \pm standard deviation. ($n = 3$)

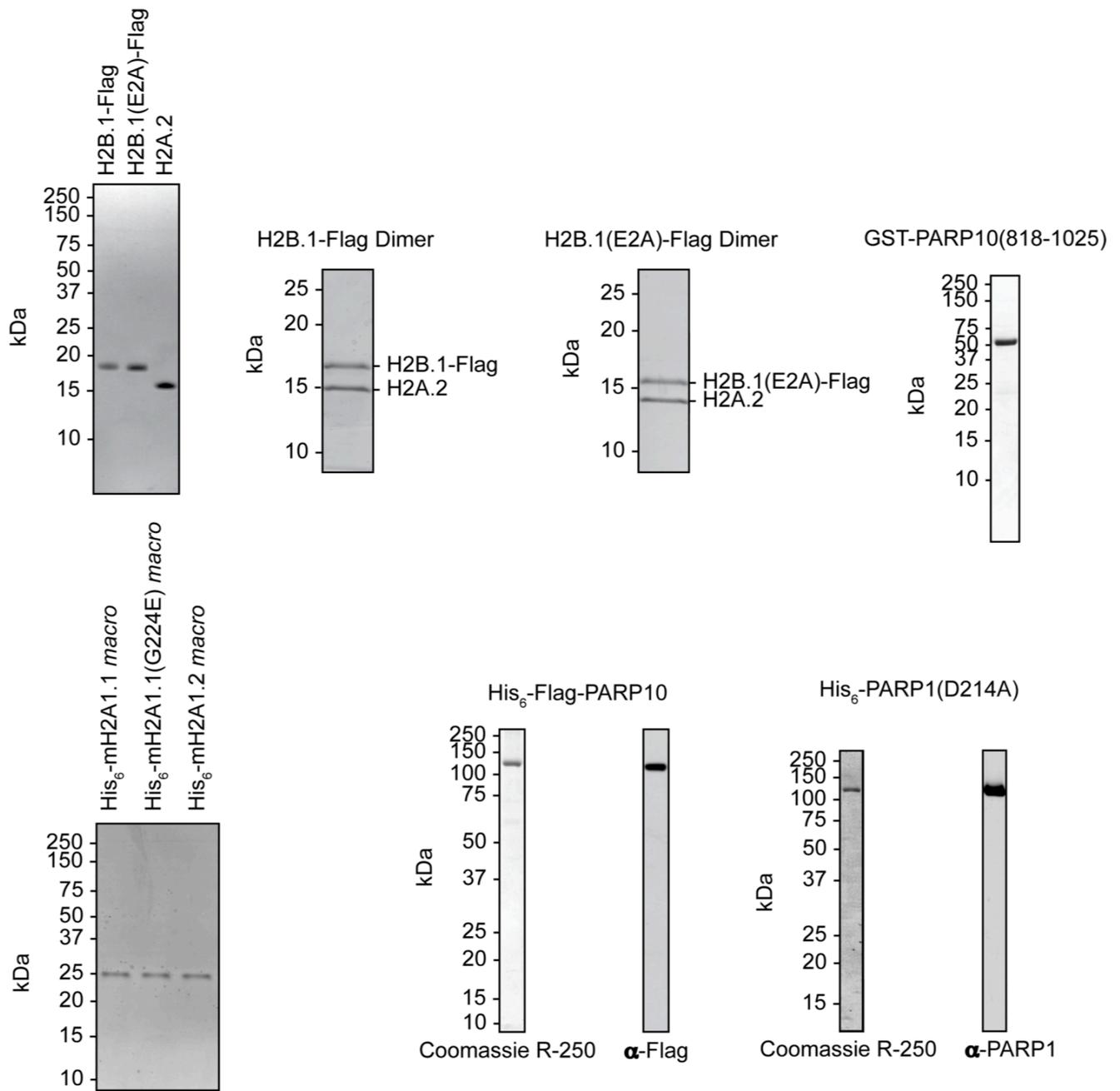


Figure S14. SDS-PAGE of expressed proteins after Coomassie R-250 staining. Western blots are presented for PARP1 and PARP10.

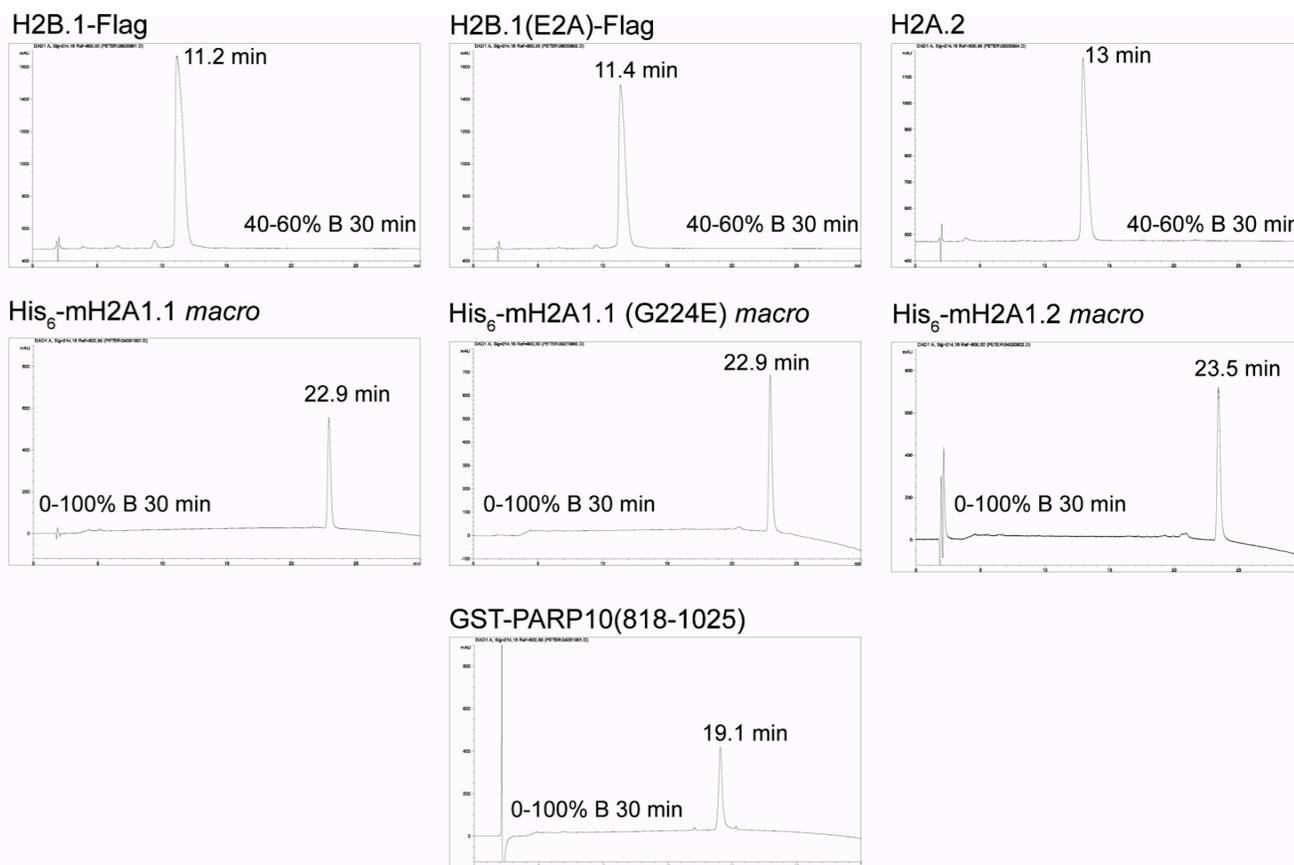
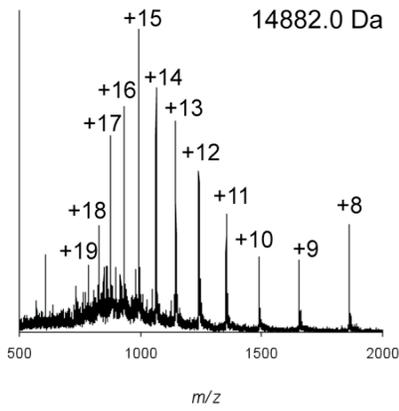
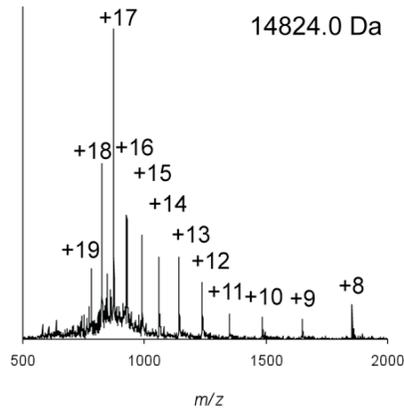


Figure S15. Analytical reverse-phase HPLC data for the purified recombinant proteins.

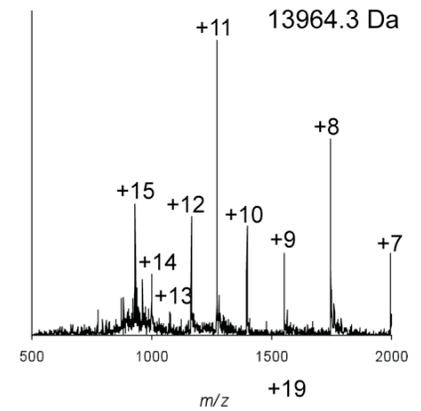
H2B.1-Flag



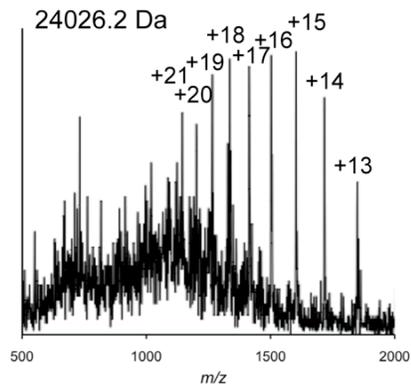
H2B.1(E2A)-Flag



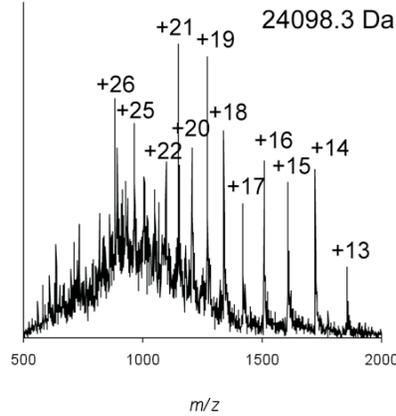
H2A.2



His₆-mH2A1.1 macro



His₆-mH2A1.1 (G224E) macro



His₆-mH2A1.2 macro

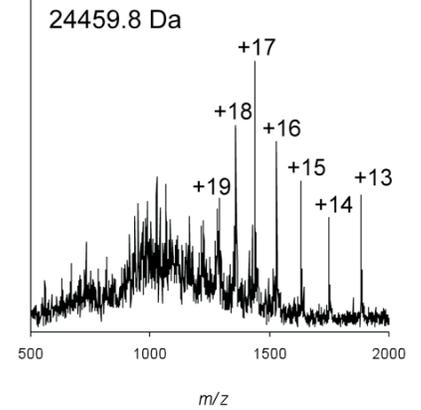


Figure S16. ESI-MS data for purified recombinant *E. coli* derived proteins, with protonated multiply charged ions indicated.